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Editorial Preface

Jordan Journal of Biological Sciences (JJBS) is a refereed, quarterly international journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS celebrated its 12th commencement this past January, 2020. JJBS was founded in 2008 to create a peer-reviewed journal that publishes high-quality research articles, reviews and short communications on novel and innovative aspects of a wide variety of biological sciences such as cell biology, developmental biology, structural biology, microbiology, entomology, molecular biology, biochemistry, medical biotechnology, biodiversity, ecology, marine biology, plant and animal biology, plant and animal physiology, genomics and bioinformatics.

We have watched the growth and success of JJBS over the years. JJBS has published 14 volumes, 60 issues and 800 articles. JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Clarivate Analytics- Zoological Record and recently has been included in the UGC India approved journals. JJBS Cite Score has improved from 0.7 in 2019 to 1.4 in 2021 (Last updated on 6 March, 2022) and with Scimago Institution Ranking (SJR) 0.22 (Q3) in 2021.

A group of highly valuable scholars have agreed to serve on the editorial board and this places JJBS in a position of most authoritative on biological sciences. I am honored to have six eminent associate editors from various countries. I am also delighted with our group of international advisory board members coming from 15 countries worldwide for their continuous support of JJBS. With our editorial board's cumulative experience in various fields of biological sciences, this journal brings a substantial representation of biological sciences in different disciplines. Without the service and dedication of our editorial; associate editorial and international advisory board members, JJBS would have never existed.

In the coming year, we hope that JJBS will be indexed in Clarivate Analytics and MEDLINE (the U.S. National Library of Medicine database) and others. As you read throughout this volume of JJBS, I would like to remind you that the success of our journal depends on the number of quality articles submitted for review. Accordingly, I would like to request your participation and colleagues by submitting quality manuscripts for review. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscripts or not, is the feedback of our review process. JJBS provides authors with high quality, helpful reviews to improve their manuscripts.

Finally, JJBS would not have succeeded without the collaboration of authors and referees. Their work is greatly appreciated. Furthermore, my thanks are also extended to The Hashemite University and the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research for their continuous financial and administrative support to JJBS.

Professor Wedyan ,Mohammed A.
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Electrophysiological Analysis of Dopamine's Modulatory Effects on the Retina and Central Visual Structures

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Abstract

This study examined the effects of electrical stimulation of the substantia nigra on dopaminergic modulation of bioelectrical activity in the retina and key structures of the visual analyzer in rabbits. These structures included the superior colliculus, the dorsal part of the lateral geniculate nucleus (pars dorsalis), and the visual cortex. Assessments were based on analyses of the electroretinogram (ERG), evoked potentials (EP), and electroencephalogram (EEG). Stimulation of the substantia nigra increased the amplitude of the ERG recorded from the retina. In contrast, it reduced the amplitude of EPs in the superior colliculus, the dorsal part of the lateral geniculate nucleus, and the visual cortex. Spectral analysis of the EEG showed a significant decrease in low-frequency rhythms (delta and theta bands) in the superior colliculus and visual cortex. No significant changes were detected in these parameters in the dorsal part of the lateral geniculate nucleus compared to the control group.

These findings indicate that the substantia nigra selectively modulates dopaminergic activity across visual system structures. This supports its critical role in the differential regulation and functional integration of retino-colliculo-cortical pathways.

Keywords: retina, dopamin, visual cortex, lateral geniculate body, superior colliculus.

1. Introduction

Dysfunction of the dopaminergic system is linked to various neurological and ophthalmological disorders, including Parkinson's disease, depression, schizophrenia, diabetic retinopathy, and Alzheimer's disease. In Alzheimer's disease, degenerative changes are frequently observed in the substantia nigra (SN) and its dopaminergic pathways (Bayer et al., 2021; Williams et al., 2019). Dopamine plays a key role not only in motor functions but also in cognitive and sensory integration, with evidence supporting its protective effects against depression (Auwal Adamu et al., 2023).

The substantia nigra, a brainstem structure rich in dopaminergic neurons, has traditionally been studied in the context of motor control. However, recent research highlights its modulatory influence on sensory systems, particularly the visual pathway. Dopaminergic projections from the SN extend to the retina and central visual structures, including the colliculus superior (CS), lateral geniculate body (LGB), and visual cortex (VC). Within the retina, dopamine regulates essential processes such as light adaptation, contrast sensitivity, and color discrimination, underscoring its importance in visual processing.

Electrophysiological techniques provide high temporal and spatial resolution to investigate how dopamine modulates neuronal connectivity and activity in visual pathways. Studies have shown that dopamine alters the response properties of retinal ganglion cells and neurons in the visual cortex, shaping their activity in response to

visual stimuli (Zhou et al., 2023). It also influences bipolar cell receptive fields and light-dark adaptation, effects that are partly mediated through GABA_A receptors (Farshi et al., 2020). Furthermore, dopamine has been shown to modulate synaptic activity in retinal ganglion cells and participate in non-image-forming visual functions (Bergum et al., 2024).

Despite these insights, the functional impact of direct dopaminergic modulation from the SN on peripheral and central visual structures remains insufficiently explored. This study addresses this gap by using an experimental rabbit model to evaluate the effects of electrical stimulation of the SN on bioelectrical activity in the retina and central visual structures, including the CS, LGB, and VC. These findings will advance our understanding of dopaminergic mechanisms in the visual system and may contribute to early diagnostic and therapeutic strategies for neurodegenerative disorders affecting vision (Miryusifova et al., 2022, 2024).

2. Materials and Methods

The experiments were conducted on Chinchilla rabbits maintained under standard vivarium conditions with a controlled 12:12 h light-dark cycle, free access to food and water, and regular veterinary monitoring.

All procedures were performed in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the Law of the Republic of Azerbaijan and

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were approved by the Institutional Animal Care and Use Committee (Approval No. 1/643-IIQD).

Under general anesthesia (urethane, 1.0 g/kg, intraperitoneally), electrodes were stereotactically implanted into the CS, LGB, VC and SN. The coordinates were determined according to the stereotaxic atlas of Fífkova et al. (1962) (Table 1).

Table 1. Structure coordinates

Structure	AP (Anterior-Posterior-mm)	ML (Medial-Lateral-mm)	DV (Dorsal-Ventral-mm)
SN	5-6	3-4	14-15
CS	9-10	2,5-3	8-9
LGB	4-5	6-7	9-10
VC	9	9	1-2

Monopolar nichrome electrodes were used, with diameters of 0.5 mm for cortical and 1.5 mm for subcortical structures. The electrodes were insulated except at the tip. A stainless-steel screw placed in the cranial bone served as the reference electrode.

Electrical stimulation of the SN was delivered using a current intensity of 200 μ A, pulse duration of 0.2 ms, frequency of 50 Hz, for 10 s per trial. Neural activity was recorded using the Neuron-Spectrum 5 system (Neurosoft, Russia). Signals were sampled at 1 kHz, band-pass filtered between 0.5 and 100 Hz, and stored for offline analysis.

Spectral and amplitude analyses of EEG and ERG, along with evoked potential (EP) parameters, were performed using the system's built-in software. Statistical analyses were conducted in Microsoft Excel using one-way ANOVA, with significance set at $p < 0.05$.

3. Results and Discussion

The study was conducted in two main stages. First, baseline activity of the visual system was recorded to establish control parameters. Then, the SN was electrically stimulated, and its effects on the ERG, EP and EEG in the CS, LGB, VC (Tab. 1, Fig. 1).

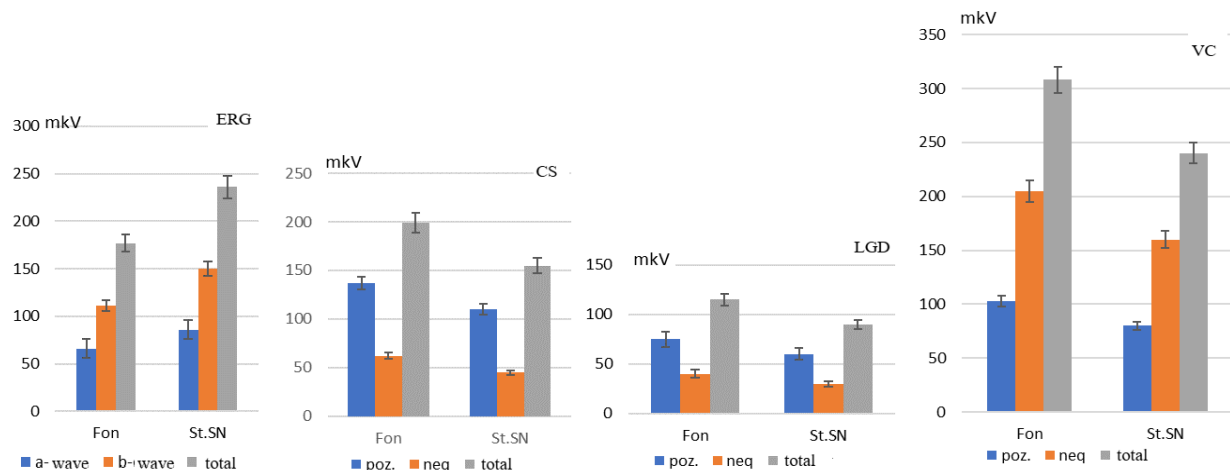


Figure 1. Effect of Substantia Nigra stimulation on the amplitude parameters of evoked potentials in the retina and central structures of the visual analyzer (CS, LGD, VC).

Table 2. Percentage changes in the amplitude parameters of evoked potentials in the retina and visual analyzer structures following stimulation of the SN.

Structure	Component	Change (%)
ERG	a-wave amplitude	+26.3%
	b-wave amplitude	+36.4%
	Total amplitude	+25.0%
CS	Positive wave amplitude	-18.5%
	Negative wave amplitude	-27.8%
	Total amplitude	-22.0%
LGD	Positive wave amplitude	-5.9%
	Negative wave amplitude	-7.1%
	Total amplitude	-6.7%
VC	Positive wave amplitude	-18.2%
	Negative wave amplitude	-14.3%
	Total amplitude	-15.6%

Immediately after stimulation, retinal responses were absent, likely reflecting a delay in neurochemical

signaling. After several minutes, ERG amplitudes increased (Fig. 1), indicating enhanced visual signal transmission at the retinal level. This facilitation is consistent with increased dopamine release from the SN and its modulatory action on photoreceptors, bipolar cells, and Müller cells (Witkovsky, 2004; Djamgoz et al., 1997). Upregulation of dopamine acting via D1/D2 receptors is known to influence Na^+/K^+ permeability and enhance visual responses (Jackson et al., 2012).

In the SC, stimulation led to a marked reduction in EP amplitudes and in the low-frequency (delta and theta) bands of the EEG (Fig. 2). These findings suggest an inhibitory modulation of SC activity by SN projections, as consistent with earlier anatomical and electrophysiological studies showing dopaminergic and GABAergic regulation of this midbrain structure (Appel & Behan, 1990; Kayama & Koyama, 1984).

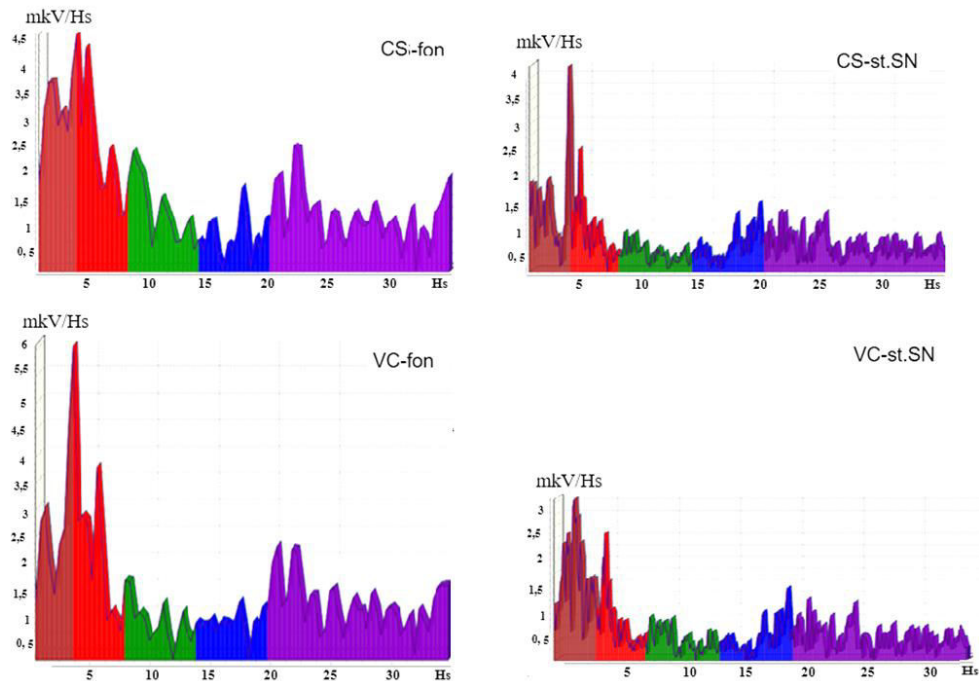


Figure 2. Effect of substantia nigra stimulation on the amplitude parameters of evoked potentials in the central structures of the visual analyzer (superior colliculus, visual cortex).

In the LGB, changes were minor and not statistically significant, indicating relatively stable functional activity or possible compensatory mechanisms. The laminar organization and strong inhibitory interneuron network in the LGB (Sherman & Guillery, 2002) may contribute to this stability.

In the VC, a moderate reduction in EP amplitudes was observed, along with decreased low-frequency EEG power (Fig. 2). This suggests that SN stimulation modulates cortical excitability, potentially through dopaminergic and GABAergic pathways that influence cortical network dynamics (Lewis et al., 2001). Reduced low-frequency activity is consistent with increased cortical inhibition, possibly mediated by D1-receptor-dependent mechanisms (Seamans & Yang, 2004).

This study demonstrated that electrical activation of dopaminergic neurons in the SN exerts a selective and multidirectional modulatory influence on the visual system of rabbits. At the retinal level, a marked enhancement of bioelectrical activity was observed, whereas in the SC and VC, a reduction in the amplitude of evoked potentials and a decrease in the power of low-frequency EEG rhythms were detected. No significant changes were found in the LGB, which may reflect the stability of its neuronal networks or the engagement of compensatory mechanisms in this structure.

The novelty of this work lies in the comprehensive, integrated assessment of ERG, EP, and EEG within a single experimental model to evaluate functional changes in both peripheral and central components of the visual system under SN stimulation. These findings broaden current understanding of the neuromodulatory role of dopamine in sensory integration and visual pathway plasticity, aligning with modern research priorities in experimental neurobiology and neurophysiology.

The practical significance of the study stems from its potential to provide an experimental basis for developing

methods of early diagnosis and targeted therapy for neurodegenerative disorders such as Parkinson's and Alzheimer's diseases, where disturbances in dopaminergic transmission and visual function are prominent. These results also lay the groundwork for future studies aimed at exploring strategies for modulating dopaminergic systems to improve cognitive and sensory functions.

4. Conclusion

This study demonstrates that dopaminergic stimulation of the substantia nigra selectively modulates neuronal activity across different levels of the rabbit visual system. Retinal responses were enhanced, while activity in the CS and VC decreased, indicating region-specific modulation. The LGB, by contrast, showed relative stability, suggesting compensatory or homeostatic regulation.

These results provide new experimental evidence that the substantia nigra exerts differential **neuromodulatory** control over retino-colliculo-cortical pathways. This highlights the importance of dopaminergic mechanisms in visual information processing and offers insights relevant to the early diagnosis and potential therapeutic targeting of visual and cognitive dysfunctions associated with neurodegenerative diseases.

5. Conflict of interests

The authors declare no competing interests.

6. Ethical approval

All procedures described in this study were carried out following the recommendations of the Institutional Animal Ethics Committee (IAEC) of the institute.

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Photosynthetic Performance and Stomatal Plasticity of Six Vegetable Plants in Vegetative Phases under Controlled Hydroponic Conditions

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Abstract

In the vegetative phase, shoot and root development are influenced by genetic and environmental factors. This research investigates changes in plant growth and stomatal plasticity among six crops grown under controlled floating raft hydroponic conditions: *Brassica chinensis* var. *parachinensis* (BC), *Brassica oleracea* var. *sabellica* (BO), *Ipomoea aquatica* L. (IR), *Brassica rapa* subsp. *chinensis* (BR), *Lactuca sativa* L. var. *crispa* (LS), and *Brassica narinosa* L. (BN). During the vegetative phase (42 days), plants adapted their stomatal development in response to growth needs, leading to increased stomatal density across all cultivars. However, this increase did not significantly affect stomatal opening width or the number of subsidiary cells, which are likely genetically controlled. A strong positive correlation was found between stomatal density, photosynthetic performance, and growth in most cultivars (BC, BR). The BN cultivar highlighted that high stomatal density does not necessarily guarantee growth functionality, emphasizing the importance of coordination with leaf area. Observations from LS and BN suggest a dynamic adaptation to balance photosynthetic demand and water use efficiency. Additionally, the consistency in stomatal aperture size and the number of subsidiary cells underscores genetic stability. Overall, the plasticity of stomatal traits demonstrates how these species optimize gas exchange and photosynthetic performance in hydroponic conditions. These findings help identify key characteristics for distinguishing plant performance, such as a photosynthetic indicator of better plant growth under hydroponic conditions, which is potentially valuable for breeding or stress resilience studies.

Keywords: horticulture, hydroponic, stomatal characteristics, urban plant, vegetative

1. Introduction

The hydroponics market is poised for notable growth, particularly in addressing the demand for food and vegetables in areas where cultivation space is limited and soil media cannot be utilized (Wang *et al.*, 2019). This positive trend has been further supported by advancements in various systems, including Nutrient Film Technique (NFT), Deep Flow Technique (DFT), vertical farming, and floating raft hydroponics. These innovative methods are increasingly embraced in urban environments to cultivate leafy greens such as lettuce, spinach, pak choi, and basil, with biomass harvests typically occurring during the mid to late vegetative stages (Palmitessa *et al.*, 2024; Vega *et al.*, 2023).

During the vegetative phase, plants primarily focus on developing their shoots, which include the stem, new

shoots, and leaves, as well as root growth (Jia *et al.*, 2023). In hydroponic systems, this stage is optimized through controlled environmental conditions—such as light, humidity, and temperature—as well as a balanced nutrient solution that is particularly rich in nitrogen, phosphorus, and potassium to promote leaf growth. Additionally, efficient water usage and oxygenation are crucial for ensuring healthy root development (Al-Gaadi *et al.*, 2024; Palmitessa *et al.*, 2024). Research indicates that hydroponics accelerates vegetative growth because plants have access to readily available nutrients and more favorable growing conditions, with fewer pathogens compared to traditional soil systems (Silva *et al.* 2018; Palsha *et al.* 2024).

Cultivating leafy greens requires a well-controlled vegetative period to maximize biomass production. This phase is the period of growth following germination and preceding flowering. During the vegetative phase, plants

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** **Abbreviations:** BC, *Brassica chinensis* var. *parachinensis*; BN, *Brassica narinosa* L.; BO, *Brassica oleracea* var. *sabellica*; BR, *Brassica rapa* subsp. *chinensis*; IR, *Ipomoea aquatica* L.; LS, *Lactuca sativa* L. var. *crispa*; DFT, deep flow technique; NFT, nutrient film technique; WUE, water use efficiency.

primarily focus on expanding their leaf area to enhance photosynthesis, developing shoots and roots to facilitate nutrient uptake (Jha, 2019), and performing various metabolic processes (Kadam *et al.*, 2015). The efficiency of these processes is significantly influenced by the characteristics of the plant's leaves, particularly the stomata. Stomatal properties are vital for regulating gas exchange and conserving water through transpiration, both of which are essential for optimal growth (Chua & Lau, 2024).

Stomatal development is governed by genetic pathways involving the SPCH, MUTE, and FAMA genes (Ortega *et al.*, 2019; Verma *et al.*, 2020). Additionally, environmental factors such as light, carbon dioxide concentration, humidity, and nutrient availability significantly influence the expression of these genes. During the vegetative phase, light not only promotes stomatal development but also facilitates an increase in leaf area (Chua & Lau, 2024; Ekeke *et al.*, 2017). Furthermore, the presence of nitrogen enhances stomatal density and supports greater leaf expansion (Chen *et al.*, 2020). Water availability also plays a critical role in modulating stomatal aperture activity and density, primarily in response to variations in water content within plant tissues (Chen *et al.*, 2020; Chua & Lau, 2024).

Research has shown a positive correlation between stomatal density and biomass accumulation during the vegetative phase, which subsequently affects photosynthetic efficiency (Stinziano *et al.*, 2017; Yin *et al.*, 2020). However, it is important to note that excessively high stomatal density can negatively impact the water use efficiency (WUE) of leaves, resulting in increased transpiration rates (Bian *et al.*, 2019). Additionally, different cultivars display distinct responses and growth patterns, even when subjected to identical environmental conditions (Parvin *et al.*, 2017).

In this study, we aimed to conduct a thorough growth analysis during the vegetative phase of several plant species, specifically *Brassica chinensis* var. *parachinensis*, *Brassica oleracea* var. *sabellica*, *Ipomoea aquatica* L., *Brassica rapa* subsp. *chinensis*, *Lactuca sativa* L. var. *crispa*, and *Brassica narinosa* L. Previous research, as noted by Silva *et al.* (Silva *et al.*, 2018), has indicated that, within the *Brassica* genus, vegetative growth—quantified through leaf area expansion—follows a logistic growth pattern that positively responds to specific controlled conditions, including a temperature of around 35 °C, moderate nutrient levels (5 mS/cm EC), and high light intensity.

Conversely, a study by Zhou *et al.* (Zhou *et al.*, 2020) highlighted that in *L. sativa* var. *crispa*, relative growth rates (RGR) and various other growth components tend to fluctuate with nutrient availability, showcasing the plant's remarkable morphological plasticity during the vegetative expansion phase. Moreover, it is essential to acknowledge that several additional factors influence the growth of these six plant species, including hormonal regulation, photoperiod, sunlight intensity, environmental stimuli, mechanical signals, and the genetic control of plant architecture and anatomical traits, such as stomatal density (Araújo *et al.*, 2011; Chua & Lau, 2024).

This study seeks to enhance our understanding of the changes in plant growth during the vegetative period, as well as the stomatal plasticity of six different vegetable

crops cultivated under controlled hydroponic conditions. Over a 42-day observation period, we measured various growth parameters to elucidate the growth patterns of these crops. The focus is particularly on the stomatal plasticity exhibited by the crops during three distinct phases of vegetative growth, all within optimal hydroponic conditions. We anticipate that the insights gained from this research will be beneficial for improving plant biomass yield in hydroponic systems.

2. Materials and methods

2.1. Plant materials

This research with hydroponic sets was carried out simultaneously using several types of vegetable plants, including Caisim (*Brassica chinensis* var. *parachinensis*-BC), Kale (*Brassica oleracea* var. *sabellica*-BO), water spinach (*Ipomoea aquatica* - IR), Pakcoy (*Brassica rapa* subsp. *chinensis*-BR), Lettuce (*Lactuca sativa* L. Var. *crispa*-LS), and Pagoda mustard (*Brassica narinosa* L.-BN), derived from seeds obtained from farm shops. The seeds of each cultivar were germinated in water with nutrient media consisting of AB mix. The components in AB-mix consist of macro nutrients such as NO₃ (9.9%), NH₄ (0.48%), P₂O₅ (4.83%), K₂O (16.5%), MgO (2.83%), CaO (11.48%), SO₃ (3.81%) and micro nutrients such as B (0.0013%), Mn (0.025%), Zn (0.015%), Cu (0.002%), Mo (0.003%), and Fe (0.037%).

2.2. Experimental site

This study was conducted in a greenhouse in an urban environment with temperatures between 29 °C and 32 °C between 10 am and 2 pm, and air humidity ranging from 80% in the dry season to 100% in the rainy season. The planting location was on the second floor, and a block research design was used. Each plant was planted in a different box according to its type and placed randomly, and its position was exchanged every two weeks. The number of plant replications was 3 blocks, with each block containing nine individual plants. The design for each group followed a completely randomized blocked design (CRBD).

2.3. Maintain hydroponic conditions

Cultivation and maintenance were conducted weekly, with plant ages ranging from seedling to harvest at 42 days old. The sterilized seeds were first soaked in a 1% chlorine solution for one minute, then rinsed three times with distilled water. They were subsequently soaked for 24 to 48 hours in trays kept in the dark. After germination, the seeds from the trays were placed into hydroponic pots, with two sprouts in each pot, with a diameter of five cm. These pots were then placed in tubs containing three liters of water, along with a growing medium that had been mixed with AB mix nutrients. The AB mix was applied by combining tap water with the AB mix solution at a ratio of 10 ml of AB mix solution to one liter of water. A total of 30 ml of AB mix was introduced into the three liters of water, which was replaced once a week. Once the plants reached the mid-vegetative stage, between 14 and 21 days after planting, the frequency of nutrient and water applications increased to twice a week until the plants entered the final vegetative period.

2.4. Measurement of growth characteristics

The observed growth parameters included plant height, number of leaves, leaf area of the third leaf, and leaf circumference. Plant height was measured from the base to the tip of the longest leaf. The total number of leaves was counted for each clump of plants, including both the youngest leaves that had begun to grow and the oldest leaves that had turned brown. Leaf area measurements were taken using a Leaf Area Meter (LI-3000C) and were also verified with two-dimensional digital measurements using Image Raster 3.0.

2.5. Observation of stomatal characteristics

Stomata were observed by taking imprints of the outer layer of the abaxial epidermis from the third leaf of each treatment group ($n=3$). Each imprinted leaf was placed on an object glass and covered with several drops of water before being examined under a binocular light microscope (Boeco BM-180/SP, Germany). Observations were recorded using Optilab software (Miconos 1.0) at magnifications of 4x, 10x, and 40x. All images were subsequently analyzed using Image Raster 3, with initial calibration completed. Stomatal characteristics were measured based on several parameters, including the number and density of stomata, the width of stomatal pore openings, and the height and width of the stomata.

2.6. Photosynthetic and gas exchange measurement

Gas exchange and photosynthetic performance were observed during the vegetative period using the LI-6800 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA) to evaluate the CO_2 response of A (photosynthetic rate) employing a traditional method. The measurement parameters for the chamber were established as follows: CO_2 concentration at $410 \mu\text{mol mol}^{-1}$, photon light intensity at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, flow rate at $700 \mu\text{mol s}^{-1}$, temperature at 28.0°C , and H_2O at $20.93 \text{ mmol mol}^{-1}$. Measurements were conducted between 9 am and 1 pm, using leaves that had been expanded before being placed in the leaf chamber. Measurements were taken only when environmental parameters—including flow rate, fan speed, CO_2 concentration, air humidity, light intensity, and temperature—remained stable. Data corrections were applied to align with the CO_2 concentration and H_2O levels

in the leaf chamber after each measurement for each treatment group. Data capture occurred once fluctuations had stabilized, typically within 30 seconds to 2 minutes after the leaves were placed in the chamber. The photosynthetic characteristics measured included the photosynthetic rate (A), transpiration rate (E), intercellular CO_2 concentration (C_i), and total stomatal conductance to CO_2 (g_{sw}).

2.7. Statistical analysis

This research employed a completely randomized design, with three replications from different blocks of the same plant cultivar. Significant differences among all treatments for each parameter were tested using One-way ANOVA, followed by the Tukey range test, at a 95% confidence interval. Additionally, the correlation among all parameters was analyzed using the Pearson Correlation Test with GraphPad Prism software (version 10.3, 2025).

3. Results

In this study, observations made during the early, middle, and late vegetative phases provided data on changes in plant phenotypes, specifically concerning plant height, the number of leaves, and variations in leaf size.

3.1. Floating raft hydroponic system

The study utilized a floating raft hydroponic system, which included a five-liter container and a rack with 5 cm diameter pots. Figure 1 illustrates the model of the plants grown in this study, which were 21 days old at the time of observation. Each plant cultivar exhibited distinct shoot cover, and the number of tillers varied among them. In this hydroponic model, nutrient replacement was conducted periodically, once a week, to ensure that the plants' optimal nutritional needs were met. The growth of each cultivar in the hydroponic box was observed during three phases: early vegetative (21 days after sowing), mid vegetative (35 days after sowing), and late vegetative (42 days after sowing). Several parameters were measured to compare the changes that occurred, including plant morphological growth, leaf area, and leaf stomatal characteristics observed microscopically.

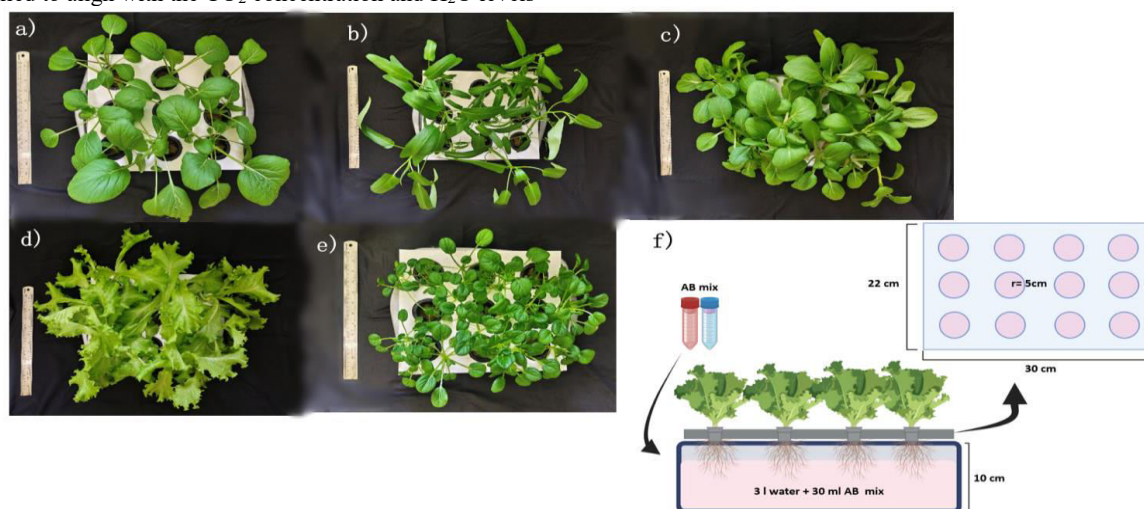


Figure 1. A box of a 21-day-old vegetable planting model using a floating raft hydroponic system with plants a) *Brassica chinensis* var. parachinensis, b) *Ipomoea aquatica* Poir., c) *Brassica rapa* subsp. Chinensis, d) *Lactuca sativa* L. var. crispa, e) *Brassica narinosa* L., and f) rack arrangement and growing media used—bar: 30 cm.

3.2. Plant Morphological Changes

Figure 2a illustrates the changes in plant height. The cultivars BC, IR, LS, and BN exhibited significant differences in height among the three phases. In contrast, cultivars BO and BR maintained similar heights throughout the early, mid, and late vegetative phases. Although there were significant differences in height across phases, the hydroponic vegetable cultivars did not show notable differences in height growth between the early and mid-vegetative phases. Figure 2c depicts the changes in plant height across the three stages: I, II, and III represent early vegetative, mid vegetative, and late vegetative, respectively.

Observations of growth characteristics were made based on changes in the number of leaves at each

developmental phase (see Figure 2b). All cultivars exhibited an increase in leaf count from the early vegetative stages, which is expected as plants grow. The cultivars IR and BR demonstrated a significant increase in leaf numbers across all stage comparisons. In contrast, cultivar LS showed the weakest trend, with no significant difference between the early and middle stages or between the middle and late stages. Notably, cultivar IR had the highest leaf count during the late vegetative stage, averaging around 35 leaves, followed by BR and BN. Additionally, cultivars BC, BO, BR, LS, and BN showed no significant differences between the early vegetative and middle vegetative stages, indicating a slower rate of growth during the early vegetative phase.

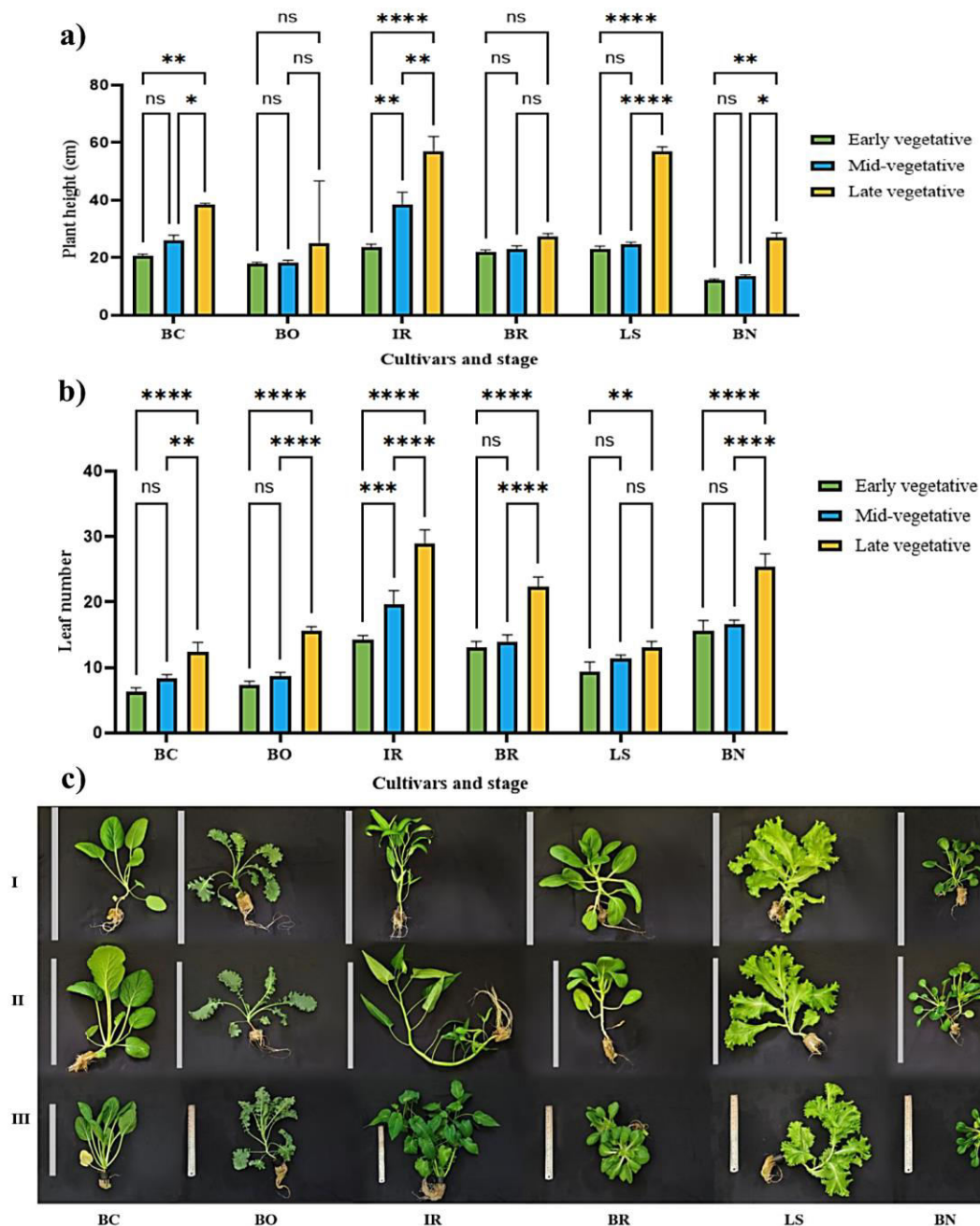


Figure 2. Plant growth at three vegetative phases in parameters a) plant height, b) number of leaves, c) visualization of plant growth with bar = 30 cm in plants: BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L.

3.3. Leaf area and perimeter

During the vegetative phase of growth: early, middle, and late, plants experience significant changes in leaf area. Figure 3a illustrates the leaf morphology of six vegetable crops as they progress through this phase. The data indicate that leaf development, specifically in terms of area and perimeter, is notably influenced by both cultivar and growth phase factors. Cultivar LS exhibited the highest levels of leaf area and perimeter growth, with substantial variations observed across different phases (Figure 3b and 3c). This makes LS particularly suitable for studying changes in leaf biomass. Cultivars BC, BO, and IR also showed considerable vegetative growth, indicating their potential for improving leaf yield traits. In contrast,

cultivar BN demonstrated the smallest rate of leaf expansion. This aligns with its growth characteristics, which prioritize bud formation over increasing leaf area. Statistical analyses (Figure 3b and 3c) confirmed that leaf traits differ significantly across developmental phases for all cultivars, except for BN and, in some cases, BR.

Growth in plant height and leaf number was statistically correlated with each other. However, in some cultivars, no significant changes were observed in leaf area as the growth phase progressed. This lack of change is thought to contribute to variations in the size, distribution, and opening of leaf stomata, particularly during the vegetative phase. To confirm this, the stomatal characteristics were measured.

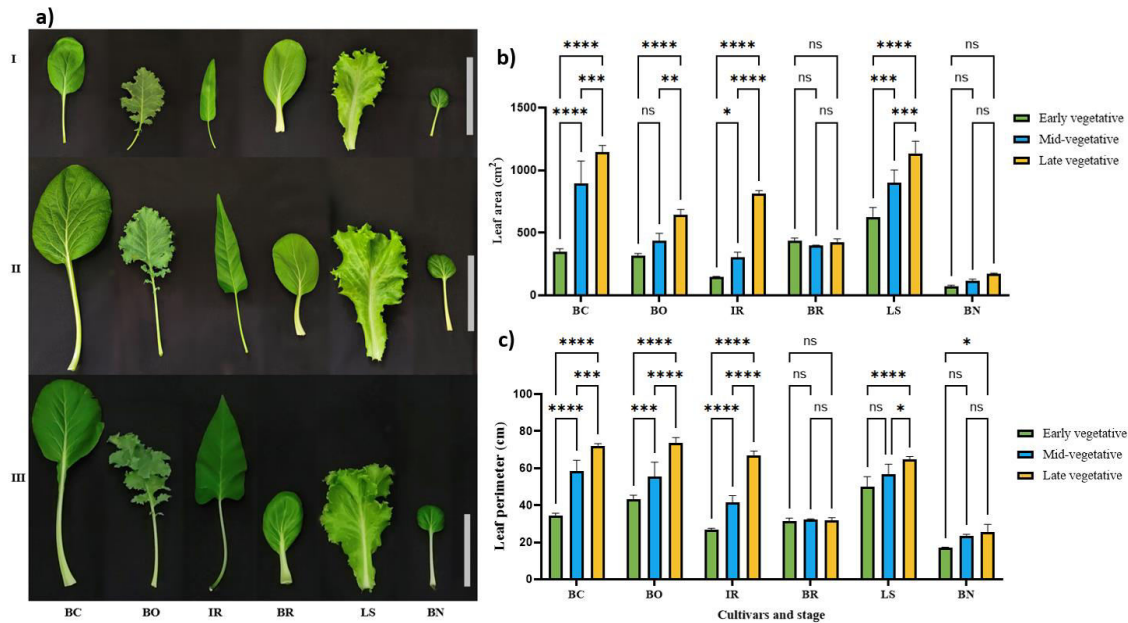


Figure 3. Leaf growth at three vegetative phases (I, II, and III) was observed in parameters a) visualization of leaf changes at three phases with bar = 10 cm, b) leaf area, c) leaf perimeter in BC plants: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. I: early vegetative, II: mid-vegetative, and III: late vegetative.

3.4. Stomatal size and auxiliary cell types

Figure 4 provides an overview of the shape of stomata from six different vegetable species. Each species exhibits distinct stomatal characteristics, which are characterized by the positions of guard cells (which control stomatal opening), subsidiary cells (which assist guard cell movement), chloroplasts, and epidermal cells (the cells surrounding the stomata and subsidiary cells). According to the observations presented in Figure 4, cultivars BC, BR, and BN display anisocytic stomatal characteristics, characterized by unequal cells surrounding the stomata. Cultivar IR has paracytic stomatal characteristics, with

subsidiary cells arranged in parallel, while cultivar LS shows anomocytic stomatal characteristics, featuring irregular subsidiary cell structures. Additionally, cultivar BO also exhibits unique stomatal characteristics, with either diacytic or anisocytic stomatal complexes. Overall, all species share a similar stomatal arrangement but differ in terms of density, complex type, and the size of their subsidiary cells. Understanding these stomatal characteristics is essential for identifying their function and their relationship with physiological responses such as transpiration, gas exchange, and adaptation to stress.

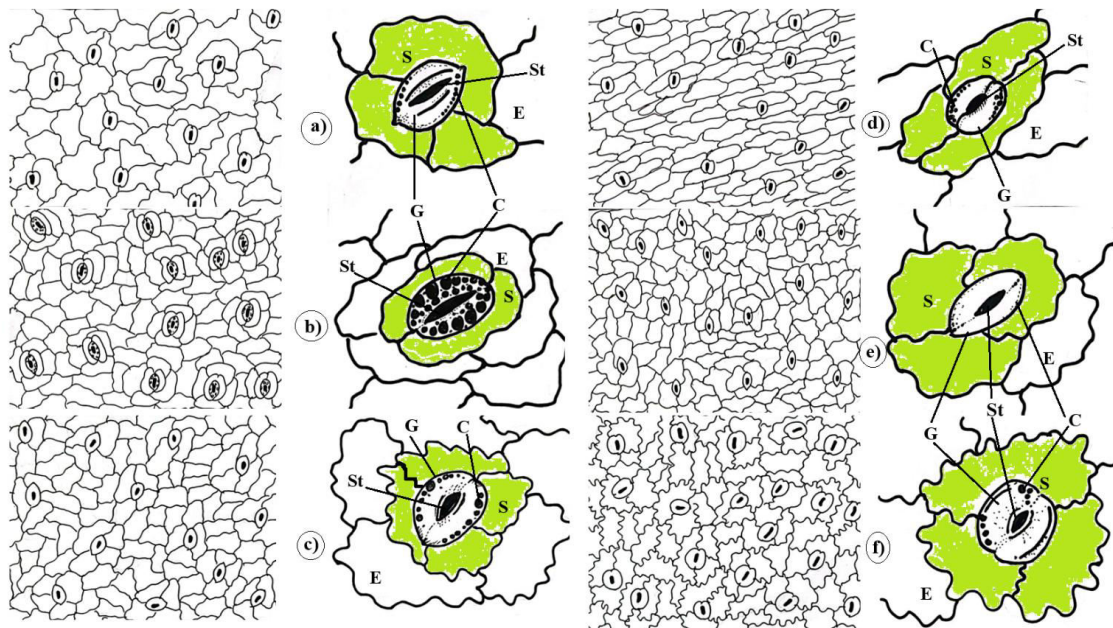


Figure 4. Stomatal characters based on observations on hydroponic media in plants of a) *Brassica chinensis* var. parachinensis (BC), b) *Ipomoea aquatica* Poir (IR), c) *Lactuca sativa* L. var. crispa (LS), d) *Brassica oleracea* var. sabellica (BO), e) *Brassica rapa* subsp. Chinensis (BR), and f) *Brassica narinosa* L. (BN) with the Symbols: C: chloroplast, E: epidermal cell, G: guard cell, S: subsidiary cells, St: stomatal aperture

To differentiate between stomatal characteristics during the vegetative phase of development, we measured stomatal length (Figure 5a), stomatal width (Figure 5b), stomatal aperture width (Figure 5c), and the average number of subsidiary cells (Figure 5d). The results indicated that the cultivars LS and BN exhibited a significant increase in stomatal length during the vegetative phase. Specifically, LS showed a rise from mid to late stage, while BN experienced significant growth from the early/mid stage to the late stage. In contrast, the other cultivars (BC, BO, IR, and BR) did not demonstrate any significant differences across the phases ($p > 0.05$). This suggests that LS and BN are more adaptable in terms of stomatal elongation as development progresses.

It is noteworthy that no significant differences were observed across all cultivars and stages in the width of stomatal pore openings (Figure 5c). This finding suggests

that stomatal openings remain stable during the vegetative and growth phases under optimal environmental conditions. Consequently, variations in age and cultivar only influence other characteristics, not the width of the stomatal pore openings. These results indicate that different cultivars exhibit specific physiological adjustments in stomatal morphology during plant growth, particularly in the vegetative stage.

Regarding subsidiary cells (Figure 5d), uniformity was seen in the BC, BO, BR, and BN cultivars, each with three subsidiary cells surrounding the guard cell. In contrast, the LS cultivar had four subsidiary cells, while the IR cultivar had two subsidiary cells per stoma. Notably, there was no variation in the number of subsidiary cells across all vegetative stages, implying that this trait is likely genetically determined and remains consistent during vegetative development.

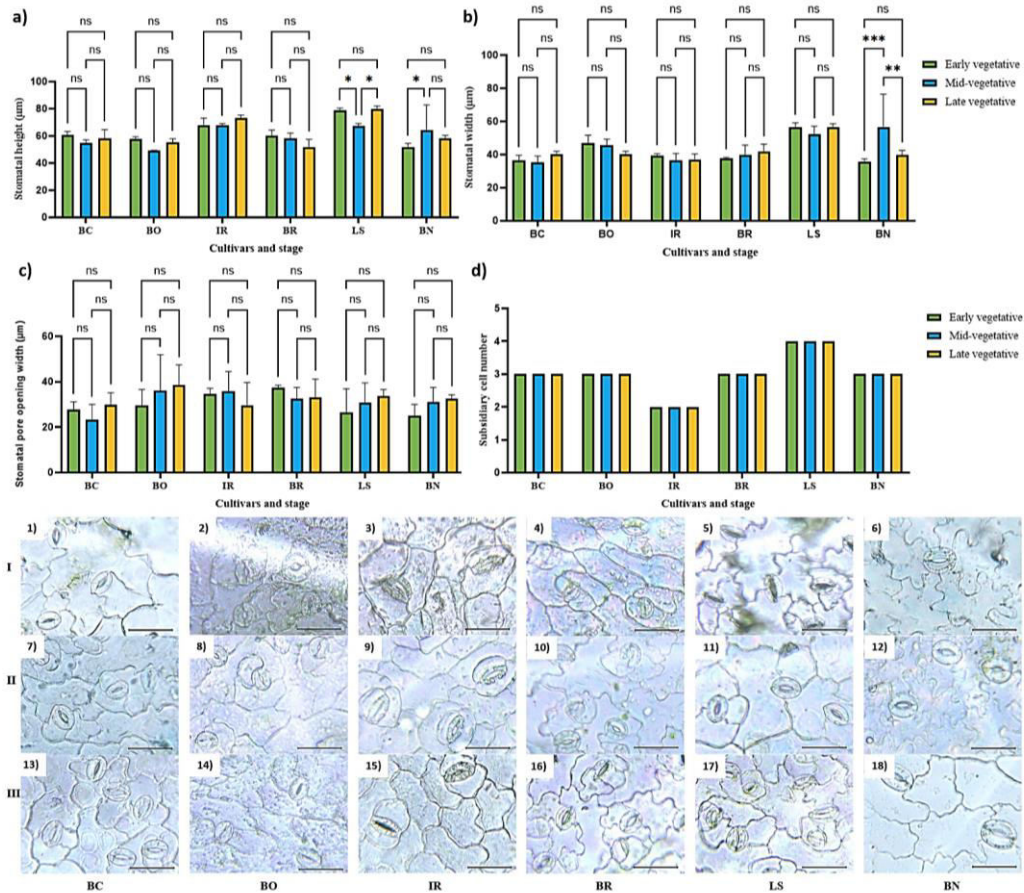


Figure 5. Changes in leaf abaxial stomatal characters with parameters a) stomatal length, b) stomatal width, c) stomatal opening width, and d) number of subsidiary cells per stoma in BC plants: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. in the vegetative phase consisting of I: early vegetative (1-6), II: mid-vegetative (7-12), and III: late vegetative (13-18). Bar: 100 μm. Significance tests were conducted using the Tukey method with a confidence level of 95%.

In this study, we observed notable differences in stomatal density among the various cultivars during the early, middle, and late vegetative phases (see Figure 6). Stomatal density typically indicates how plants respond to their developmental and environmental conditions. According to Figure 6, the BC cultivar showed a slight increase in stomatal density from the early to the mid-vegetative phase; however, there was no significant difference between the mid and late stages. In contrast, the BO, IR, BR, LS, and BN cultivars exhibited a significant increase ($P < 0.05$) in stomatal density from the early to

mid-phases and continued to rise toward the late stage. Notably, the BN cultivar had the highest final stomatal density among all the cultivars studied. Interestingly, the IR cultivar experienced a reduction in stomatal density during the late stage, which may indicate a water-conserving strategy or a shift in growth focus towards leaf expansion as compared to changes in leaf area characteristics. The photosynthetic parameters were then measured to determine the effect of stomatal configuration on physiological performance.

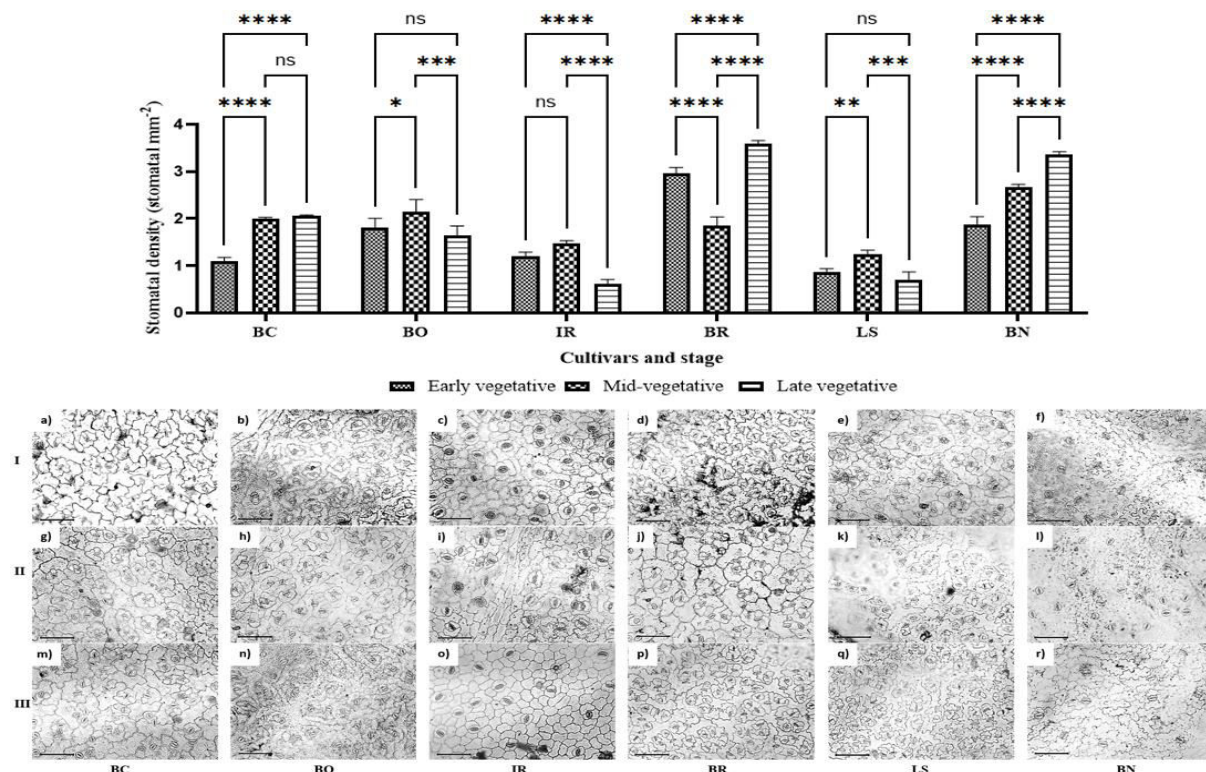


Figure 6. Leaf abaxial stomatal density in BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispata, and BN: *Brassica narinosa* L. in its vegetative phase, consisting of I: early vegetative (a-f), II: mid-vegetative (g-l), and III: late vegetative (m-r). Bar: 100 µm.

3.5. Photosynthetic performance of six different species under hydroponics

Figure 7 provides the physiological responses of plants at three vegetative growth stages. Intercellular CO₂ concentration remained relatively stable across all cultivars and stages (Figure 7a), with no significant differences marked as 'ns'. This suggests that *C_i* is not significantly affected by growth stage or cultivar in this dataset. Figure 7b shows significant variations observed across growth stages in several cultivars. BC and BO showed a sharp decrease in *g_{sw}* (stomatal conductance) from early to late stages (* to ****). LS and BN exhibit higher *g_{sw}* during mid stages with significant reductions in the late stage (****). Cultivar IR and BR showed less variation, primarily indicated by 'ns'. This shows that stomatal regulation is active and variable depending on developmental stage and cultivar. A similar trend to stomatal conductance is shown in Figure 7c in the transpiration rate. BC and BO cultivars had the highest transpiration in the early stage, then declined sharply (****). IR and BR had moderate differences showing a decline at late stages, while LS and BN maintained relatively high transpiration until late stages, where reductions occurred (* or ns). Meanwhile, photosynthetic rate (Figure 7d) shows a significant increase in all cultivars from early to later stages (mostly with *** or ****). The highest photosynthetic rate was observed during the late vegetative stage for most cultivars. This suggests enhanced carbon assimilation capacity in the *g_{sw}* and transpiration rate.

All the parameters tested in this study led to changes in the photosynthetic performance of the plant under hydroponic conditions. To know the correlation between each parameter in six species tested along three different

vegetative stages, the Pearson correlation test is performed, followed by Principal component analysis (Figure 8) to know the clustering pattern of species and stages through the parameters measured. Figure 8a shows that photosynthetic rate correlates strongly with stomatal conductance (*r*~0.72), transpiration rate (*r*~0.68), and leaf number and area (moderately). Stomatal density shows a negative correlation with stomatal size traits, indicating a trade-off between number and size. Plant height correlates positively with leaf area and perimeter, while subsidiary cell number and stomatal number are positively associated, reflecting a shared developmental pathway.

The PCA scores plot (Figure 8b-d) projects individual observations (e.g., cultivar-stage combinations) onto the first two principal components (PC1 and PC2). Samples cluster by cultivar and growth stage, suggesting genotype and stage influence trait variability. For example, the IR and BR-late stage (3) clusters are far left, suggesting distinct morpho-physiological profiles, while the LS and BN groups are toward the center, indicating similar intermediate trait patterns. Figure 8c specifically displays how traits contribute to the first two principal components. PC1 (horizontal axis) is dominated by stomatal density, leaf number, epidermal cells around stomata, and leaf perimeter, while PC2 (vertical axis) is dominated by leaf area, plant height, and stomatal height. Traits further from the origin have a stronger influence on component variance. Figure 8d shows a bar chart of the proportion of variance explained by each principal component. PC1 and PC2 together explain ~52.4% of total variation (PC1 ~35.4%, and PC2 ~17.0%). The cumulative variance reaches ~72% by PC3 and ~83% by PC4, thus 90% by PC 5-6, suggesting up to 6 PCs are needed to explain most variation.

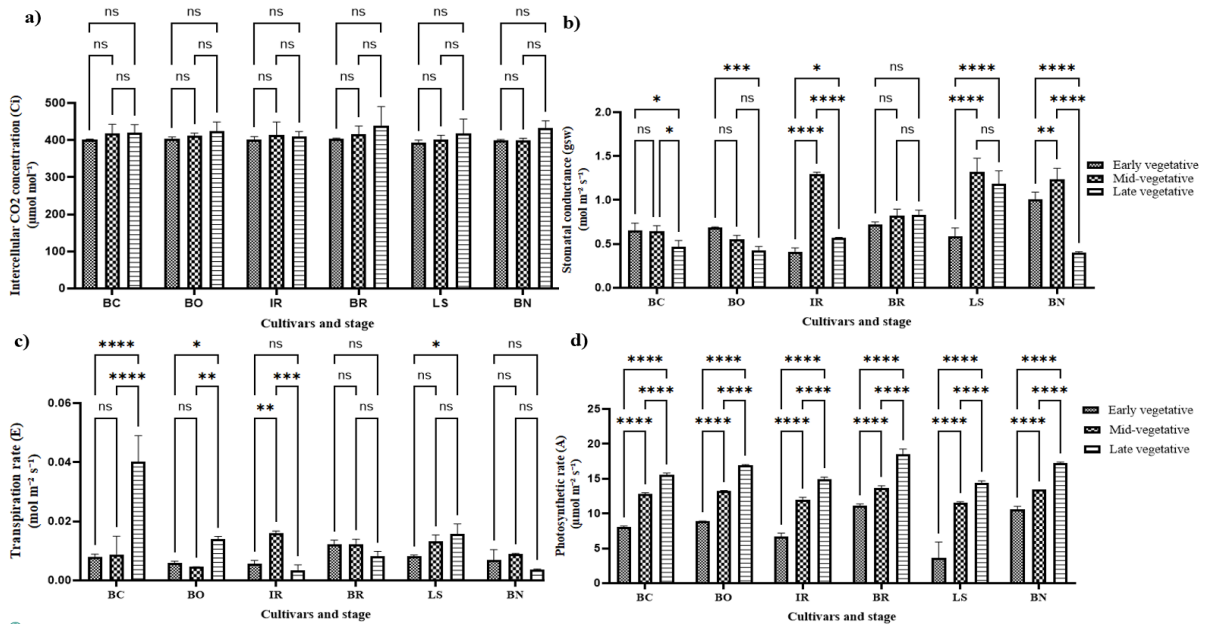


Figure 7. Leaf photosynthetic characteristics shown from a) intercellular CO₂ concentration, b) stomatal conductance, c) transpiration rate, and d) photosynthetic rate in BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. in its vegetative phase.

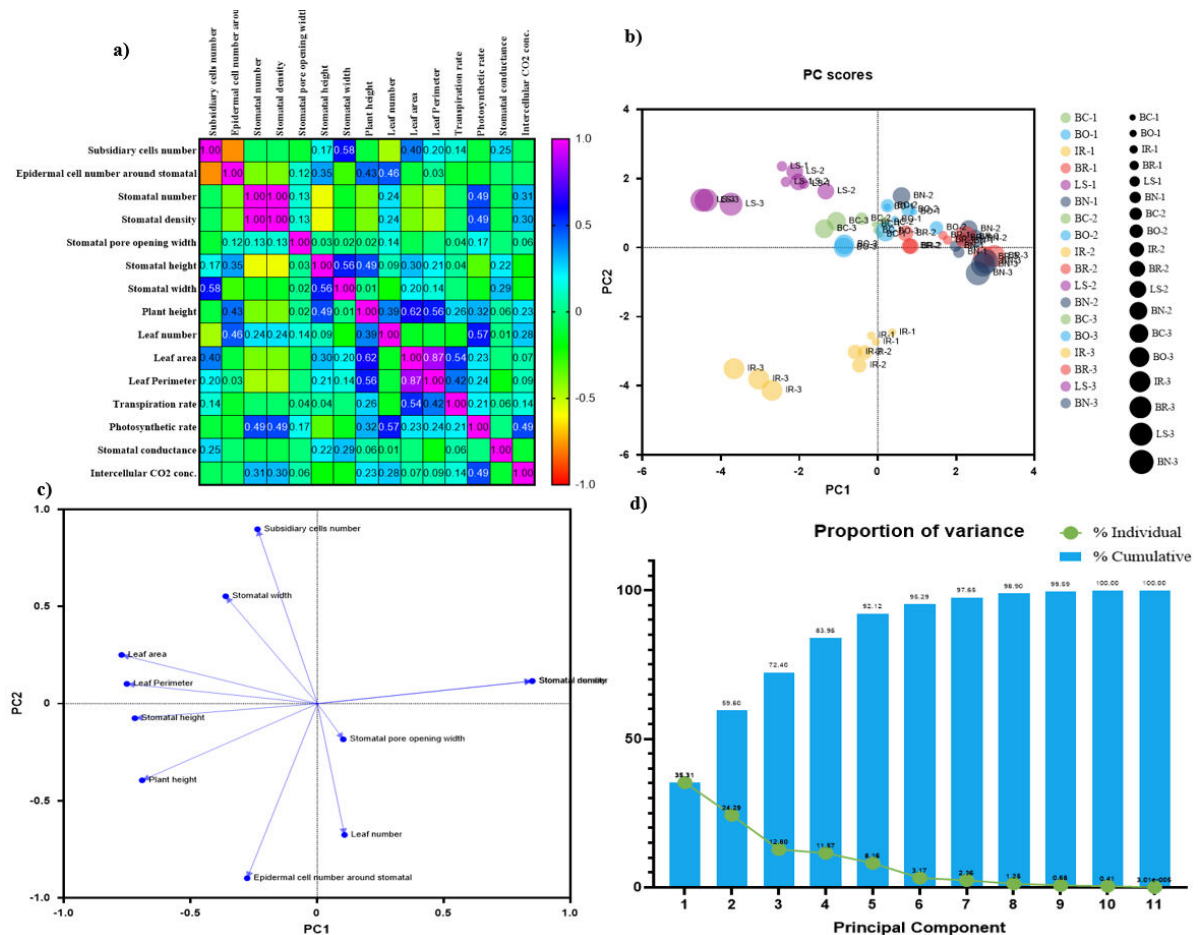


Figure 8. a) Pearson correlation analysis and b) principal component analysis (PCA), c) PCA loading plot and d) Eigenvalues of all leaf parameters tested in this study in BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. in its vegetative phase.

4. Discussion

This study evaluated the changes in morphology—including plant height, leaf number and area, and stomatal traits—length, width, stomatal aperture width, number of subsidiary cells, and stomatal density—among vegetable plant species (BC, BO, IR, BR, LS, and BN) during the early, middle, and late vegetative phases. Stomata are microscopic pores surrounded by guard cells that regulate gas exchange and plant transpiration. (Torii, 2021). Their size and density significantly influence plant water-use efficiency (WUE), photosynthetic capacity, and adaptability to environmental changes (Kitta & Katsoulas, 2020; Qiao *et al.*, 2024).

In this study, optimal environmental conditions were achieved using a floating raft hydroponic system, which ensures that each plant receives the appropriate amount of macro and micro nutrients. Under this condition, to understand the effects of age and planting method on plant growth, we analyzed plant morphology alongside stomatal anatomical characteristics to explain the growth patterns of each species, particularly during the vegetative phase.

Figure 2 and 3 clearly visualize the quantitative data showing that, during the early vegetative stage, all cultivars exhibited shorter plant heights, fewer leaves, and smaller leaf areas compared to the middle and late vegetative stages. This size developed over time, as did the number of leaves of each cultivar. The IR and BR cultivars demonstrated superior vegetative growth across all phases. Notably, in the late vegetative stage, almost all cultivars showed increased plant height, number of leaves, and leaf area. Interestingly, both the BR and BN cultivars maintained a leaf area that did not significantly differ ($p > 0.05$) across the vegetative phases. This observation is closely related to the characteristics of leaf stomata and their distribution on the abaxial surface of the leaves, prompting an analysis of stomatal traits, including size, aperture opening, and stomatal density.

Stomatal size, including both length and width, reflects a plant's physiological adaptations to its environment and growth stages. As a plant advances through its vegetative stages, larger stomata can enhance CO₂ uptake for photosynthesis, but they may also increase water loss (Ouyang *et al.*, 2017). In contrast, smaller stomata or reduced aperture width can limit water loss during later growth stages or under environmental stress (Maricle *et al.*, 2009). Surprisingly, the stomatal size of almost all cultivars in this study undergoes stability without any significant change in size along the vegetative growth phase.

The stomatal characteristics observed among the six plant species are theoretically distinct, but are primarily governed by the genetic expression inherent to each cultivar (Akbar & Begum, 2020; Chen *et al.*, 2020). For example, in the previous research, *B. rapa* subsp. *chinensis* demonstrates a stomatal density that varies across different accessions (Akbar & Begum, 2020). Furthermore, it is noteworthy that *B. oleracea* possesses a stomatal pattern of the cruciferous type, which consists of two guard cells and two unequal subsidiary cells. The density of these stomata can differ based on genotype and environmental adaptability. In addition, cultivars of the *Ipomoea* species exhibit an amphistomatic arrangement, featuring stomata

on both the upper and lower surfaces of the leaves, alongside paracytic complexes. In contrast, BN typically showcases cruciferous-type stomata (Akbar & Begum, 2020; Ye *et al.*, 2024).

According to Figure 5b, BN showed a significant decrease in stomatal width from the early to the late stage and also from the mid to the late stage. The other cultivars did not exhibit any significant changes ($p > 0.05$). This may indicate that BN reduces its stomatal aperture size during development, possibly as a water-saving adaptation. Generally, stomatal size—particularly height and width—reflects a plant's physiological adaptations to developmental and environmental conditions.

In this study, the LS and BN cultivars displayed a significant increase in stomatal length during the late vegetative stage. This suggests a developmental adjustment that could potentially enhance photosynthetic capacity as leaf area increases. However, the BN cultivar also demonstrated a notable reduction in stomatal width during this period, which can be interpreted as a compensatory mechanism to restrict excessive transpiration, despite the increase in stomatal height. These findings align with previous studies (Chua & Lau, 2024; Han *et al.*, 2021), indicating that stomatal plasticity—both in size and aperture—is a critical characteristic for development during the vegetative phase and for acclimatization to stress (Salsinha *et al.*, 2021).

Stomatal aperture opening is an important indicator of stomatal conductance. Interestingly, this study found no significant variation in stomatal aperture width across all stages and cultivars, suggesting that pore aperture may be tightly regulated and less responsive to developmental stages alone (Demicheli *et al.*, 2023; Han *et al.*, 2021). This stability reflects the plant's efforts to balance gas exchange and water conservation during vegetative growth. When examining the characteristics of subsidiary cells, it is clear that these cells play a crucial role in the rapid movement of guard cells, enabling more efficient opening and closing of stomata in response to changes in the external environment (Araújo *et al.*, 2011; Daszkowska-Golec & Szarejko, 2013). Data in this study indicate that the number of subsidiary cells remains constant across different cultivars throughout the vegetative development phase. This observation supports the notion that the formation of subsidiary cells is likely genetically determined, as indicated by previous studies (Chen *et al.*, 2020) on stomatal development.

In cultivars such as IR and BR, genetic factors play a significant role in determining stomatal characteristics, leading to variations in vegetative growth. Cultivars with optimized stomatal traits show greater adaptability and better biomass performance. During the vegetative phase, especially from the mid to late stages, plants adjust their stomatal development to meet transpiration demands as well as growth requirements, such as height increase and leaf expansion (Chatterjee *et al.*, 2016; Fayed *et al.*, 2020). Generally, a period of vigorous growth is accompanied by an increase in stomatal density (Chatterjee *et al.*, 2016; Verma *et al.*, 2020). However, this increase does not affect the width of stomatal openings or the number of subsidiary cells surrounding the likely genetically regulated stomata.

Stomatal conductance and transpiration rates are positively correlated as the stomatal pores widen, leading to increased water loss through transpiration. Although

there is a decline in both g_{sw} and transpiration rates in later stages, photosynthetic rates exhibit an increase (Figure 7). This suggests an enhancement in mesophyll efficiency, a better utilization of intercellular CO_2 , and potentially improved chloroplast activity or enzyme regulation during the late vegetative growth phase (He *et al.*, 2015; Parvin *et al.*, 2017). In this study, the stable intercellular CO_2 levels across different stages imply that variations in assimilation and gas exchange are primarily attributed to stomatal and biochemical adjustments rather than fluctuations in C_i .

The C_i remains stable regardless of the growth stage or cultivar, indicating that internal CO_2 availability is not a limiting factor during vegetative growth. This is in line with findings (Pan *et al.*, 2023; Yang & Li, 2016) where C_i remained relatively constant under non-stress conditions. Opposite to the C_i patterns, the decline in g_{sw} at later stages may reflect stomatal closure or reduce stomatal aperture to conserve water due to the changes in endogenous hormonal signaling (Lucas *et al.*, 2013). Another study found that the strong correlation of g_{sw} was found with transpiration rate at later stages, suggesting an adaptive response to limit water loss, possibly due to changes in stomatal behaviour or leaf surface characteristics, supporting the idea of a functional linkage between transpiration and stomatal regulation (Merewitz *et al.*, 2011; Verma *et al.*, 2020).

Despite the decline in g_{sw} and transpiration, the photosynthetic performance observed in this study across six cultivars is enhanced. This indicates that while intercellular CO_2 concentrations remain stable, stomatal conductance and transpiration rates decline from early to late vegetative stages in most cultivars. In contrast, photosynthetic rates significantly increase. This data suggests an improvement in photosynthetic efficiency during the later stages, along with cultivar-dependent variations in stomatal regulation. This performance shows an increased mesophyll conductance or biochemical efficiency. This implies greater internal CO_2 use efficiency, which is consistent with the findings (Han *et al.*, 2021; He *et al.*, 2015) that suggest increased photosynthetic capacity at advanced growth stages.

The data on Pearson correlation and PCA reveal strong interrelationships between physiological traits (photosynthesis, g_{sw} , and transpiration) and anatomical traits (stomatal density, size, and leaf traits). PCA separates cultivar-stage combinations based on these traits, showing how growth stages and genotypes influence functional and structural parameters. The first two components are mainly driven by stomatal density and leaf dimensions (PC1) and plant height and leaf area (PC2).

5. Conclusion

This study shows the main contribution in providing stomatal plasticity data among six species tested under hydroponic conditions, giving a new insight into plant responses, especially in leaf photosynthetic matters, to the total growth under hydroponic conditions. This study shows consistency in stomatal aperture size, and the number of subsidiary cells highlights the developmental stability of these traits. Overall, the integrated plasticity of stomatal traits shows how vegetable plants optimize gas exchange and photosynthetic performance throughout the vegetative growth phase in hydroponic conditions. These

findings help identify key characteristics for distinguishing plant performance, such as a photosynthetic indicator of better plant growth under hydroponic conditions, which is potentially valuable for breeding or stress resilience studies. This study only highlights plant growth in the vegetative stage, so further analysis on the generative stage should be done.

6. Author contributions

YCFS, idea and conceptualization, observation and analysis, manuscript writing; DYWL, data analysis, visualization, manuscript writing; DSR, research design, observation and measurement, statistical analysis; SP, literature review, microscopic measurement; SS, manuscript writing and evaluation; KT, observation and manuscript writing; NK, data analysis, manuscript writing and literature review.

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ITS-Based technique characterization of Goosefeet weed (*Chenopodium* spp) Across Different Regions in Al-Anbar Province, Iraq

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Abstract

The biodiversity of the Goosefoot weed plant (*Chenopodium* spp) was investigated across thirteen ecologically diverse regions in western Iraq during the growing season of 2024. These regions cover diverse environments, such as agricultural lands, riverbanks, and arid zones. The Internal Transcribed Spacer (ITS) marker was employed in this study to investigate the characterization of this plant at the molecular level. The Internal Transcribed Spacer Ribosomal DNA (ITS rDNA) region sequencing and phylogenetic relationships revealed three major species, namely *Chenopodium murale*, *Chenopodium hybridum*, and *Chenopodium album*. Phylogenetic analysis divided the collected samples from the thirteen regions of Al Anbar governorate into two major clusters. The first cluster consolidated the arid regions, while the second cluster consolidated the agricultural and riverbank lands. Species such as *Chenopodium murale* reflected halophytic adaptation as it grouped in the arid zones such as Hit and Qa'im, while *Chenopodium album* was dominant in the agricultural zones. Meanwhile, *Chenopodium hybridum* reflected high ecological plasticity in diverse environments. The genetic diversity was consistent with the effect of the environments on the structure of the goosefoot weed population, where minimum divergence was observed in the river-connected areas in comparison with isolated arid regions. The heterogeneity of goosefoot populations was shaped by the divergence of environments, which in turn suggests that the characterised species could act as reservoirs of stress-tolerant traits. In addition, findings also highlight the importance of ITS barcoding in the study of plant diversity. Further investigations regarding integrating geographical, climatic and phenological studies are required to better elucidate the biodiversity of this weed.

Keywords: Goosefoot, ITS barcoding, Biodiversity, phylogenetic analysis, *Chenopodium* spp.

1. Introduction

Fundamentally, biodiversity is the most crucial pillar in protecting ecosystems. It contributes to maintaining stability and health, and a high level of biodiversity leads to high resilience in ecosystems against climate change. Biodiversity must also preserve natural resources, which are essential not only for the ecosystem but also to maintain a high level of sustainability. Although weed plants are considered a nuisance source for farmers and growers, they are important to nature and biodiversity. In addition, weed plants are beneficial in protecting the soil from erosion and can be used in grazing (Mousa et al., 2024). However, some weeds can be used in human nutrition. Weed plants contain various active compounds that might be utilized in pharmaceutical industries; this is further illustrated by studies on native Jordanian species such as *Chiliadenus montanus*, which exhibits notably high levels of Terpinen-4-ol and potent antibacterial activity, highlighting the pharmacological promise of wild flora in arid environments (Reham, 2024). Additionally,

most weed plants are ancestors of domesticated plants. Therefore, maintaining them is essential as a gene bank for the domesticated plants. Hence, understanding their biodiversity leads to a better understanding of the growth and development of their counterparts, domesticated plants, which aligns with broader conservation strategies, such as cryopreservation, which have proven effective for preserving rare plant species and their genetic material (Saifan et al., 2024). Among these species is goosefoot weed (*Chenopodium* spp.). Based on the Angiosperm Phylogeny Group-APG-III, it belongs to the Amaranthaceae family within the Chenopodioideae subfamily (Bremer et al., 2009). This family is one of the most widespread families around the globe (Raven & Wu, 2003) and contains numerous flowering plant species, some of which are perennial and others are annual. Chenopodioideae subfamily contains around 100 genera and 1700 species (Fuentes-Bazan et al., 2012) growing in temperate and tropical regions worldwide. Also, many species belonging to this subfamily are widespread in a wide range of environments, including arid and semi-arid areas, and most of them are halophytes and can grow in

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desert regions (Fuentes-Bazan et al., 2012). Based on the aforementioned variability of the Amaranthaceae family and subfamily (Chenopodioideae), the taxonomy of species in a particular area is fundamental, primarily if DNA-based techniques are used. The use of molecular methods to evaluate the genetic diversity of crop wild relatives and landraces has revolutionized plant taxonomy, resulting in many updates to angiosperm orders and families (Al Khateeb et al., 2024; Al-Kiyyam et al., 2024; Chase et al., 2016; Müller & Borsch, 2005). The diversity of nucleotides in the organism's genome can be exploited in the characterisation and taxonomy of organisms, including plants, and this is so-called DNA barcoding. DNA barcodes are beneficial as they can employ DNA fragments, besides their availability in all organisms' cells during all growth stages (Hebert et al., 2003). These barcodes are located in the conserved regions of the genome in the cells of the organisms. Therefore, they actively explore the diversity and distinguish between progenies (Wattoo et al., 2016). Many molecular methods exist to investigate the DNA barcodes, including the Internal Transcribed Spacer (ITS) and Ribulose Biphosphate carboxylase large chain/maturase K (rbcL/matK), etc. The Internal Transcribed Spacer (ITS) marker was chosen for this study because it is a widely used and highly reliable molecular tool for plant species identification and phylogenetic analysis. ITS regions, located within the ribosomal RNA gene cluster, are highly conserved yet exhibit sufficient variability among species, making them ideal for distinguishing closely related plant species, such as those within the Chenopodiastrium genus.

Additionally, ITS barcoding is effective in assessing genetic diversity, particularly in multi-part environments like western Iraq (Al Anbar province), where the ecological and environmental factors might affect the genetic diversity of plant populations (Chen et al., 2010; Zhu et al., 2022). Based on the information mentioned above, the current study highlighted the understanding of the biodiversity of goosefoot weed in different regions of the Al Anbar province, which is located in western Iraq, by employing the ITS method and studying the phylogenetic relationships between the various areas. The outcome of the study can help the policy makers in preserving the biodiversity and agricultural development in the specified region.

2. Materials and Methods

2.1. Study Area and Plant Samples:

The study included 13 regions in Al Anbar governorate, as shown in Table 1 and the weather data during the period of the study, as shown in Table 2. These regions belong to various ecological zones, including deserts, riverbanks, and agricultural lands. Several field trips were done during 2024 from January to April to collect plant samples at the flowering stage. Samples of goosefoot weed were collected from each region by taking a small part of its leaves and placing them directly into a tube full of TRIzol, then they were immediately placed in the fridge ($4 \pm 1^\circ\text{C}$) before further molecular analysis.

Table 1. The Geographical Coordinates, Symbols and Names of the 13 Regions of Al Anbar Governorate

No	Location		Climatic Region	Symbol	English Name	Arabic Name
	Latitude	Longitude				
1	33°23'55" N	43°16'39" E	Semi-Arid	T1	Ramadi-Tash	الرمادي - الطاش
2	33°26'56" N	43°17'57" E	Semi-Arid	T2	Ramadi-Al-Jazeera	الرمادي-الجزيرة
3	34°22'26" N	41°59'11" E	Semi-Arid	T3	Anah	عنه
4	34°28'49" N	41°54'52" E	Semi-Arid	T4	Rawa	راوة
5	33°38'42" N	42°49'31" E	Semi-Arid	T5	Hit	هيت
6	33°29'14" N	43°22'39" E	Semi-Arid	T6	Ramadi-Albu Aath	الرمادي-البوعيث
7	33°27'26" N	43°23'43" E	Semi-Arid	T7	Ramadi-Al-Hamdhiyah	الرمادي-الحمضية
8	33°27'03" N	43°17'00" E	Semi-Arid	T8	Ramadi-Albu Faraj	الرمادي-البوفراج
9	34°07'51" N	42°22'26" E	Semi-Arid	T9	Haditha	حديثة
10	33°02'16" N	40°17'00" E	Desert	T10	Rutba	الرطبة
11	34°19'23" N	41°09'37" E	Desert	T11	Qa'im	القائم
12	33°20'50" N	43°46'52" E	Semi-Arid	T12	Al Fallujah	الفلوجة
13	33°25'03" N	43°27'16" E	Semi-Arid	T13	Khalidiya	الخالدية

Table 2. Data on Weather during the period of study (Nov 2023 to May 2024) showing the monthly mean for each parameter in Al-Anbar Governorate, Iraq

Parameter	2023			2024			
	November	December	January	February	March	April	May
Temperature	17.4	11.97	10.34	10.36	14.04	22.35	25.87
Relative Humidity(%)	49.73	62.02	63.55	62.22	53.01	41.61	34.57
Precipitation (mm day ⁻¹)	0.52	1.22	0.22	1.09	1.05	0.46	0.28
Wind speed (m sec ⁻¹)	2.42	2.36	2.42	2.58	2.88	2.53	3.12
Soil Moisture in the root zone (%)	0.42	0.45	0.46	0.47	0.48	0.46	0.43

Ref: <https://power.larc.nasa.gov/data-access-viewer/>

2.2. Molecular study

2.2.1. DNA Extraction

The preserved samples from goosefoot were homogenized in liquid nitrogen using a mortar and pestle. Total genomic DNA was extracted with CTAB reagents following the manufacturer's protocol. Finally, the quality was assessed through 1.5% agarose gel electrophoresis in 1x TEB buffer and stained with ethidium bromide dye.

2.2.2. Identification of ITS using PCR

Primers used for amplification of the internal transcribed spacer (ITS) were provided by Macro Gene (South Korea), including: ITS1F: 5'-TCCGTAGGTGAACCTGCGG-3' and IT4R: 5'-TCCTCCGCTTATTGATATGC-3' (Wang et al., 2015).

The reaction mixture consisted of 12.5 µl ddH₂O, 2.5 µl Thermopol (10X), 2.5 µl MgCl₂ (25 mM), 5 µl dNTPs (10 mM), 0.5 µl each primer (10 µM), 0.5 µl Taq polymerase (5 U/µL), and 1 µL of DNA sample. The mixture was then well vortexed and subjected to the following thermal cycles: Initial denaturation at 95 °C for 5 min, followed by 30 cycles involving denaturation at 95 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. A final extension of 1 cycle was carried out at 72 °C for 7 min, after which the reaction was held at 4 °C.

The PCR product was visualized by 1.5% agarose gel (Figure 1). The product was then cleaned to remove the proteins, primers-dimers and dNTPs. DNA (ITS1 and ITS4) extracted from the gel was sequenced to determine the phylogenetic relationship between the plant samples according to Macrogen Company, Korea, using a Genetic

Analyzer. DNA sequences were then subjected to the Multivariate Statistical Package (MVSP v.3.22) to construct the phylogenetic tree.

3. Results

Goosefoot (*Chenopodium* spp) from thirteen locations in Al Anbar governorate was subjected to molecular analysis. Figure 1 shows that all amplified bands located between 600 and 700 bp validated the quality and integrity of DNA extracted from goosefoot weed plants collected from the above-mentioned regions. Therefore, the gel electrophoresis analysis confirms the successful ITS region amplification, and this will provide the fundamental data for the subsequent analysis, e.g. sequencing, and phylogenetic relationship.

Table 3 showed that 10 out of 13 samples of goosefoot weed plants were registered in the NCBI database. The analysis also revealed that there was considerable biodiversity among them. ITS regions of rDNA Sequences identified three species, including *Chenopodium album*, *Chenopodium murale* and *C. hybridum* (Table 3). Table 3 results indicated a high identity percentage (97-100%) associated with a low expected value (E value), and this confirms the close genetic relationship to the reference sequence. It also appeared that *Chenopodium murale* dominated regions such as Ramadi-Tash, Hit, and Qa'im, while *Chenopodium hybridum* spread in regions like Anah and Haditha. However, Ramadi-Al-Hamdhiah and Rutba were dominated by *Chenopodium album*. This suggests the adaptability to various ecological zones.

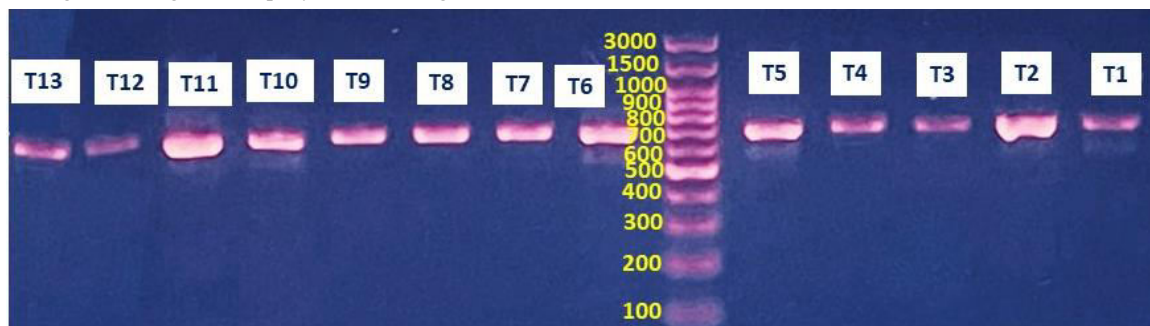


Figure 1. Amplification of ITS region of rDNA (Agarose gel 1.5%) Extracted from Goosefoot weed Collected from 13 Regions of Al Anbar Governorate. (T1-Ramadi-Tash, T2-Ramadi-Al-Jazeera, T3-Anah, T4- Rawa, T5-Hit, T6-Ramadi-Albu Aath, T7- Ramadi-Al-Hamidiyah, T8-Ramadi-Albu Faraj, T9-Haditha, T10- Rutba, T11- Qa'im, T12- Al Fallujah, T13- Khalidiya).

Table 3. Accession number of 10 out of 13 plant samples collected from 10 regions of Al Anbar Governorate.

Locations	Accession No.	Identified Species	Identity(%)	Query Coverage (%)	E-value
Ramadi-Tash	PV418216.1	<i>Chenopodium murale</i>	99.4	100	0
Anah	PV418217.1	<i>Chenopodium hybridum</i>	98.9	98	3×10 ⁻¹²⁰
Rawa	PV418218.1	<i>Chenopodium album</i>	97.8	99	1×10 ⁻⁹⁵
Hit	PV418219.1	<i>Chenopodium murale</i>	99.1	100	0
Ramadi-Al-Hamdhiah	PV418220.1	<i>Chenopodium album</i>	98.5	97	2×10 ⁻¹¹⁰
Haditha	PV418221.1	<i>Chenopodium hybridum</i>	99	99	0
Rutba	PV418222.1	<i>Chenopodium album</i>	98.2	96	5×10 ⁻¹⁰⁰
Qa'im	PV418223.1	<i>Chenopodium murale</i>	99.3	100	0
Al Fallujah	PV418224.1	<i>Chenopodium album</i>	98.7	98	1×10 ⁻¹⁰⁵
Khalidiya	PV418225.1	<i>Chenopodium hybridum</i>	99.2	100	0

The phylogenetic analysis indicated that plant samples from different regions were ordered into two clusters. The top cluster consisted of T7, T12, T4, T3, T1, T11, T2, and T9, indicating that the goosefoot population in those regions is genetically similar because of the gene flow since they are located close to the Euphrates riverbank. The second cluster included T10, T8, T6, T5, and T13. Goosefoot weed plants from Ramadi-Al-Hamidhiyah and Fallujah scored the minimal distance (close to 0.00), indicating the same ITS sequences. Goosefoot collected from Hit and Rutba were grouped in one cluster, which is located in the desert region, and this reflects the unique genetic background.

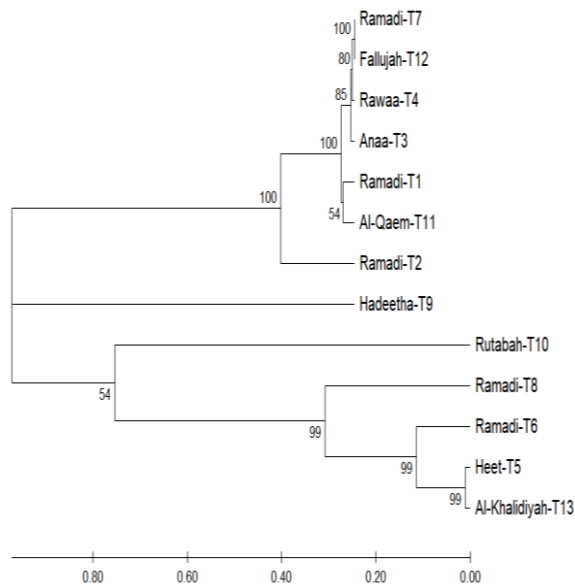


Figure 2. Phylogenetic relationship of ITS sequences among goosefoot weed from 13 regions of Al Anbar Governorate using UPGMA Method (MEGA v. 3.22)

Table 4. Matrix of Distances among ITS region sequences collected from the 13 regions of Al Anbar Governorate using MEGA 12 (v. 3.22)

	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Ramadi-Tash-T1												
Ramadi-Al-Jazeera-T2	0.31											
Anah-T3	0.06	0.31										
Rawa-T4	0.06	0.31	0.02									
Hit-T5	1.70	1.70	1.70	1.70								
Ramadi-Albu Aath-T6	1.70	1.70	1.70	1.70	0.23							
Ramadi-Al-Hamdhiyah-T7	0.06	0.31	0.02	0.01	1.70	1.70						
Ramadi-Albu Faraj-T8	1.70	1.70	1.70	1.70	0.61	0.61	1.70					
Haditha-T9	1.45	1.45	1.45	1.45	1.70	1.70	1.45	1.70				
Rutba-T10	1.70	1.70	1.70	1.70	1.51	1.51	1.70	1.51	1.70			
Qa'im-T11	0.05	0.31	0.06	0.06	1.70	1.70	0.06	1.70	1.45	1.70		
Al Fallujah-T12	0.06	0.31	0.02	0.01	1.70	1.70	0.00	1.70	1.45	1.7	0.06	
Khalidiya-T13	1.70	1.70	1.70	1.70	0.02	0.23	1.70	0.61	1.70	1.51	1.7	1.7

4. Discussion

Goosefoot weed (*Chenopodium* spp) is a widely spread weed plant, especially in Al Anbar governorate, which is characterized by various ecosystems ranging from arid and semi-arid areas to riverbanks (Alfalahi & Saleh, 2018; Dawood et al., 2020). Plant biodiversity plays a

crucial role in sustaining ecosystem functions, particularly in arid and semi-arid environments. Goosefoot weed species (*Chenopodium* spp.) contribute significantly to biodiversity maintenance in Al Anbar governorate by stabilizing soil and preventing erosion in desert regions. These species, belonging to the Chenopodiaceae family, are well-adapted to arid conditions and are among the most abundant plant families in desert ecosystems, highlighting

their importance in maintaining species diversity and ecosystem stability (Sun et al., 2023). In addition, they provide suitable habitats for the microorganisms and insects. Therefore, the availability of those plants improves the ecosystem's resistance to climate change. *Chenopodium murale* can easily grow in soil affected by salinity. Hence, this makes it vital to exploit degrading soils. Besides, some species of goosefoot are considered wild ancestors of the domesticated plants such as spinach and quinoa (Areces-Berazain, 2022; Ihuoma, 2021). This could make the Al Anbar regions as a gene bank for breeding important crops, particularly for traits such as drought and salt tolerance (Sharef et al., 2024; Alfalahi et al., 2022). Such genetic reservoirs are increasingly critical, especially when combined with conservation biotechnologies like cryopreservation to prevent biodiversity loss (Saifan et al., 2024). The current investigations on goosefoot weed (*Chenopodium spp*) from 13 regions of Al Anbar governorate revealed important biodiversity, which was shaped by the ecology's heterogeneity (Salih et al., 2023). ITS-based molecular analysis uncovered mainly three goosefoot species, including *Chenopodium murale*, *Chenopodium hybridum*, and *Chenopodium album*, which originated in distinct regions of Al Anbar. The use of ITS-based molecular markers in this study aligns with previous investigations that utilized DNA markers to reveal eco-geographical genetic diversity in wild plant species *Gundelia tournifortii* in Jordan (Saifan et al., 2020). Furthermore, phylogenetic relationships and genetic distance highlight the effect of environmental elements on the genetic backgrounds. This, in turn, gives an insight into the evolution and adaptation in the diverse regions of Al Anbar. The species *Chenopodium murale* was dominant in the arid regions such as Hit and Qa'im. The survival of this species in such areas provides it with halophytic traits (Flowers & Colmer, 2008). On the contrary, *Chenopodium album* prevailed in agricultural lands such as Ramadi-Al-Hamidiyah, indicating that this species adapted to grow in such soils, while *Chenopodium hybridum* occupied regions such as Anah and Haditha, which reflects a wide range of ecological plasticity. The habit specialization within the subfamily Chenopodiaceae was consistent with the global pattern observed in the family of Amaranthaceae (Bremer et al., 2009). On the other hand, phylogenetic analysis indicated two main clusters, including the riverbank and agricultural lands group and the arid and semiarid group. Also, the minimal genetic distance within the first cluster between samples collected from Fallujah and Ramadi-Al-Hamidhiyah suggests that gene flow was facilitated by the Euphrates River, which connects the two regions, and this maintains the genetic homogeneity in the agricultural lands. In contrast, the second group showed higher divergence between Rutba and Ramadi-Tash (1.70), indicating the isolation of environments between those two regions. It also showed high adaptability to the local habitat.

It is worth mentioning that the lower genetic distance (0.02) between Hit and Khalidiya, despite the geographical variation, might reflect convergent evolution due to the similar desert environment or anthropogenic activities. The genetic diversity of the goosefoot population in arid regions such as Rutba highlights the role of these plants as

a gene bank of adaptive traits, which is necessary for tolerance against climate change (Bhargava et al., 2006). While the homogeneity in agricultural lands increases the risk of genetic erosion, this urges policymakers to preserve ecosystems by promoting sustainable agriculture, which will ultimately protect biodiversity. ITS-based DNA barcoding has proven to be an effective method to highlight the genetic relationships among species belonging to the same genus in various geographical regions (Chen et al., 2010). Alternatively, the 97.8% identity found in Rawa may be a result of intraspecific variation or frequent interbreeding, which is common in species such as *Chenopodium* (Mandák et al., 2018). The biodiversity observed in the current investigations highlights the importance of maintaining those species as a gene bank for domesticated plants, particularly in regions affected by climate change.

5. Conclusions

The results of the present study underscore the influence of environmental heterogeneity in Al Anbar on the genetic diversification of goosefoot weed (*Chenopodium spp.*), suggesting that such ecological variability may drive adaptive evolutionary processes across arid and agricultural habitats. In addition, findings provide the foundation for preserving genetic resources, and this is vital for sustaining biodiversity in the study area, which is characterized by insubstantial ecosystems. It is recommended to incorporate more advanced DNA barcoding approaches, such as *matK* or genome-wide SNP analysis, to further resolve biodiversity patterns (Ancona et al. 2023). Further investigations integrating geographical, climatic, and phenological data are essential to comprehensively elucidate the biodiversity and ecological adaptation of this weed.

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Bacteriome Diversity Inside the Cemetery Soils: A Metagenomic Approach

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Abstract

Cemetery soil represents a distinctive environment characterised by elevated microbial degradation activity associated with decomposing organic matter, which significantly influences the bacterial community (bacteriome). Despite their significance for numerous purposes, data on the bacteriome of cemetery soil in Indonesia are scarce. This research sought to explore the bacteriome of cemetery soil in Surakarta, Indonesia, obtained from three different sampling locations at depths of 20 cm (Sample A) and 140 cm (Sample B). Soil samples were subjected to chemical characterisation, after which total DNA was extracted and sequenced via next-generation sequencing on the Illumina HiSeq 2500 PE250 platform. The results revealed that Sample B (140 cm depth) exhibited a higher number of operational taxonomic units (OTUs) and greater diversity compared to Sample A (20 cm depth). Proteobacteria was the dominant bacterial phylum at 20 cm depth, whereas Actinobacteriota predominated at 140 cm depth. Within the Proteobacteria phylum, the genera *Pseudomonas*, *Sphingomonas*, and *Microvirga* were consistently present across all samples. The phylum Actinobacteriota consistently showed the presence of the genera *Gaiella*, *Pseudoarthrobacter*, *Streptomyces*, and *Micromonospora*. According to Principal Component Analysis (PCA), the composition of bacterial communities in cemetery soil was found to be affected by the depth at which sampling took place. Biomarker analysis discovered *Actinomadura* sp. and *Nocardioidea* sp. to be the predominant discriminating genera at depths of 20 cm and 140 cm, respectively. Research in this area provides valuable insights into the diversity of the bacteriome in cemetery soil across both shallow and deeper levels.

Keywords: Endophytic bacteria, Enterobacter, Bacillus, 16S rRNA gene, IAA

1. Introduction

Burial sites in cemeteries are designated areas for the interment of human corpses. In Indonesia, bodies are usually buried at depths ranging from 1.5 to 2 metres. Following a burial, decomposition starts, transforming cemeteries into highly active sites for organic decomposition (Keenan et al., 2018). The decomposition process in cemeteries involves a diverse array of organisms including bacteria, fungi, and insects, primarily those from the order Diptera (Goff, 2010; Horenstein et al., 2012). Factors influencing decomposition include external elements like temperature, oxygen levels, and humidity, as well as internal factors such as soil acidity, the time and cause of death, and the presence of diseases within the deceased body (Ioan et al., 2017; Javan et al., 2019).

In cemetery soil, the primary decomposers are lipolytic and proteolytic bacteria. About 20% of human remains are composed of proteins, approximately 10% are lipids, around 5% are minerals, 4% are water, and roughly 1% are carbohydrates (Janaway et al., 2009; Dent et al., 2004; Ioan et al., 2017). The decomposition process yields a range of organic compounds, such as amino acids, fatty

acids, ammonia, ammonium, methane, mercaptans, sulphates, nitrates, and calcium. The decomposition process also enhances electrical conductivity (Fineza et al., 2014; Taylor et al., 2023) and leads to the emission of heavy metals (Egbimhanlu et al., 2020; Franco et al., 2022). Research using culture-dependent methods on cemetery soil in Surakarta has isolated proteolytic and lipolytic bacteria, with a proteolytic index (IP) of 4.2 (Salsabilla et al., 2024) and a lipolytic index (IL) of 5.43 (Rini et al., 2023).

Temporal fluctuations in the bacterial community of cemetery soil occur during corpse decomposition, with such changes being influenced by the unique decomposition environment (Garcia et al., 2020; Singh et al., 2018; Keenan et al., 2018). In the initial phases of decomposition, bacterial families like Actinomycetaceae, Aerococcaceae, Bifidobacteriaceae, Campylobacteriaceae, Corynebacteriaceae, Lactobacillaceae, Micrococcaceae, and Staphylococcaceae are commonly found, whereas families such as Alcaligenaceae, Planococcaceae, and Pseudomonadaceae prevail in later stages (Garcia et al., 2020).

Extensive research on bacterial communities associated with corpse decomposition has been conducted globally,

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primarily for forensic purposes (Wei et al., 2022; Garcia et al., 2020; Javan et al., 2019; Singh et al., 2018), alongside investigations into the risks of pathogenic contamination (Finley et al., 2015; Abia et al., 2019). In contrast, such studies are notably scarce in Indonesia. Existing research in this domain has primarily employed culture-dependent approaches, isolating bacteria from various soil depths and identifying potential proteolytic (Całkosiński et al., 2015; Salsabilla et al., 2024) and lipolytic (Rini et al., 2023; Mandiri et al., 2023) bacterial species. Proteolytic bacteria produce proteases, which play significant roles in industries such as food processing, waste management, detergents, biofilm degradation, leather processing, silver recovery, dairy, baking, beverages, and pharmaceuticals (Saputri et al., 2023; Matter et al., 2023). Similarly, lipases produced by lipolytic bacteria have applications in treating oily wastewater, oil degradation, leather processing, detergent production, biodegradation of oil and organic waste, food processing, oil manufacturing, biodiesel production, and the medical industry (Solanki et al., 2021; Ali et al., 2023; Chandra et al., 2020).

Bacteriome metagenomic data from the cemetery soils were described by Yao et al. (2021). This study is necessary to obtain the cemetery soil bacteriome data that

can be utilised in multiple biotechnological sectors. This study sought to examine a bacterial community (bacteriome) isolated from cemetery soil in Surakarta, Central Java, Indonesia.

2. Materials and methods

2.1. Sample collection and DNA extraction

The selected sites for soil sample collection in Surakarta, Central Java, Indonesia, were two cemeteries: Pracimaloyo (P) and Bonoloyo (B) (See Figure 1). Soil was excavated to a depth of 20 cm at each site for Group A and 140 cm for Group B. Six samples were collected in total: two from the P site at depths of 20 cm and 140 cm, labelled as P12A and P12B, and four from the B site, also at depths of 20 cm and 140 cm, labelled as B18A, B23A, B18B, and B23B. Figure 1 illustrates the sampling locations. Comprehensive chemical and physical analysis was conducted on the collected soil samples (Dauletkul et al., 2025), and the total genetic material was isolated from the samples using the ZymoBIOMICS DNA Miniprep Kit, following the manufacturer's protocol (Rakhmawati et al., 2024).

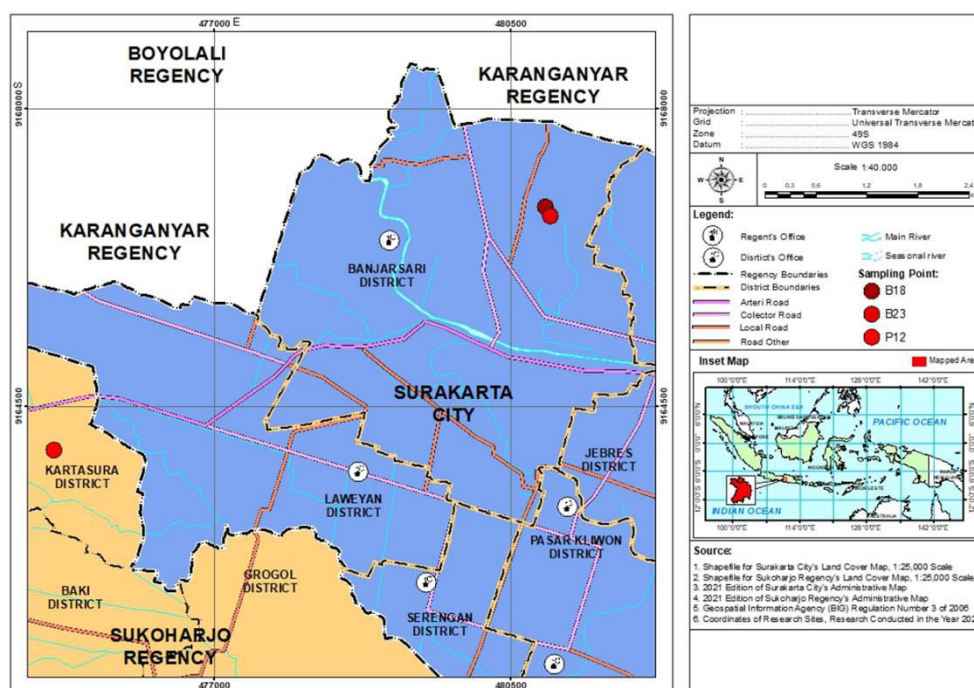


Figure 1. Sampling sites of cemetery soils in Surakarta City, Indonesia

2.2. Library Construction, Quality Control and Sequencing

PCR amplification was conducted on the target regions, V3-V4 regions, using the following primer sequences 341F 5'-CCTAYGGGRBGCASCAG-3' and 806R 5'-GGACTACNNGGTATCTAAT-3' (Asriatno et al., 2023; Rahayu et al., 2024; Rakhmawati et al., 2024). The correct size of the PCR products was identified through agarose gel electrophoresis using a 2% gel. PCR products from each sample were then combined, end-paired, A-tailed, and further linked with Illumina adapters in equal amounts. The resultant libraries were sequenced using a paired-end Illumina system to produce 250bp

paired-end unprocessed reads. The library's quality was verified using Qubit and real-time PCR quantification, along with bioanalyzer analysis to determine its size distribution. Quantified libraries will be grouped together and sequenced on the Illumina platform, with the sequencing adjusted in accordance with the effective library concentration and the desired amount of data.

2.3. Sequencing Data Processing

Paired-end reads were selected based on their distinct barcodes, and subsequent trimming involved cutting off the barcode and primer sequences, after which they were merged using FLASH (V1.2.7) (Rahayu et al., 2024), a tool capable of rapid and accurate analysis, intended for

merging paired-end reads with overlapping sequences from the opposing end of the same DNA fragment, which were then referred to as raw tags. Raw tags underwent quality control under specific filtering conditions to produce high-quality clean tags, as described in the QIIME (V1.2.7) (Bokulich et al., 2013) quality control process (Magoč and Salzberg, 2011). The tags were compared with the reference database via the UCHIME algorithm (Caporaso et al., 2010).

2.4. OTU Cluster and Taxonomic Annotation

Sequence analysis was carried out utilizing Uparse software, which processed all relevant tags. Sequences with a similarity of 97% or higher were grouped into the same operational taxonomic unit (OTU). Sequences corresponding to each operational taxonomic unit were chosen for further annotation. The QIIME platform, which utilises the Mothur method, was employed to annotate each chosen sequence against the SSUrRNA database from SILVA138, with species annotation undertaken at each taxonomic rank, using a threshold of 0.8–1. The phylogenetic relationships among all OTU representative sequences were determined using MUSCLE (Version 3.8.31), a program renowned for its high accuracy and processing speed. The OTU abundance data were normalised relative to the sample with the fewest sequences. The subsequent analyses of alpha and beta diversity were conducted using the normalized dataset.

2.5. Alpha Diversity

The complexity of biodiversity in a sample was analyzed using 6 indices, specifically Observed-species, Chao1, Shannon, Simpson, ACE, and Good-coverage, as per the methods outlined by Thakur et al. (2022). All of these indices in our samples were calculated using QIIME (Version 1.7.0) and visualised with R software (Version 2.15.3).

2.6. Beta Diversity

An assessment of beta diversity was conducted to evaluate the differences in species diversity among samples. The QIIME software, version 1.7.0, calculated beta diversity using both weighted and unweighted unifracs methods. Prior to cluster analysis, principal component analysis (PCA) was conducted using the FactoMineR and ggplot2 packages in R software (version 2.15.3) to reduce the dimensionality of the original variables. For obtaining principal coordinates and simplifying visualisation of intricate, multi-dimensional data, Principal Coordinate Analysis (PCA) was utilised. A previously collected set of samples was transformed, with a distance matrix of weighted or unweighted UniFrac being converted into a new set of orthogonal axes, the first principal coordinate of which represents the greatest variation, the second the next highest, and so on. Permutational multivariate analysis of variance (PcoA) analysis was depicted with the aid of the WGCNA package, statistical software, and the ggplot2 package in R software, version 2.15.3. The Unweighted Pair-group Method with Arithmetic Means (UPGMA) hierarchical clustering, using average linkage, was

employed with the QIIME software (Version 1.7.0) to analyze the distance matrix.

2.7. Biomarker Analysis

Linear Discriminant Analysis Effect Size (LEfSe) is a statistical approach used for biomarker analysis, combining non-parametric tests with linear discriminant analysis (LDA). Firstly, the Kruskal-Wallis test is used to identify significant differences among microbial taxa, and subsequently pairwise Wilcoxon tests are employed to verify consistency across subclasses. Ultimately, LDA calculates the effect size of each taxon, identifying those with the greatest discriminatory power as biomarkers (Marcos-Zambrano et al., 2021). Taxa with LDA scores exceeding a specified threshold (usually above 2.0) and having statistically significant p-values are considered reliable indicators that can differentiate between sample groups.

3. Results

3.1. Taxonomic Composition Analysis.

The programme QIIME classified all sequences from phylum to genus using its default settings. The sequences were categorised into 30-39 phyla, 67-87 classes, 143-190 orders, 197-395 families, and 270-516 genera. The different samples shared similar bacterial compositions but had different phylum distributions, as indicated in Table 1.

Table 1. Number of identified taxa inside the cemetery soils

Taxon	P12A	P12B	B18A	B18B	B23A	B23B
Phylum	30	37	33	39	38	34
Classis	67	80	71	87	79	84
Order	143	183	164	180	190	187
Familia	197	395	248	271	292	296
Genus	270	429	397	397	477	516

The phyla in sample A, which measures 20 cm, are ranked by their relative abundance as follows: Proteobacteria at 8.9%, Actinobacteriota at 6.5% and Firmicutes at 5.8%. In contrast to sample A, Actinobacteriota is the most prevalent phylum in sample B, making up 10.2% of the total, whereas Proteobacteria account for 6.6% and Firmicutes 5% (Figure 2). The phylum Crenarchaeota was found in almost all samples, accounting for between 1-7% of the total relative abundance. In the two samples, *Bacillus* (a type of Firmicutes phylum) dominated, with a range of OTU numbers extending from 4631 to 14392. The dominant genera isolated from sample B18B were *Marmoricola* and *Nocardioides* in the phylum Actinobacteriota, corresponding to OTU numbers 13206 and 12789, respectively. Species like *Gaiella*, *Pseudoarthrobacter*, *Streptomyces*, and *Micromonospora* were found in nearly all samples within the same phylum. Proteobacteria phylum genera including *Pseudomonas*, *Sphingomonas*, and *Microvirga* were predominantly found in all the samples. The predominant phylum Proteobacteria genera in the B18A sample were *Wolbachia* and *Buchnera*, comprising OTUs 7371 and 6658, as shown in Figure 3.

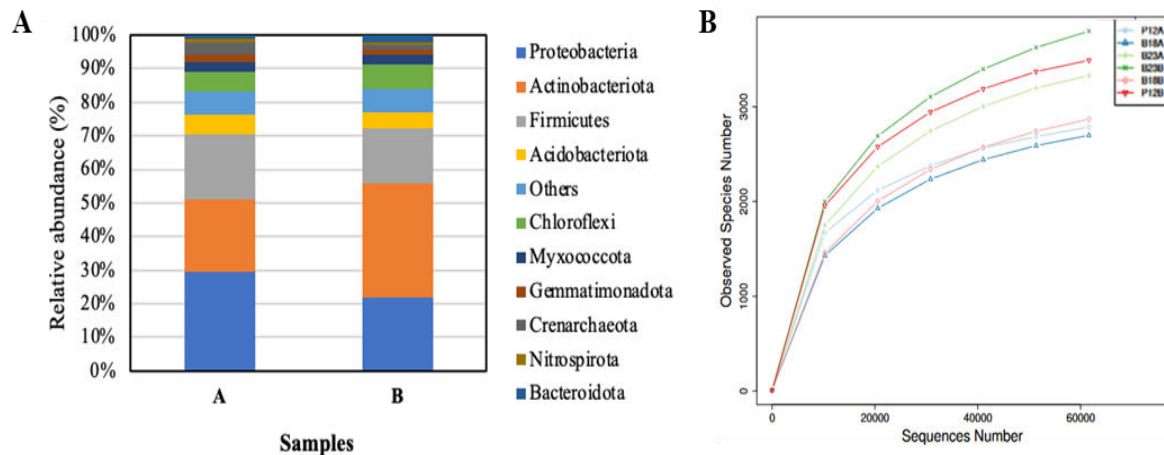


Figure 2. Relative abundance and the number of observed species. **A.** The relative abundance of soil cemetery bacterial community within phyla. **B** samples were soil from 140 cm-depth, while **A** samples were 20 cm-depth. **B.** Rarecurve of observed species number in six sampling sites.

3.2. Alpha Diversity Analysis

The average numbers for effective tags, taxon tags, and observed OTUs from the six samples were 109,942, 95,613, and 3,456, respectively, as per Table 2. In all 6 samples, the rarefaction curves typically trended towards the saturation plateau, as shown in Figure 2B. The

Figure 3. Heatmap of relative abundance of cemetery soil bacterial community within genera. Red color indicates higher relative abundance compared to white and blue. The numbers designate the number of OUT

rarefaction curves demonstrated the variability in the OTU abundance across multiple samples. Notably, the OTU numbers for samples P12B, B18B, and B23B at a depth of 140 cm (3788, 3285, 4162) exceed those for samples P12A, B18A, and B23A at a depth of 20 cm (2785, 2992, 3724) (Table 2).

Tax_detail	P12A	P12B	B18A	B18B	B23A	B23B
p_Firmicutes;c_Bacilli;g_Bacillus;	4631	4911	9577	14392	11338	6670
p_Actinobacteriota;c_Actinobacteria;g_Marmoricola;	148	308	132	13206	150	280
p_Actinobacteriota;c_Actinobacteria;g_Nocardioideis;	250	1162	344	12789	452	980
p_Proteobacteria;c_Alphaproteobacteria;g_Wolbachia;	0	504	7371	0	597	813
p_Proteobacteria;c_Gammaproteobacteria;g_Buchnera;	0	2528	6658	0	1	6
p_Proteobacteria;c_Gammaproteobacteria;g_Pseudomonas;	3	503	1442	4769	1201	1830
p_Crenarchaeota;c_Nitrososphaeria;g_Nitrososphaeraeae;	1157	556	81	1045	4709	1346
p_Proteobacteria;c_Gammaproteobacteria;g_Escherichia-Shigella;	20	184	2605	8	115	217
p_Actinobacteriota;c_Actinobacteria;g_Pseudarthrobacter;	72	2233	242	1243	771	2454
p_Actinobacteriota;c_Thermoleophilia;g_Gaiella;	1383	1734	2136	2366	1635	1616
p_Firmicutes;c_Bacilli;g_Paenibacillus;	263	163	2332	689	405	417
p_Actinobacteriota;c_Actinobacteria;g_Agromyces;	40	407	179	2040	199	297
p_Firmicutes;c_Bacilli;g_Lactobacillus;	0	1243	1963	393	1466	1711
p_Proteobacteria;c_Alphaproteobacteria;g_Asticcacaulis;	1	11	1900	0	4	17
p_Firmicutes;c_Clostridia;g_Romboutsia;	23	1018	1647	47	1150	1387
p_Proteobacteria;c_Gammaproteobacteria;g_Achromobacter;	0	0	1615	1	10	3
p_Proteobacteria;c_Alphaproteobacteria;g_Sphingomonas;	325	795	578	1174	587	1608
p_Proteobacteria;c_Alphaproteobacteria;g_Microvirga;	311	1061	354	379	784	1582
p_Firmicutes;c_Desulfotomaculia;g_Desulfurispora;	2	0	0	1535	0	0
p_Proteobacteria;c_Gammaproteobacteria;g_Pseudoxanthomonas	2	42	1530	0	81	85
p_Proteobacteria;c_Alphaproteobacteria;g_Devosia;	16	107	572	10	750	1452
p_Verrucomicrobiota;c_Verrucomicrobiae;g_Candidatus;	84	113	402	1388	141	102
p_Actinobacteriota;c_Actinobacteria;g_Intrasporangium;	65	395	138	1339	132	403
p_Proteobacteria;c_Alphaproteobacteria;g_Pedomicrobium;	585	251	153	59	1327	623
p_Actinobacteriota;c_Thermoleophilia;g_Solirubrobacter;	198	1204	398	982	414	733
p_Firmicutes;c_Clostridia;g_Sedimentibacter;	0	1160	1	5	0	2
k_Archaea;p_Crenarchaeota;g_Candidatus_Nitrososphaera;	454	255	7	128	1143	532
p_Nitrospirota;c_Nitrospira;g_Nitrospira;	1134	539	254	269	726	886
p_Firmicutes;c_Bacilli;g_Ammoniphilus;	255	104	297	1059	210	170
p_Proteobacteria;c_Alphaproteobacteria;g_Allorhizobium;	29	327	258	8	837	1005
p_Acidobacteriota;c_Vicinamibacteria;g_Vicinamibacteraceae;	952	693	232	946	998	733
p_Actinobacteriota;c_Actinobacteria;g_Microlunatus;	94	548	118	0	568	889

p_Actinobacteriota;c_Actinobacteria;g_Streptomyces;
 p_Myxococcota;c_Myxococcia;g_Anaeromyxobacter;
 p_Actinobacteriota;c_Rubrobacteria;g_Rubrobacter;
 p_Proteobacteria;c_Alphaproteobacteria;g_Pelagibacterium;
 p_Proteobacteria;c_Alphaproteobacteria;g_Dongia;
 p_Firmicutes;c_Bacilli;g_Domibacillus;
 p_Actinobacteriota;c_Actinobacteria;g_Micromonospora;
 p_Actinobacteriota;c_Actinobacteria;g_Sinomonas;
 p_Proteobacteria;c_Gammaproteobacteria;g_Steroidobacter;
 p_Proteobacteria;c_Gammaproteobacteria;g_Enterobacter;

841	327	142	863	689	740
325	161	118	824	115	194
89	247	103	83	312	799
0	0	0	0	429	796
793	433	189	68	417	634
155	461	206	131	784	591
569	678	325	757	485	772
27	142	31	707	42	54
212	330	124	13	549	701
0	698	69	171	26	76

Data on alpha diversity indicate that the soil in cemeteries has a high level of bacterial diversity. The Shannon diversity index (H') for all the samples is greater than 8 ($H' > 8$), indicating a high level of diversity. The Simpson dominance index indicates a correlation with H' values for sample A at a low diversity level (Table 2).

Table 2. OTU numbers and alpha diversity of cemetery soil bacterial community

Samples	Effective Tags	Taxon tags	Observed OTUs	Alpha diversity		
				Shannon	Simpson	Chao1
P12A	77,857	61,592	2,785	9.38	0.10	3.13
P12B	95,022	80,044	3,788	9.53	0.10	3.85
B18A	97,677	88,278	2,992	8.13	0.98	2.95
B18B	164,563	150,070	3,285	8.27	0.99	3.25
B23A	105,118	90,139	3,724	8.98	0.99	3.62
B23B	119,412	103,555	4,162	9.64	0.10	4.48
Average	109,942	95,613	3,456			

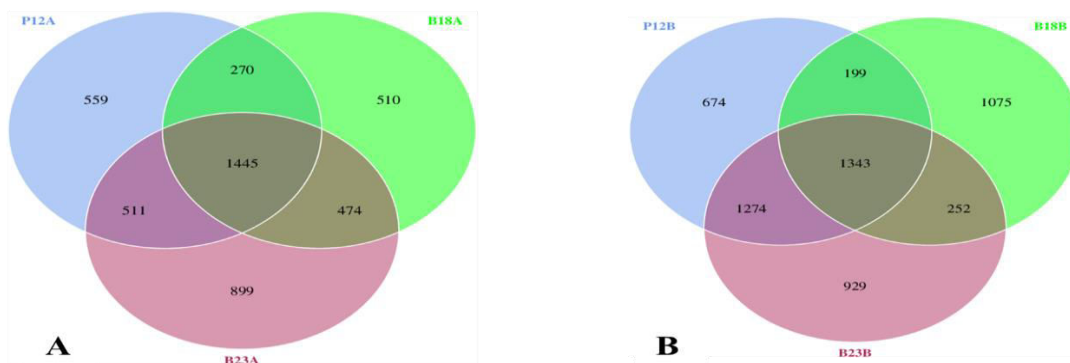


Figure 4. Venn diagram of cemetery soil bacterial community collected from 20 cm-depth (A) and 140 cm-depth (B)

Furthermore, 911 OTU are identified as the core genera for all samples, primarily consisting of *Marmoricola*, *Bacillus*, *Pseudoarthrobacter* and *Escherichia coli* were present with relative abundances of 4.55% and 4.0%, respectively, while *Pseudoarthrobacter* and *Escherichia coli* also had relative abundances of 3.6% and 3.0% respectively. Eleven phyla exhibited relative abundances exceeding 0.2% (Figure 5). Within the Phylum Actinobacteriota, 15 core genera were identified as having relative abundances greater than 0.3%. The genus *Candidatus_Nitrososphaera* within the phylum

Crenarchaeota in Kingdom Archaea had a relative abundance of 1.0% (Figure 3).

3.3. Beta Diversity Analysis

The bacterial community composition of cemetery soil was found to be affected by sampling depth, as shown by PCA. Figure 6A shows that bacterial communities collected at a depth of 140 cm (Group B) are distinct from those collected at a depth of 20 cm, with the exception of samples B18B and P12A. This finding is supported by the UPGMA cluster tree, which was constructed using Weighted UniFrac distance, as shown in Figure 6B.

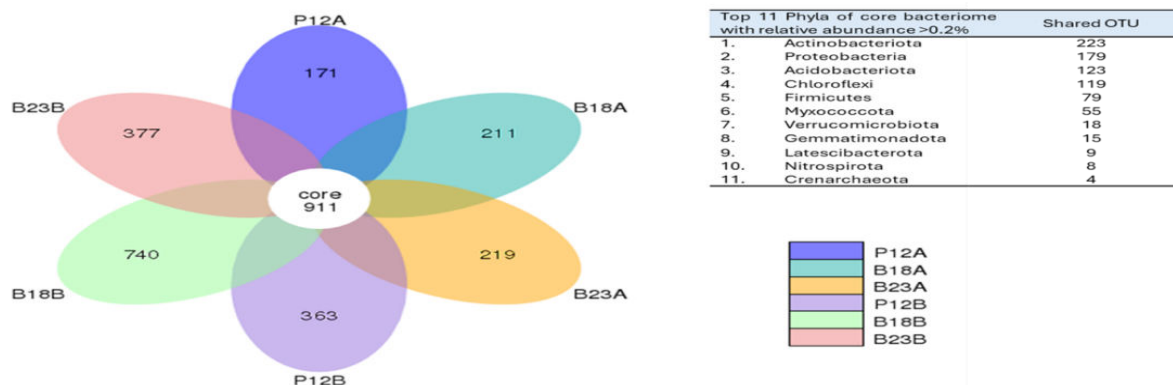


Figure 5. Distribution of genera as the main bacterial community of cemetery soil

3.4. Identification Based on the 16S rRNA Gene

The identification of two potential isolates, T5 and S6, was based on the 16S rRNA gene following extraction and amplification of their DNA genome, which comprises 1500 base pairs of the 16S rRNA gene. The findings

indicated that T5 displayed the highest similarity to the genus *Streptomyces*, with a 99.86% identity percentage, comparable to *Streptomyces sp.* VEL17. In contrast, the S6 strain showed an 83.50% identity with *Streptomyces sp.* strain ADE 004.

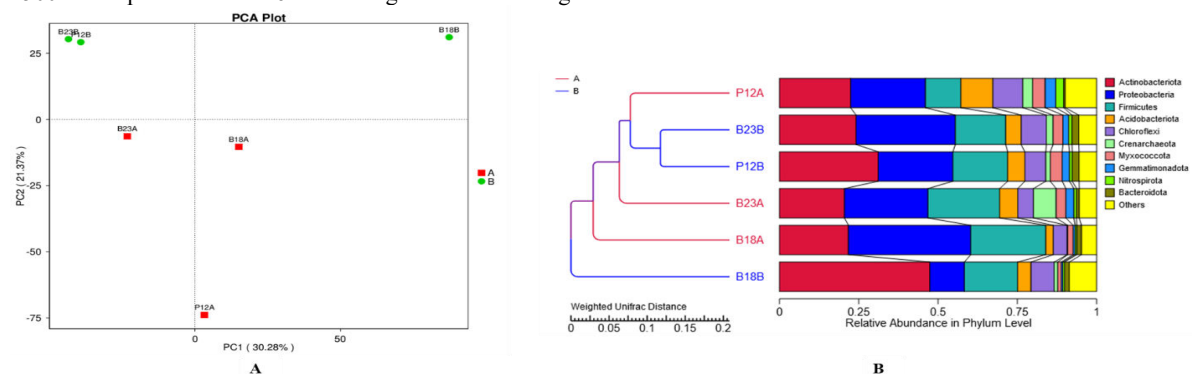


Figure 6. Principal component analysis (PCA) describes the different bacterial community in the cemetery soils in deep and shallow sites (A). UPGMA cluster tree based on Weighted Unifrac distance showed the genetic distance among samples (B).

3.5. Biomarker Analysis

The LEfSe analysis identified 26 bacterial genera as biomarkers at soil depths of 20 cm and 140 cm, as shown in Figure 7. The genera *Actinomadura sp.* and *Lysinibacillus sp.* were more abundant at 20 cm depth, whereas the other 24 genera were more prevalent at 140 cm. *Actinomadura sp.* and *Nocardioides sp.* were notably the organisms that yielded the highest Linear Discriminant Analysis (LDA) scores, 2.97 and 4.61 respectively. These two genera were predominant at different depths: *Actinomadura sp.* at 20 cm and *Nocardioides sp.* at 140 cm.

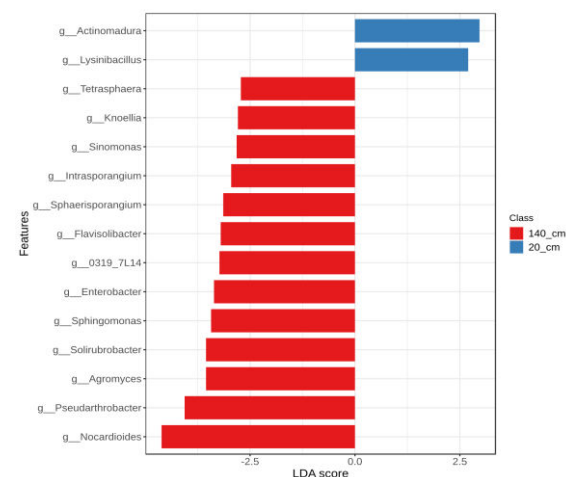


Figure 7. LEfSe analysis results of soil samples at 20-cm and 140-cm depths

4. Discussion

Samples taken from a depth of 140 cm showed greater quantities of operational taxonomic units and higher Shannon Index values than those from a depth of 20 cm, as evident in Tables 1 and 2. Previous research by Garcia et al. (2020), Keenan et al. (2018), and Singh et al. (2018) supported these findings. In Indonesia, bodies are buried at a depth of about 140 cm (Edgar et al., 2011). The bacterial abundance at this depth can be attributed to the availability

of organic substances produced from active corpse decomposition (Fineza et al., 2014; Taylor et al., 2023). Fluorescence diacetate (FDA) hydrolysis and dehydrogenase activity also demonstrated active decomposition in cemetery soil (Lumombo, 2017). Evidence has shown that these organic substrates facilitate bacterial metabolism (Emmert et al., 2021; Aya et al., 2022; Baskaran et al., 2020; Huang et al., 2020; He et al., 2020). It was also discovered that electrical conductivity significantly impacts the growth of bacteria that are positively correlated, a connection backed by the higher electrical conductivity values documented for samples collected from 140 cm depth (Table 3), according to Song et al. (2022)..

The phylum Proteobacteria predominated at 20 cm depth, whereas Actinobacteriota was dominant at 140 cm depth (Figure 2). The prevalence of Actinobacteriota at burial sites has similarly been reported in prior studies (Abia et al., 2019; Singh et al., 2018). Unique OTUs were more abundant at 140 cm depth than at 20 cm depth (Figure 4), likely due to limited oxygen availability at the greater depth despite nutrient abundance (Fineza et al., 2014). This oxygen limitation acts as a constraining factor for certain bacterial communities, thereby promoting the emergence of distinct assemblages at each sampling depth. In contrast, shared OTUs were more prevalent at 20 cm depth than at 140 cm depth (Figure 4), reflecting sufficient growth factors for bacteria without substantial variation. At 20 cm depth, the bacterial community is predominantly influenced by exogenous factors in addition to corpse decomposition.

In Sample B, the phylum Actinobacteriota is dominant and comprises one of the phyla that contribute to the shared operational taxonomic units (OTUs) across the six samples examined (Figure 5). This phylum consists of widely distributed genera that are vital in breaking down various organic materials, such as cellulose, polysaccharides, proteins, lipids, and organic acids (Ghare et al., 2023). Members of Actinobacteriota can thrive in extremely challenging environments, such as deserts, caves, deep-sea sediments, and salt lakes, thanks to their capacity to generate spores or conidia for reproduction (Mahapatra et al., 2020). This group consists of specific

taxa that have developed specialised adaptations. Thermophilic genera like *Thermomonospora*, *Streptomyces*, *Microbispora*, *Saccharomonospora*, and *Saccharopolyspora* can survive temperatures ranging from 45°C to 65°C (Fu et al., 2021). Acidophilic genera such as *Kitasatospora* and *Streptomyces* can endure pH levels between 3.5 and 6.5, as stated by Anandan et al. (2016). Halophilic genera, comprising *Rhodococcus*, *Micromonospora*, and *Streptomyces*, have adapted to salinity levels spanning 0.5 to 4 M, as noted by Hui et al. (2021). In addition, some of these species act as endophytes (Liu et al., 2020), symbionts (González et al., 2024), or endosymbionts (Matroodi et al., 2020).

The phylum Actinobacteriota contains several genera with notably high relative abundance, and *Gaiella*, *Pseudoarthrobacter*, *Streptomyces*, and *Micromonospora* stand out as prominent examples (Figure 3). Research indicates that *Gaiella* thrives in soils contaminated with polycyclic aromatic hydrocarbons (PAHs), as reported by Zahra et al. (2020). Additionally, studies have shown a positive correlation between *Gaiella* and higher concentrations of nitrate (NO₃-N), ammonium (NH₄⁺-N), and soil organic carbon (SOC) in cemetery soil, both within the soil structure and intracellularly (Meynier et al., 2020). Furthermore, the genus *Streptomyces* encompasses

Table 3. The results of chemical analysis of cemetery soils

Paremeters	Unit	Block and deep (cm)						Method
		B18		B23		P12		
		20 cm	140 cm	20 cm	140 cm	20 cm	140 cm	
pH (H ₂ O)		7.29	7.95	7.84	7.90	8.10	7.51	pH meter 1:5 IK. 5.4.c
pH (KCl)		6.69	6.27	6.54	6.87	7.39	6.45	pH meter 1:5 IK. 5.4.c
DHL	(μs/cm)	87	28	13	18	9	42	Conductometer 1 : 5
C-organic	%	1.23	1.03	1.13	0.99	0.79	0.73	Walkley & Black IK 5.4.d
N-total	%	0.08	0.07	0.04	0.05	0.06	0.03	Kjeldahl IK 5.4.e
K availability	Ppm	106	91	129	124	147	171	Morgan-Wolf
P ₂ O ₅	ppm	2	1	NA	NA	41	93	Olsen IK.5.4.h

The microbial genera represented by *Actinomadura* sp. and *Nocardioides* sp. serve as contrasting ecological indicators of different soil horizons. *Actinomadura* is primarily located in surface soils, due to its ability to adapt to oxygen-rich areas rich in organic matter (Ay et al., 2017; Lahoum et al., 2016). The organism's capacity to break down complex plant polymers like lignin and cellulose (Shi et al., 2019), as well as its production of antimicrobial compounds (Ay, 2021), gives it a competitive advantage within the diverse microbial populations of topsoil. These characteristics establish *Actinomadura* as a robust indicator of biologically active, nutrient-rich surface layers, especially in areas affected by plant residues or agricultural activities. In contrast to other species, *Nocardioides* species are more common in deeper soil layers, where their physiological adaptations allow them to survive under oligotrophic, low-oxygen, and high-pressure conditions. Their metabolic versatility allows them to survive in microaerophilic or intermittently anaerobic environments with limited nutrient availability (Sui et al., 2024; Ma et al., 2023). Their robust cellular structures and stress-tolerance mechanisms improve survival in subsoil conditions, including compaction and lower moisture levels. As a result, *Nocardioides* is consistently identified as a vital taxon within microbial communities found in deep soils, indicating ecological niches that are both stable and resource-limited.

a range of thermophilic, halophilic, and acidophilic species. The presence of Actinobacteriota, particularly *Streptomyces*, in cemetery soil underscores its capability for tasks like decomposing organic substances (including lipids, proteins, and cellulose) and producing antibiotics (Goudjal et al., 2018; Zhang et al., 2023). This genus is recognized for producing a wide variety of valuable secondary metabolites (Li et al., 2022; Ambarwati et al., 2023; Ayuningrum et al., 2022).

Research revealed a significant relative abundance in the genera *Wolbachia* and *Buchnera*. Insects and arthropods are home to symbiotic organisms, which often use decomposing remains to lay eggs or form cocoons (Wulandari & Rahayu, 2015; Arab & Lo, 2021; Kaur et al., 2021; Moran, 2021; Porter & Sullivan, 2023), a trend also observed by Sanaei et al. (2021), Hu et al. (2020). The bacterial community composition in cemetery soil appears to be impacted by the depth at which the sampling site is collected (Figure 5). Studies of soil samples, identified as Sample A and Sample B, revealed no significant differences, although electrical conductivity data showed considerable statistical significance (Table 3). The electrical conductivity has been identified as a crucial factor impacting changes in soil bacterial populations (Pittner et al., 2020; Han et al., 2021; Wang et al., 2020).

This study provides important insights into the bacteriome of cemetery soils, identifying the most abundant phyla across both shallow and deep sampling sites. Future research should focus on isolating and characterising potentially significant bacterial groups found in cemetery soil.

5. Conclusion

This study found that sample B contains a greater number of OTUs and greater diversity compared to sample A. At a depth of 20 cm, Proteobacteria is the predominant phylum, whereas at 140 cm depth, Actinobacteria takes over. Genera of the phylum Proteobacteria that were consistently found in all samples included *Pseudomonas*, *Sphingomonas*, and *Microvirga*. Concurrently, the equally well-represented genera within the phylum Actinobacteriota include *Gaiella*, *Pseudoarthrobacter*, *Streptomyces* and *Micromonospora*. Principal component analysis revealed that bacterial community composition in cemetery soil is affected by soil depth. Biomarker analysis actually showed that specific genera were present, including *Actinomadura* sp. at a depth of 20 cm and *Nocardioides* sp. at 140-cm deep. This discovery offers important insights into the variety of the bacteriome in both deep and shallow regions of the cemetery. A more in-depth examination of the detected biomarker genera within

deep and shallow soils could be a vital area of study in the near future.

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Investigating the Bioactivity of Extracellular Protease Produced by Bacterial Strains Collected from Different Soil in Egypt

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Abstract

Proteases are enzymes of considerable industrial importance, owing to their broad catalytic versatility across a wide range of applications. In this study, twenty-three bacterial isolates were obtained from cultivated soil samples. Among these, six isolates -designated B1, B7, D1, F2, G and H- exhibited notable proteolytic activity, as evidenced by clear zones of casein hydrolysis on skimmed milk agar and enhanced growth extending beyond the cultured disc boundary. Of these, isolate D1 demonstrated the most pronounced proteolytic activity, characterized by extensive degradation zones and vigorous peripheral growth. This isolate was taxonomically identified as *Alcaligenes faecalis* and subsequently deposited in the GenBank database under accession number PQ357195 (EG1-AMY).

Protease production was optimized by evaluating various submerged fermentation conditions. Following characterization of enzyme's activity profile, the protease was classified as an alkaline protease based on its optimal activity in alkaline pH. The highest enzymatic yield was achieved when all optimized parameters were combined in a single test, resulting in an approximately fourfold increase in protease activity compared to the original condition.

Partial purification of the enzyme was performed using ammonium sulphate precipitation across a range of saturation levels. Maximum purity was attained at 90% saturation, followed by dialysis to eliminate residual salts and impurities. Further purification was carried out using Fast protein Liquid Chromatography (FPLC). The molecular mass of the purified protease was determined using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). A consistent band at 57 kDa was observed in both the dialyzed crude extract and the FPLC-purified fraction.

Keywords: *Alcaligenes faecalis*, Fermentation, Skimmed milk, Alkaline protease, FPLC, Dialysis.

1. Introduction

Enzymes play a vital role in the global industrial sector, serving as highly efficient biocatalysts that accelerate chemical reactions efficiently under mild conditions, significantly lowering energy demands and reducing environmental impact (Singh and Panesar, 2023). Thanks to their precision and versatility, enzymes become the key for improving product quality, enhancing process efficiency and minimizing waste across industries such as food and beverage, textiles, pharmaceuticals, biofuels, and detergents (Allied, Market Research, 2023; Khan, 2025). Enzymes not only boost economic performance but also pave the way for more sustainable and innovative manufacturing practices across diverse applications worldwide.

Proteases (EC 3:4, 11-19, 20-24, 99) are a diverse group of enzymes that play a crucial role in proteolysis - the hydrolysis of peptide bonds in proteins (Watanabe and Hayano, 1994; Singhal *et al.*, 2012, and Jisha *et al.*, 2013). They are widely distributed across all forms of life, including plants, animals and microorganisms (Polaina and Mac Cabe, 2007). Intracellular bacterial proteases, for instance, are responsible for degrading proteins that are

either damaged or functionally obsolete. This targeted breakdown prevents the accumulation of toxic protein aggregates and replenishes the amino acids pool, supporting the synthesis of new proteins and maintaining cellular homeostasis (Olivares *et al.*, 2016). These enzymes are also essential for protein metabolism in human; for instance, they can be used as dietary supplements to enhance protein digestibility (Paulussen *et al.*, 2024).

Due to the growing industrial demand for proteases, microbial sources have become economically favorable for large-scale production (Rani *et al.*, 2012 and Abdul Razzaq *et al.*, 2019). Proteases are generally classified into three main categories: alkaline, acidic and neutral. Additional classifications have also been proposed by Berrett, (1999). These enzymes have numerous industrial applications, as in detergents, leather processing and pharmaceuticals (Singh *et al.*, 2001; Shafee *et al.*, 2005, and Liu *et al.*, 2010).

The genus *Alcaligenes* was first identified in fecal samples due to its presence in the intestines and respiratory tract of vertebrates. Subsequently, members of this genus have been isolated from a wide range of environments, including soil, marine ecosystems, wastewater and other habitats (Rehfuss and Urban, 2005; Tena *et al.*, 2014;

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Olusola-Makinde *et al.*, 2021; Aullybux *et al.*, 2022, and Uttaratotai *et al.*, 2022). Bacteria of this genus are aerobic, rod-shaped, Gram-negative, and motile due to the presence of flagella (Busse and Stolz, 2006). *Alcaligenes faecalis* is one of the seven species formally recognized in the 9th edition of Bergey's Manual of Determinative Bacteriology (Holt, 1994). Alkaline proteases (EC. 3.4.21-24, 99), in particular, are produced by various bacterial species, the *Alcaligenes faecalis* being a notable producer.

2. Materials and Experimental Methods

2.1. Selection of protease-producing bacteria

2.1.1. Bacterial isolation

Soil samples were aseptically collected in sterile polyethylene bags from various crop-cultivated fields in Giza, Egypt. Under sterile conditions, 5g of each sample was mixed with 100 ml of peptone water (pH 7.2 at 25 °C) prepared as described by Atlas, (2010). The mixtures were shaken at 150 rpm for 1 hour, then allowed to settle for 30 minutes. Serial dilutions (10^{-1} - 10^{-5}) were prepared. From each dilution, 100 μ l was spread onto nutrient agar (NA) plates prepared following Atlas, (2010) and supplemented with nystatin to inhibit fungal growth. Plates were incubated at 37 °C for 48 hours, and distinct colonies were selected and sub-cultured.

2.1.2. proteolytic activity screening

Selected bacterial isolates were cultured using the inverted agar disc method on skimmed milk agar (SKM-A) prepared according to Atlas, (2010), then plates were incubated. Proteolytic activity was assessed by measuring the diameter of the clear zones around the discs and the growth zones of the isolates.

2.1.3. Assay for protease activity

Under submerged fermentation conditions, 49 ml of autoclaved skimmed milk broth (SKM-B) was dispensed into sterile 250 ml capped bottles, and inoculated with 1 ml of bacterial suspension (1.8×10^{-8} CFU) standardized using the 0.5 McFarland method. Cultures were incubated at 37 °C and 150 rpm for 48 hours in a shaking incubator. Post-incubation, samples were centrifuged at 8000 rpm and 4 °C for 10 minutes, and the supernatants were collected for protease analysis.

Protease activity was determined using casein as the substrate, following a modified protocol based on Chimbekujwoa *et al.*, (2020), incorporating principles from Kunitz, (1946) and Boethling, (1975). The enzyme extract was diluted 1:1 with 0.2 M sodium phosphate buffer (pH 7.0), then 1 ml of this solution was mixed with 2 ml of 1 % casein (prepared in the same buffer) and incubated at 37 °C for 15 minutes. The reaction was stopped by adding 2.5 ml of 5% trichloroacetic acid (TCA), and the precipitate was removed via Whatman filter paper. For spectroscopic analysis, 1 ml of filtrate was mixed with 2.5 ml of 0.5 M sodium carbonate and 1 ml of Folin -Ciocalteu reagent (Alpha Chemika, India), vortexed for 30 seconds, and incubated at 37 °C for 20 minutes. A blank was prepared by substituting buffer for the enzyme extract, and the standard curve was generated using 5.5 mM tyrosine. Absorbance was measured at 660 nm using a UV-VIS spectrophotometer (photolab 7600

,WTW), and protease activity was calculated as per Septianingrum *et al.* (2023):

$$\text{Enzyme activity (unit ml}^{-1}\text{)} = \frac{\text{tyrosine conc (ppm)} \times (\text{substrate volume} + \text{enzyme volume}) (\text{ml})}{\text{enzyme volume (ml)} \times \text{incubation time (min)}}$$

Eq. nu.

(1)

Under the described assay conditions, one unit of protease activity (U/ml) is defined as the amount of enzyme that releases 1 μ g of tyrosine per minute.

2.2. Identification and microscopic examination of isolate

2.2.1. Identification of the selected proteolytic isolate

Molecular identification was performed using 16S rRNA gene sequencing method. DNA was extracted following Macrogen lab protocols, and the 16S rDNA was amplified via PCR using the sequencing primer 785F in conjunction with 907R (Zhang *et al.*, 2000). The resulting sequence was compared against the GenBank database, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA11 software.

2.2.2. Light microscopy examination

A Gram-staining bacterial slide was prepared from a 24- hour SKM-A slant culture, following the America Society for Microbiology protocol (Gerhardt *et al.*, 1981) with minor modifications. Sterile distilled water replaced tap water for washing, and crystal violet was applied post-fixation. Morphological features were observed using an Olympus CHBS Laboratory Binocular Microscope (160/0.17) provided with camera.

2.2.3. Scanning Electron Microscopy (SEM)

SEM slides were prepared using a modified method based on Odedina *et al.*, (2015). A loopful of 24-hour culture was spread on a clean slide and fixed with 2.5% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7). After 90 minutes, the slide was washed thrice (10 minutes each) with buffer and dehydrated through graded ethanol (30-100%). The dried slide was examined using a FESEM (QUANTA FEG 250).

2.3. Optimization of cultural condition for protease production

2.3.1. Optimization of pH, temperature and incubation time

To evaluate the impact of fermentation condition on protease activity, the isolate was cultured in SKM-B medium under varying pH ranges (3, 5, 5.3, 7, 9 and 11), temperature ranges (4, 20, 37, 40, 50 and 60 °C) and incubation time ranges (0, 3, 6, 24, 48, 72, 96, 120, 144 and 168 hour). Protease activity was measured post-incubation to identify optimal conditions.

2.3.2. Effect of Carbon and Nitrogen sources

To assess the influence of media composition on enzyme production, the isolate was cultured in SKM-B supplemented with various Carbon sources (glucose, sucrose, maltose, fructose, lactose, carboxymethylcellulose and starch) and Nitrogen sources (yeast extract, urea, ammonium sulphate and sodium nitrate). Protease activity was quantified to determine the most effective nutrient sources.

2.3.3. Comparison of optimized vs. original culturing conditions

Optimal parameters from each test were combined into a single optimum test. Protease activity under these conditions was compared to that of the original setup to evaluate enhancement.

2.4. Extraction and purification of protease

2.4.1. Ammonium sulphate fractionation

Protease was precipitated from the crude extract-obtained under optimized culture conditions- by the salting out method using ammonium sulphate at varying saturation levels (20-100 %), following a slightly modified method based on Wang *et al.*, (2022). The solution was stirred at room temperature for complete salt dissolution, then incubated at 4 °C for 2 hours. Precipitates were recovered by centrifugation (10,000 rpm, 15 minutes, 4 °C), dried at 45 °C, weighted, and redissolved in 50 mM tris-HCl buffer (pH 9). Ammonium sulphate saturation was calculated using the equation described by Wingfield, (2016):

$$\text{Weight (g)} = \frac{G_{\text{sat}} (S_2 - S_1)}{1 - (P S_2)} \quad \text{Eq. nu. (2)}$$

Where: G_{sat} : A constant = 515.35

(It is the grams of ammonium sulphate /L of saturated solution at 0 °C).

S_2 : Final saturation (i.e.: the target saturation, for example: a 20% saturation is expressed as 0.2.).

S_1 : Initial saturation (= Zero).

P : Density (g/mL) = 0.2722 at 0 °C

2.4.2. Fractions evaluation

Collected fractions were evaluated through multiple steps, starting with protease activity assessment. Total protein content was measured using the Kjeldahl method (Jamal *et al.*, 2020) with a fully automated analyzer equipped with an auto-sampler (BKN-986). Specific protease activity was calculated using Equation number (3) (Mega *et al.*, 2020), while the yield percentage and purification fold were determined using Equations (4) and (5) (Islam *et al.*, 2009; Arshad *et al.*, 2017), respectively as follows :

$$\text{Specific enzyme activity (unit/mg protein)} = \frac{\text{protease activity (unit/ mL)} }{\text{total protein (mg/mL)}} \quad \text{Eq. nu. (3)}$$

$$\% \text{ yield} = \frac{(\text{activity in the fraction/ activity in crude extract}) \times 100}{\text{Eq. nu. (4)}}$$

$$\text{Purification fold} = \frac{\text{specific activity / specific activity of crude extract}}{\text{Eq. nu. (5)}}$$

2.4.3. Dialyses and Anion Exchange Chromatography

The purified protease fraction was dialyzed for 24 hour at room temperature (23-17 °C ± 1) with gentle stirring (80 rpm). The extract was processed using an ÄKTA Avant 150 FPLC system (GE Healthcare Life Sciences), following a modified protocol based on Ullah *et al.*, 2022.

Prior to chromatography, the sample was mixed with 50 mM tris-HCl buffer (pH6), and 1ml loaded onto a HiTrap DEAE FF weak anion exchange column. Elution was performed with a linear gradient of sodium chloride (0-1 M) in buffer B at constant flow rate of 1mL /min. Fractions exhibiting absorbance at 280 nm were collected and assayed for protease activity. The fraction displaying the highest enzymatic activity was stored at - 23 °C for further analysis.

2.4.4. SDS-PAGE analysis

The molecular weight of purified protease that gave the highest activity for both the dialyzed crude extract and FPLC fraction was assessed via SDS-PAGE on a 15% (w/v) polyacrylamide gel, following Laemmli's method (1970) as detailed by Banik *et al.*, (2018). Protein bands were visualized using Coomassie Brilliant Blue staining (Merck, Germany), and molecular weight markers (15-260 KDa) served as references.

2.5. Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics (version 27). All experiments were performed in triplicate (n=3) to ensure reproducibility and reliability of the results. Protease activity assays were evaluated using one -way analysis of variance (ANOVA). Additionally, comparisons of total protein content, protease activity and specific protease activity across fractions obtained via ammonium sulphate precipitations, and FPLC fractions were analyzed using one- way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc test to determine pairwise differences. Data are presented as mean ± standard deviation (SD). Statistical significance was reported where applicable, and error bars representing SD are included in the corresponding graphical representations.

3. Results and Discussion

Most biochemical reactions within living cells rely on enzymes for their functionality and survival (Pandey, 2024). Among these, proteases are a class of enzymes that catalyze the hydrolysis of proteins by cleaving peptide bonds, thereby generating polypeptides or free amino acids. These enzymes are produced both intracellularly and extracellularly by a wide range of microorganisms (Moo-young and Chisti, 1994, and Gupta *et al.*, 2002). In the present study, twenty-three bacterial isolates - previously cultured on nutrient agar (NA)- were evaluated for their ability to produce protease using Skimmed milk agar (SKM-A) medium supplemented with casein as the substrate. Of these, six bacterial isolates demonstrated clear zones surrounding the culture discs, accompanied by visible growth beyond the disc area (Figure 1a & b), indicating proteolytic activity. This approach served as a qualitative assessment of protease production. Subsequently, the protease activity of these six isolates was quantitatively analyzed to identify the isolate producing the highest enzyme concentration (Figure 3).

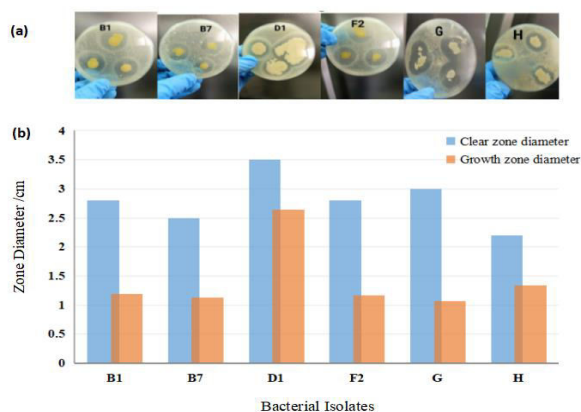


Figure 1. The clear zone and growth zone diameters for the six protease-producing bacterial isolates.

Protease enzymes hydrolyze the protein substrate casein, releasing its constituent amino acid tyrosine, which was quantitatively measured to assess protease activity (Benimana *et al.*, 2020). A standard curve for tyrosine concentration was prepared as illustrated in Figure (2). Among the tested isolates, isolate D1 demonstrated the highest level of protease activity (Figure 3). This observation was statistically validated, with a p-value of 0.003 (< 0.05) indicating strong evidence of significant variation in protease activity among the isolates. Furthermore, the F-value (= 6.8) for the test reinforcing the conclusion that the differences observed were not due to random variation but reflect genuine biological differences in enzyme production across the isolates.

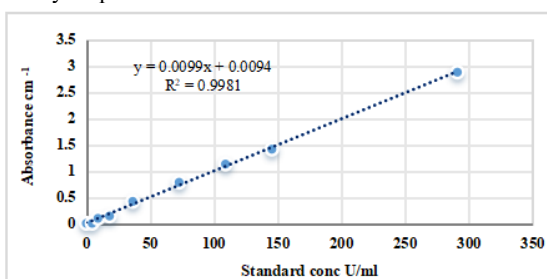


Figure 2. Tyrosine Standard curve.

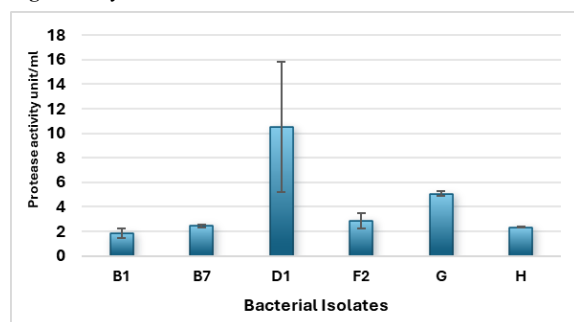


Figure 3. Screening of protease activity for the six protease-producing isolates.

The identification of isolate D1 was confirmed as *Alcaligenes faecalis* through DNA sequencing using the 16s rRNA method. BLAST analysis revealed a 98%

sequence identity with *Alcaligenes faecalis* strain P156, indicating a high level of homology. According to Janda and Abbot, (2007), bacterial species identification is considered valid when the 16S rRNA gene sequence similarity is $\geq 97\%$, supporting the classification of isolate D1 as *Alcaligenes faecalis*. The 16S rDNA sequence of the strain was submitted to the GenBank database under the accession number PQ357195 (EG1-AMY). A phylogenetic tree was subsequently constructed to illustrate its evolutionary relationship with closely related strains (Figure 4).

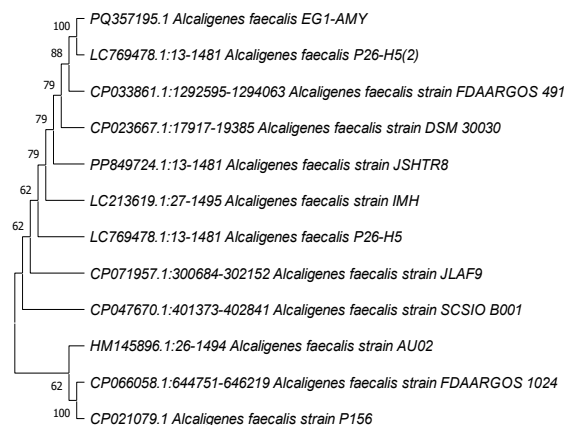


Figure 4. The phylogenetic tree for *Alcaligenes faecalis* PQ357195 (EG1-AMY)

Alcaligenes faecalis is a Gram-negative, rod-shaped bacterium observed to form long chains under scanning electron microscopy (Figure 5b). This species is characterized by its motility, facilitated by peritrichous flagella which were visible under light microscopy (Figure 5a) (Fatima *et al.*, 2020; Quach *et al.*, 2023). In general, flagellar-driven motility provides bacteria with a significant adaptive advantage, allowing them to actively move toward nutrient-rich environments or sites and steer away from harmful conditions. This locomotion enhance their capacity to locate, colonize, and persist within ecological niches that are support their growth and long-term survival (Creppy *et al.*, 2019; Makarchuk *et al.*, 2019; Colin *et al.*, 2021, and Zheng *et al.*, 2021). Such motility may explain the wide growth zone observed for the strain (isolate D1) as presented in Figures 1a and 1b.

Motile bacterial cells generally outperform non-motile ones in liquid media due to their ability to navigate the environment using flagella. Initially, *Alcaligenes faecalis* (isolate D1) was incubated in a shaking incubator to enhance aeration and facilitate uniform nutrient distribution throughout the medium. However, this method was later discontinued, and the cultures were subsequently maintained in a static incubator to better suit the growth characteristics of the strain.

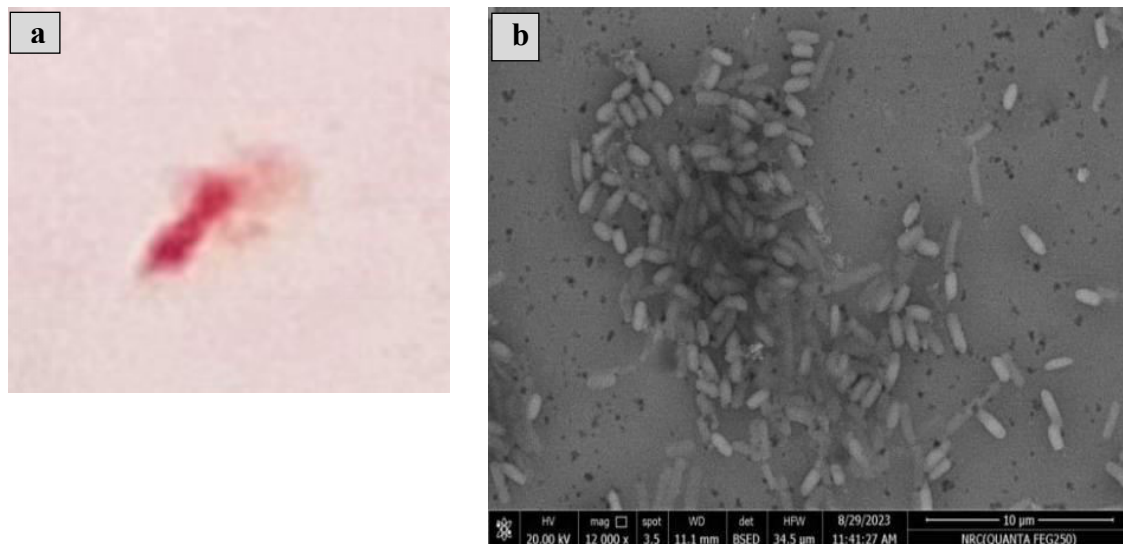
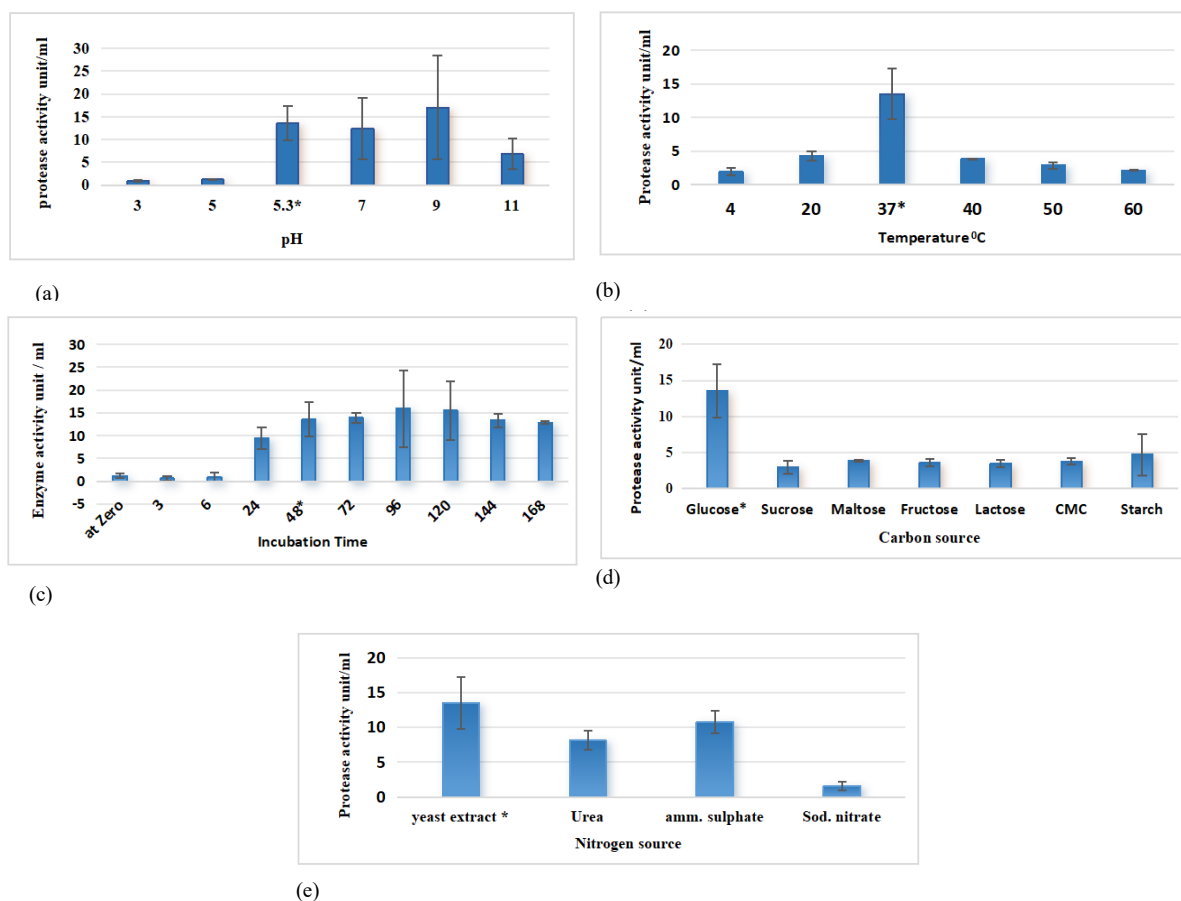


Figure 5. Examination of *Alcaligenes faecalis* PQ357195 (EG1-AMY) under light microscopy (a) and scanning electron microscopy (b).

In this study, the cultivation conditions for *Alcaligenes faecalis* and its protease production were evaluated under various selected parameters. The impact of these parameters was assessed by measuring enzyme activity. Maximum protease activity was recorded at pH 9, indicating that the isolate performs best under alkaline conditions. However, enzyme activity declined sharply at pH 11, which exceeds the optimal range (Figure 6a). These findings align with previous reports identifying *A. faecalis* as a potent producer of alkaline protease (Marathe *et al.*, 2018 and Obichi and Ezebuio, 2021). Temperature optimization experiments were conducted across a range of 4-60 °C (Figure 6b). The highest protease activity was observed at 37 °C, which corresponds to the original culturing temperature. This result is consistent with findings by Annamalai *et al.*, (2011). Regarding incubation time, 96 hours yielded the highest protease activity (Figure 6c). Prolonged incubation beyond this point led to a notable decline in enzyme activity. Interestingly, this result contrasts with several studies that reported optimal activity at 72 hour (Srividya, 2011 and

Khatoon *et al.*, 2023). *Alcaligenes* species are chemoorganotrophic bacteria capable of utilizing a broad spectrum of carbon sources (Robinson, 2014). To assess the influence of carbon sources, glucose in the original medium was substituted with various alternatives. All tested carbon sources supported bacterial growth, but glucose resulted in the highest protease activity (Figure 6d). Similarly, when nitrogen sources were varied, yeast extract produced the highest enzyme activity (Figure 6e). These outcomes are consistent with previous findings by Marathe *et al.*, (2018) on alkaline protease produced by *A. faecalis*. The statistical analysis confirmed that all tested parameters had a significant impact on protease production by *A. faecalis*. Variations in pH ($F = 4.1$, $p = 0.022$), temperature ($F = 23.1$, $p < 0.001$), incubation time ($F = 8.9$, $p < 0.001$), carbon source ($F = 12.5$, $p < 0.001$), and nitrogen source ($F = 16.8$, $p < 0.001$) each produced statistically significant differences in protease activity. These results underscore the importance of optimizing environmental and nutritional factors to enhance protease yield.



* : indicates the used parameter in the original test.

Figure (6): Optimization of cultivation conditions for protease production by *Alcaligenes faecalis* PQ357195 (EG1-AMY).

The combined impact of all optimized parameters were assessed by applying them in a single test and comparing the results to the original conditions. As illustrated in Figure 7, the optimized setup led to marked increase in protease activity, confirming the effectiveness of the optimization. The statistical analysis yielded an F-value of 55 and a p-value of 0.002, indicating a strong and reliable improvement in protease yield due to the applied optimization strategy.

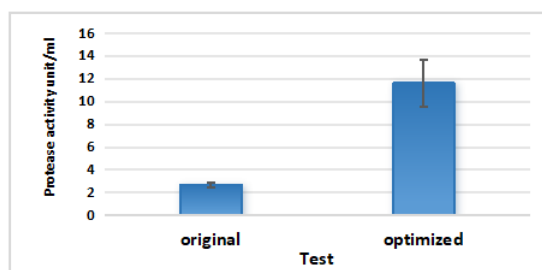


Figure 7. Comparative analysis of protease yield under original and optimized cultivation conditions in *Alcaligenes faecalis* PQ357195 (EG1-AMY).

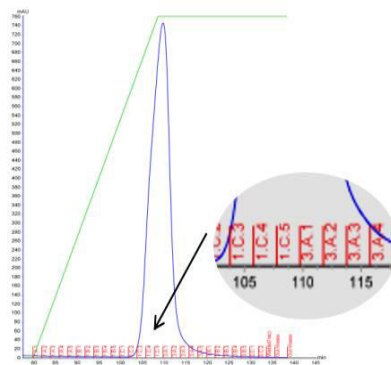
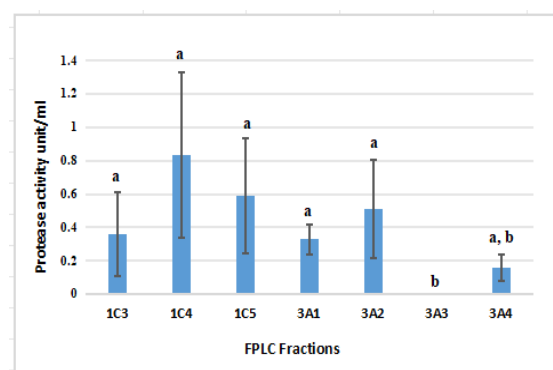
The crude extract containing the extracellular alkaline protease was successfully subjected to successive purification steps. The initial partial purification was achieved by using the salting-out method at varying ammonium sulphate saturation levels (Table 1). Protein

precipitation using ammonium sulphate had a statistically significant impact on total protein concentration ($F = 8667.0$ and $p < 0.001$), protease activity ($F = 548.8$ and $p < 0.001$) and enzyme specific activity ($F = 171.4$ and $p < 0.001$). These effects were highly significant ($p < 0.05$), as further evidenced by the high F - statistic values, which indicate substantial between- group differences. The highest protease recovery was achieved at 90 % saturation as presented in Table 1. The ammonium sulphate precipitation method relies on the principle that the ions of the salt compete for water molecules, thereby reducing protein solubility. This reduction promotes the interaction among the proteins molecules resulting their aggregation and subsequent precipitation (Vetal & Rathod, 2014 and Urgessa *et al.*, 2019). Although 100% saturation is theoretically intended to precipitate all proteins, it may induce protein unfolding and subsequent enzymatic inactivation. This is evidenced by the observed decline in protease activity, specific activity, yield and purification fold compared to 90% saturation as shown in Table 1. The observed variations in specific protease activity across different saturation levels likely reflect the heterogeneous distribution of enzymatic and non-enzymatic proteins as well as protease inhibitors, each exhibiting different solubility profiles at varying salt concentrations (Scopes, 1994; Meza-Espinoza *et al.*, 2018; Khadka *et al.*, 2024).

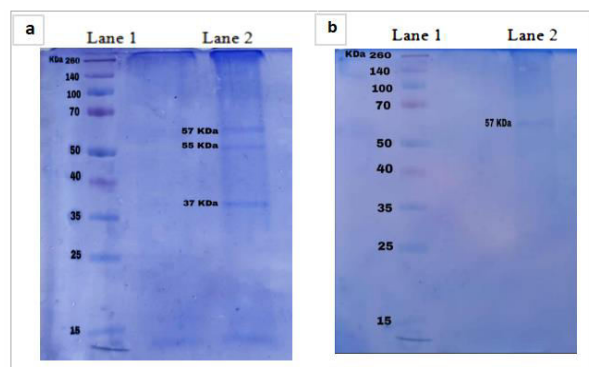
Table 1: Summary of alkaline protease fractions obtained via precipitation by ammonium sulphate.

Ssaturation (%)	Ammonium sulphate (g/mL)	Precipitate (g)	Total protein (mg/mL)	Protease activity (unit/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
crude	-	-	14.0 ± 0.12	11.6 ± 0.03	0.83 ± 0.006	-	-
20%	10.9	0.25	4.70 ± 0.03 ^a	0.03 ± 0.05	0.01 ± 0.011	0.8	0
30%	16.8	1.58	4.80 ± 0.0 ^a	0.50 ± 0.06 ^d	0.11 ± 0.012	4.3	0.1
40%	23.1	1.84	5.50 ± 0.03	1.36 ± 0.34 ^d	0.25 ± 0.060 ^c	11.7	0.3
50%	29.8	1.98	6.00 ± 0.1	1.58 ± 0.09 ^d	0.26 ± 0.011 ^c	13.6	0.3
60%	38.8	2.41	7.00 ± 0.09	2.82 ± 0.17 ^c	0.40 ± 0.029 ^a	24.3	0.5
70%	44.5	2.67	7.70 ± 0.03	3.46 ± 0.28 ^{b,c}	0.45 ± 0.034 ^a	29.8	0.5
80%	52.6	3.28	11.4 ± 0.01	4.32 ± 0.03 ^{a,b}	0.38 ± 0.002 ^{a,b}	37.3	0.5
90%	61.3	5.14	12.4 ± 0.1	4.97 ± 0.54 ^a	0.40 ± 0.047 ^a	42.8	0.5
100%	70.6	9.97	13.5 ± 0.05	4.73 ± 0.29 ^a	0.35 ± 0.02 ^{a,b}	40.7	0.4

The protein precipitated at 90% ammonium sulphate saturation was dialyzed to remove residual salts and impurities, followed by further purification using FPLC. Based on the absorbance at optical density 280 nm, seven distinct fractions were collected (Figure 8) and subsequently assayed for protease activity. Among these, the 1C4 fraction exhibited the highest protease activity (Figure 9). Notably the overall enzyme yield following the purification was relatively low, likely attributed to protease autolysis occurring during the successive purification steps (EL-Eskafy *et al.*, 2016). This may account for the observed variation in protease activity across the FPLC fractions, showing a marginally significant difference ($p=0.055$) suggestive of potential fraction-specific enrichment. The corresponding F-value ($F=2.8$) indicates that the observed differences among the fractions are meaningful but not pronounced.

**Figure 8:** Elution profile from FPLC at optical density 280 nm.**Figure 9:** Evaluation of protease activity in the FPLC fractionations.

Alkaline proteases purified from various bacterial species exhibit considerable wide range of molecular mass (Hashmi *et al.*, 2022; Ullah *et al.*, 2022 ; Salim *et al.*, 2023; Santos *et al.*, 2024 ; Punnarath *et al.*, 2025). In this study, SDS-PAGE analysis of the dialyzed crude protease extract obtained at 90% ammonium sulphate saturation revealed three bands at approximately 37, 55 and 57 KDa as shown in Figure 10a. Subsequent purification using FPLC confirmed the enzyme's purity, as evidenced by a single band at approximately 57 KDa, corresponding to its molecular mass as appeared in Figure 10b.



Lane 1 : the marker molecular weight and Lane 2 :The eluted fraction

Figure 10: SDS-PAGE analysis of dialyzed crude protease extract and FPLC- purified 1C4 fraction.

4. Conclusion

This study aimed to isolate bacterial strain capable of producing protease with high concentration and activity. Among the isolates, the isolate D1 - later identified as *Alcaligenes faecalis* and registered in GenBank under accession nu. PQ357195 (EG1-AMY) - exhibited the most prominent clear zone and growth zone diameters, indicating superior proteolytic potential.

Following a series of optimization experiments, all optimized parameters were combined and applied in a single test. Protease production was markedly improved under conditions utilizing glucose and yeast extract as carbon and nitrogen sources, respectively, at pH 9 and incubated at 37 °C for 96 hours. Compared with the original conditions, this approach significantly enhanced protease production. The enzyme produced was classified as an alkaline protease. The crude extract, precipitated at 90% ammonium sulphate saturation, was subsequently purified using FPLC. SDS-PAGE analysis revealed that the purified alkaline protease had a molecular mass of approximately 57 KDa.

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Identification and Genetic Diversity of Wild Mushroom Strains Using Nuclear Genes and Molecular Markers

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Abstract

Wild mushroom species are integral to the steppe and mountain ecosystems in Iraq's Kurdistan area. Despite extensive investigation, the evolutionary relationships among the various strains remain mostly unknown. Three molecular techniques have been used to test sixty-three samples for identification and genetic diversity. Phylogenetic analysis using Internal Transcribed Spacer (ITS) sequences identified two primary clades. The BLASTn results identified seventeen *Agaricus litoralis*, two *Pleurotus columbinus*, three *Pleurotus eryngii*, three *Agrocybe dura*, eight *Amanita lividopallescens*, nine *Amanita crocea*, ten *Amanita vittadinii*, five *Agaricus pseudolutosus*, three *Agaricus campestris*, two *Agrocybe aegerita*, and one *Agaricus bisporus*. A substantial aggregation of taxa was associated with the genera *Agaricus*, *Agrocybe*, and *Amanita*. A total of 373 polymorphic fragments were identified using conserved DNA-derived polymorphism (CDDP) and start codon target (SCoT) markers. The CDDP and SCoT markers exhibited effects analogous to those of genetic diversity indices across several genera and species of mushrooms. CDDP (18 bands) and the SCoT (18 bands) markers exhibited high numbers of polymorphic bands per primer. CDDP exhibited a greater level of species-specific variance (23.74%) than SCoT markers (20.81%). The average polymorphism information content was 0.39 for both the CDDP and SCoT markers. In the clustering analysis, both methodologies categorized 63 samples into seven distinct groups. Three classes of mushroom species and genera were delineated using both markers. *A. lividopallescens* and *A. aegerita* showed the greatest genetic distance (0.48) for CDDP markers, while *A. aegerita* and *P. columbinus* showed the highest genetic dissimilarity (0.36) for SCoT markers. The combinatorial methods could effectively tell the difference between different strains of mushrooms. The significant genotypic diversity and sequenced genomes will give an ideal basis for functional genomics studies of the mushroom strains.

Keywords: Fungi, Species, Genus, DNA Identification, Molecular markers

1. Introduction

Eukaryotic heterotrophs called fungi have an estimated million species and are considered to be of tremendous economic and ecological importance (Blackwell, 2011; Hobbett *et al.*, 2016). The distribution of many fungal species is quite extensive, and it is possible that some of these species are truly cryptic or comprised of numerous genetic lineages. The fruiting bodies of macrofungi were vulnerable to environmental influences; therefore, morphological assessments typically cannot identify closely related genetic groupings (Zhao *et al.*, 2016). In the life cycle of *Basidiomycetes* and certain *Ascomycetes*, mushrooms are seen as visible fungi with unique carpophores, also called basidiocarps or fruiting bodies. They can be edible, inedible, or poisonous, and they are morphologically characterized as stinkhorns, puffballs, brackets, and gilled fungi (Adeniyi *et al.*, 2018). Mushrooms, which are fruiting bodies that develop from a subterranean mycelium and are widely consumed around the world, are produced by certain members of the macrofungi group (Kalač, 2013). Rural areas and developing nations often gather wild mushrooms for culinary purposes or economic gain (Kotowski, 2016; Boa, 2004).

Meanwhile, a select few mushroom species are farmed commercially and sold worldwide. The current culinary trends that prioritize health and sustainability are well aligned with mushrooms; thus, they are gaining in popularity. In reality, they are suitable for vegetarian diets, have ideal nutritional properties, and are compatible with a sustainable food supply (Giusti *et al.*, 2020).

Cultivated mushrooms are widely imported into Iraqi Kurdistan since mushroom growing cannot meet the high national demand. Wild mushrooms, on the other hand, have a long history of recreational collecting in Iraqi Kurdistan, and their users tend to value them more than the cultivated varieties. The months in concern are designated for this gathering. Mushrooms are not completely risk-free to eat. As a matter of fact, poisoning from mushrooms is a major kind of toxin-induced illness that can impact the neurological, respiratory, digestive, and liver systems (Giusti *et al.*, 2021; Govorushko *et al.*, 2019). When very toxic species are present, the liver sustains permanent damage that can be lethal. In addition, there are edible species that, if not handled correctly during collection, transportation, storage, and cooking, might turn hazardous (Gawlikowski *et al.*, 2015; Nieminen and Mustonen, 2020). They thrive on a variety of substrates and are often

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abundant during the rainy season in most regions. The many ecological, nutritional, physiological, and medicinal benefits of mushrooms have earned them the status of important bioresources (Ayimbila and Keawsompong, 2023). They help the ecosystem with nutrient recycling by breaking down dead organic materials, especially those that include lignin and cellulose (Kumla *et al.*, 2020). In addition to being rich in protein, crude fiber, vitamins, and minerals, they also have medicinal properties that make them useful in treating a variety of conditions, including high cholesterol, tumors, infections, cancer, ageing, anti-inflammatory, antidiabetic, antihyperglycemic, antiparasitic, anti-inflammatory, and antihypertensive conditions (Bhambri *et al.*, 2022; Titilawo *et al.*, 2022).

The majority of morphological and agronomic features, however, are quantitative variables that are highly responsive to environmental variation (Tahir *et al.*, 2021). Consequently, the genetic variety of germplasm resources cannot be directly reflected by phenotypic data (Sleman and Tahir, 2025; Lin *et al.*, 2022; Rokni *et al.*, 2019). Fungal morphological traits are highly variable and influenced by environmental factors, leading to overlapping or indistinct features among genetically distinct groups, as evidenced by the lack of consistent morphological traits among molecularly defined fungal species and intraspecific groups (Lin *et al.*, 2023). Conventional methods for identifying fungal species are typically characterized by indiscrimination, inconsistent morphology, and inconspicuousness; however, modern molecular techniques have mitigated these issues (Abdullah and Yasin, 2025). For precise species identification of mushrooms, however, DNA analysis in conjunction with phenotypic data is preferred because of its speed and low cost. Analyses using PCR-amplified nuclear rRNA genes, and more specifically the Internal Transcribed Spacer (ITS) region, which has been selected as a DNA barcode identifier for fungi because of its significant interspecies variation, constitute the majority of current approaches. The small subunit 5.8S rRNA gene separates the two non-coding parts (ITS-1 and ITS-2), which are in the ITS region and are about 600 to 800 kb in size. Genome sequencing at this marker and subsequent comparison to publicly available reference DNA libraries constitute the highest standard for taxonomic identification (Badotti *et al.*, 2017; Creedy *et al.*, 2020; Lücking *et al.*, 2020; Yuwa-Amornpitak *et al.*, 2023). Molecular markers, including inter simple sequence repeat (ISSR), sequence related amplified polymorphic (SRAP), random amplified polymorphic DNA (RAPD), and cleaved amplified polymorphic sequences (CAPs), are powerful methods for analyzing the genetic variation and strain relationships of organisms. It is also environmentally independent because of the high levels of detectable polymorphisms (Gu *et al.*, 2024; Hao *et al.*, 2022; Tahir, 2008; Tahir and Karim, 2011). In the past, different researchers employed molecular techniques for the identification and estimation of genetic variation within different mushroom strains (An *et al.*, 2021; Jiao *et al.*, 2024; Lin *et al.*, 2022; Liu *et al.*, 2018; Rokni *et al.*, 2019; Zhao *et al.*, 2016). The use of

conserved DNA-derived polymorphism (CDDP) molecular markers is capable of capturing information uniquely offered by the targeted application of a single primer to a single locus within a gene of transcription factors (Ahmed *et al.*, 2023; Aziz and Tahir, 2023; Tahir *et al.*, 2023; Rasul *et al.*, 2022). Many studies have used SCoT markers, which are usually polymorphic and depend on shorter conserved regions close to the ATG start codon, to uncover genetic diversity and aid in conservation efforts. In this technique, similar to RAPD or ISSR markers, a single primer acts as both the forward and reverse primer (Majeed *et al.*, 2024; Tahir *et al.*, 2023; Rasul *et al.*, 2022).

Our literature review reveals an absence of published data about the in-silico analysis of the frequency of CDDP and SCoT markers across all fungal genomes. To our knowledge, this is a novel occurrence both regionally and globally for certain species. Iraqi Kurdistan possesses a wealth of mushroom genetic resources, despite having only one cultivated species. The presence of several names for identical or closely related strains results in difficulties in classification and ambiguity concerning genetic distinctiveness and relationships among strains. These challenges have complicated the categorization and preservation of genetic resources, the selection of appropriate breeding parents, and the advancement of improved cultivars. Therefore, the assessment of genetic diversity and the appraisal of germplasm resources are thus crucial. The aim of this study was to identify and assess the genetic diversity of 63 mushroom strains (62 wild and 1 cultivated) from the Iraqi Kurdistan region using the ITS gene and molecular markers (CDDP and SCoT). This comprehensive approach enabled the analysis of genetic variation data and improved understanding of the genetic diversity present in the germplasm resources from 63 mushroom strains. The identification of superior genetic resources established an outline for subsequent research aimed at enhancing and broadening current germplasm resources, breeding materials, and conservation strategy. To fully comprehend the potential of wild mushrooms and to strengthen their preservation, more research in this area is required.

2. Materials and Methods

2.1. Mushroom strains samples

In February 2024, at the end of winter, we collected sixty-two samples of wild mushrooms from eight different sites in the Kurdistan area of Iraq (Figure 1 and supplementary file, Figure S1). The distance between the sampling areas ranged from eighty to two hundred kilometers. To avoid sampling the same genotypes multiple times, we obtained each isolate at a minimum distance of 1 km from each other. For each geographical population, table S1 in the supplementary file displayed the sample size along with their names, strains, accession numbers, and altitudes. During sampling, the samples were maintained in the bag and placed in the chilled box. Afterwards, they were stored at 4 °C in the laboratory.

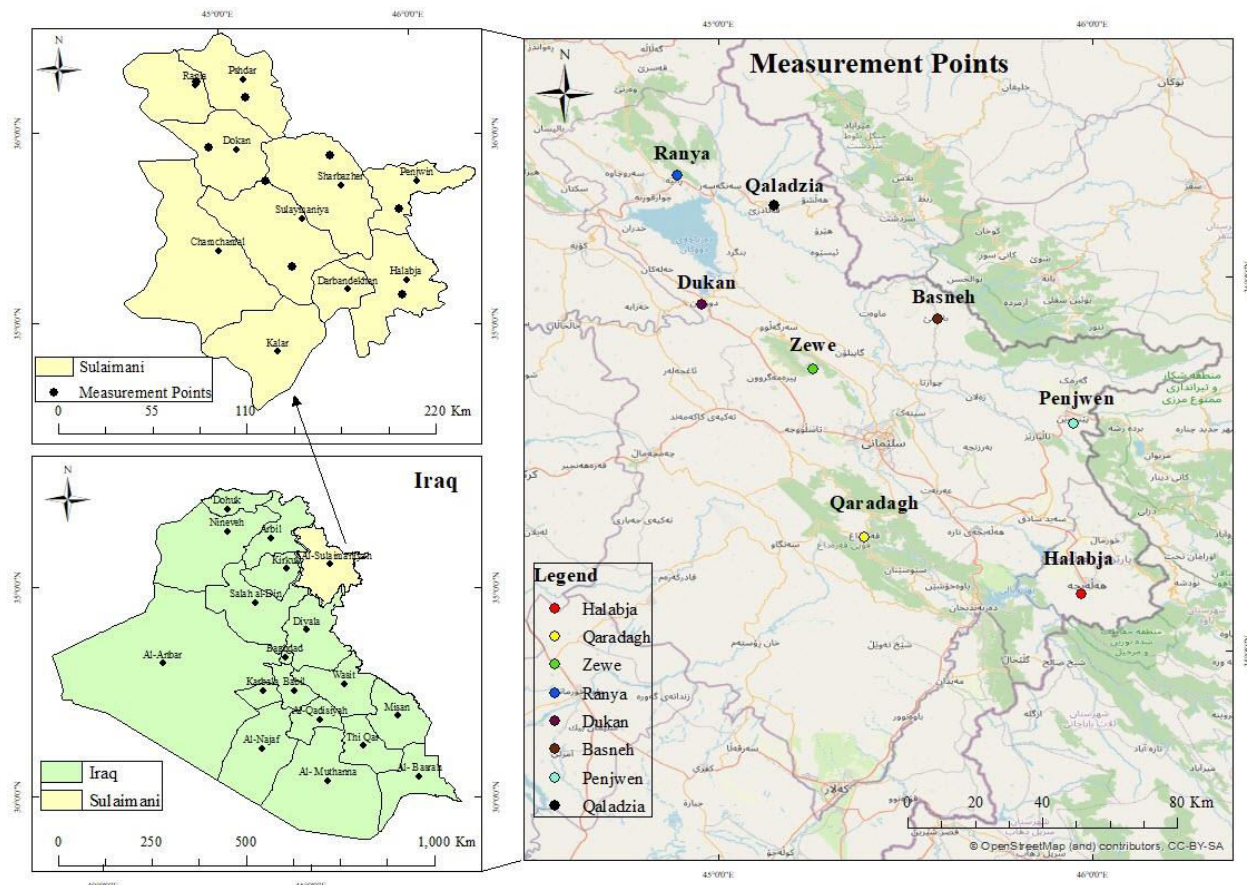


Figure 1. A map depicting the locations where distinct mushroom strains are harvested.

2.2. DNA extraction

The cap mushroom of samples (0.5 g) was ground using liquid nitrogen. DNA was extracted using the method described in Ahmed *et al.* (2023) and Aziz and Tahir (2023). The purity of genomic DNA was checked using gel electrophoresis on a 1.1% agarose gel (Addbio, Korea), and the quality was determined using Nanodrop spectrophotometry (NanoPLUS, MAALANLAB, Sweden). The DNA solution was maintained at a temperature of -17°C.

2.3. Polymerase chain reaction, purification, and DNA sequencing

The complete ITS1-5.8S-ITS2 region (supplementary file, Table S2) was amplified using PCR using a master mix kit (AddBio, Daejeon, Republic of Korea) in accordance with manufacturer protocols. The ITS primers, PCR circumstances, DNA separation, and DNA purification from agarose gel were detailed in our published article by Khal *et al.* (2023). The Macrogen Company in South Korea then sequenced the DNA and compared the results to those already recorded in the NCBI collection. GenBank assigned the sequences an accession number.

2.4. Genetic diversity analysis

Two molecular techniques, SCoT and CDDP markers, were employed to assess genetic diversity among various samples. A total of 24 PCR primers were used in the PCR method for SCoT and CDDP, comprising 12 primers for each marker type (Supplementary file, Table S2) (Aziz and Tahir, 2023; Collard and Mackill, 2009a; Collard and

Mackill, 2009b; Majeed *et al.*, 2024; Rasul *et al.*, 2024). The conditions for PCR, DNA separation, and visualization are clarified in our published works by Tahir *et al.* (2023) and Ahmed *et al.* (2022).

2.5. Phylogenetic tree and statistical analysis

To ensure the highest quality of sequences, we implemented chromatograms of sequencing in the SnapGene version 3.1 software to verify the sequences and replicate them as necessary. The sequences were aligned using the Clustal W utility in Molecular Evolutionary Genetics Analysis 11 (MEGA 11). A phylogenetic tree was constructed with 1000 bootstrap replicates using the neighbor-joining tree technique to establish pretrial reliability. The detectable PCR products produced with SCoT and CDDP were subjected to an analysis. We only considered strong, distinct, and repetitive bands to decrease the chance of inaccuracy. We utilized numbers 1 and 0 to indicate the presence and absence of amplified bands, respectively, in order to generate a binary matrix for each sample. The radar chart was generated by XLSTAT version 2020.1.3. Principal component analysis and cluster analysis were conducted using the Ward method to generate the cluster diagram with JMP version 18. Using GenAIEx version 6.51b2, genetic distance, diversity indices, and analysis of molecular variance (AMOVA) were calculated. The polymorphism information content (PIC) was calculated using the formula outlined by Muhammad and Tahir (2026) and Aziz and Tahir (2023).

3. Results

3.1. Identification and phylogenetic analysis of mushroom specimens through nuclear DNA

The BLASTn results identified seventeen *Agaricus* *litoralis*, two *Pleurotus* *columbinus*, three *Pleurotus* *eryngii*, three *Agrocybe* *dura*, eight *Amanita* *lividopallescens*, nine *Amanita* *crocea*, seventeen *Agaricus* *litoralis*, ten *Amanita* *vittadinii*, five *Agaricus* *pseudolutosus*, three *Agaricus* *campestris*, two *Agrocybe* *aegerita*, and one *Agaricus* *bisporus* (Figure 2).

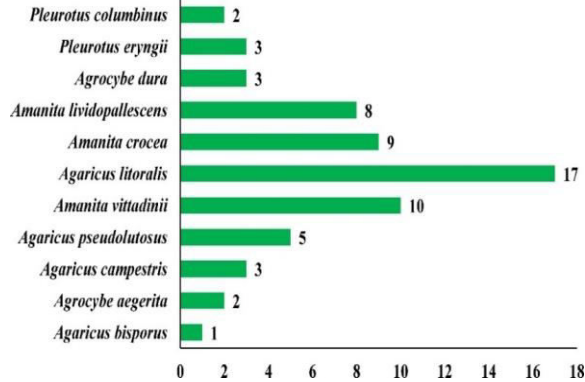


Figure 2. Number of identified strains in a collection of 63 samples through the ITS nuclear gene.

Phylogenetic analysis showed that there were two main groups of 63 isolates (Figure 3A). Five different strains of *P. columbinus* and *P. eryngii*, which belong to the genus *Pleurotus*, made up the first group (green line). In the second set of 58 isolates (red line), nine species were represented across three genera: *A. litoralis*, *A. dura*, *A. lividopallescens*, *A. crocea*, *A. vittadinii*, *A. pseudolutosus*, *A. campestris*, *A. aegerita*, and *A. bisporus*. After strain identification, the dendrogram (Figure 3B) divided the eleven species into three distinct categories based on the frequency of the nucleotides A, T, C, and G. *A. bisporus*, *A. litoralis*, *A. pseudolutosus*, and *A. aegerita* formed the initial group of taxa (red line). *A. vittadinii*, *A. crocea*, and *A. lividopallescens* were the members of the genus *Amanita*, forming the second clade (green line), while *P. columbinus*, *P. eryngii*, *A. dura*, and *A. campestris* were the members of the third clade (blue line). The four genera were categorized into two groups based on the frequency of four nucleotides. The first group consisted of three genera: *Agaricus*, *Agrocybe*, and *Amanita*; the second group was created by the genus *Pleurotus* (Figure 3C). As seen in figure 4, the range of C+G percentage was 0.43 to 0.50, whereas the A+T percentage was 0.50 to 0.57.

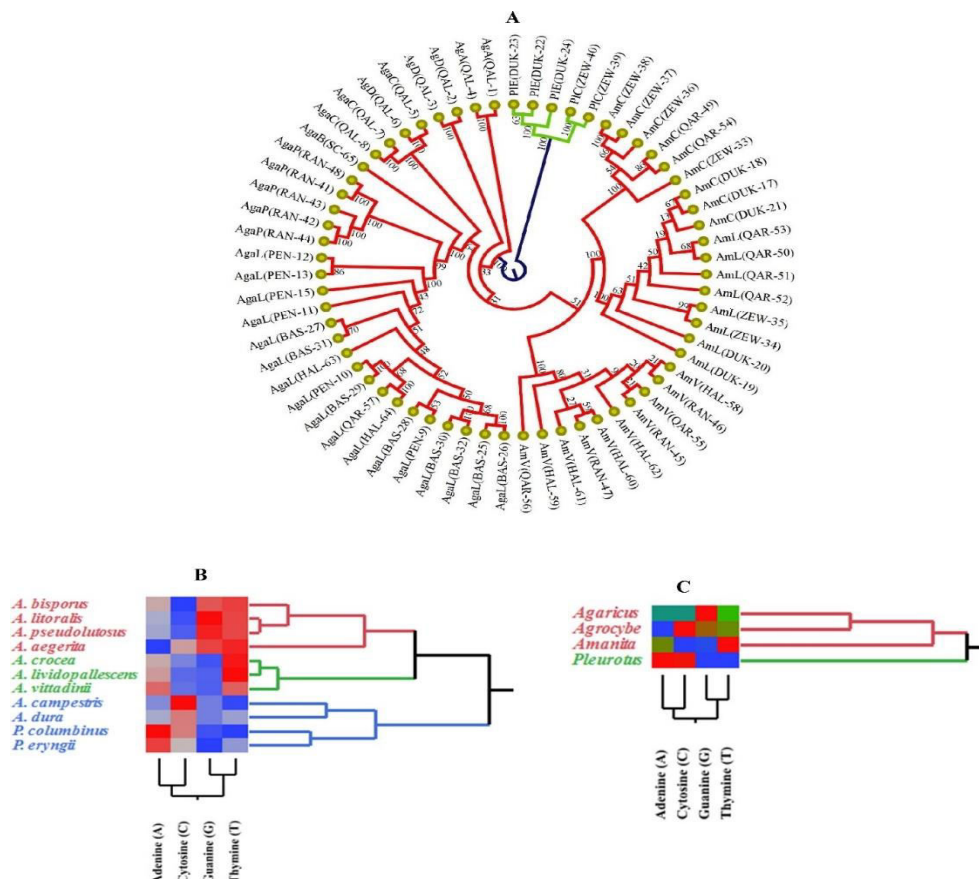


Figure 3. Clustering of mushroom specimens based on ITS gene sequencing data. A. Phylogenetic tree of all isolated samples. B. Classification of various mushroom species according to the prevalence of adenine, cytosine, guanine, and thymine. C. Clustering of diverse genera with respect to the frequency of adenine, cytosine, guanine, and thymine. The numbers on the node indicate the bootstrap percentage.

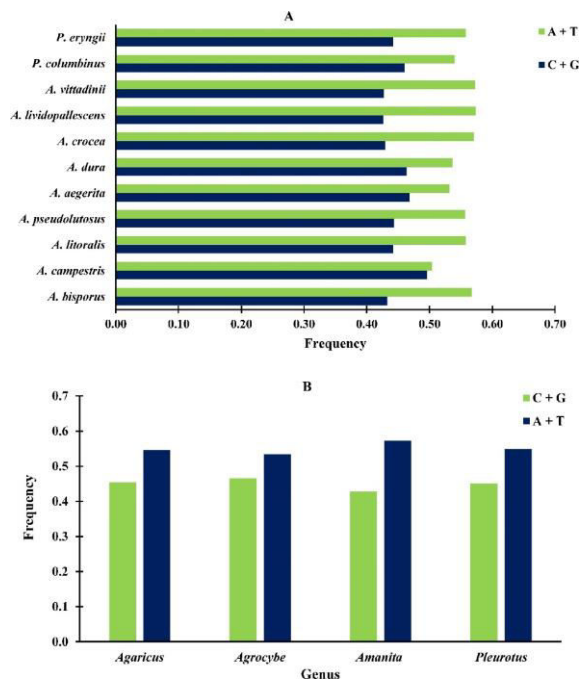


Figure 4. Illustrating the GC and AT contents in various species (A) and genera (B) of both wild and cultivated mushrooms.

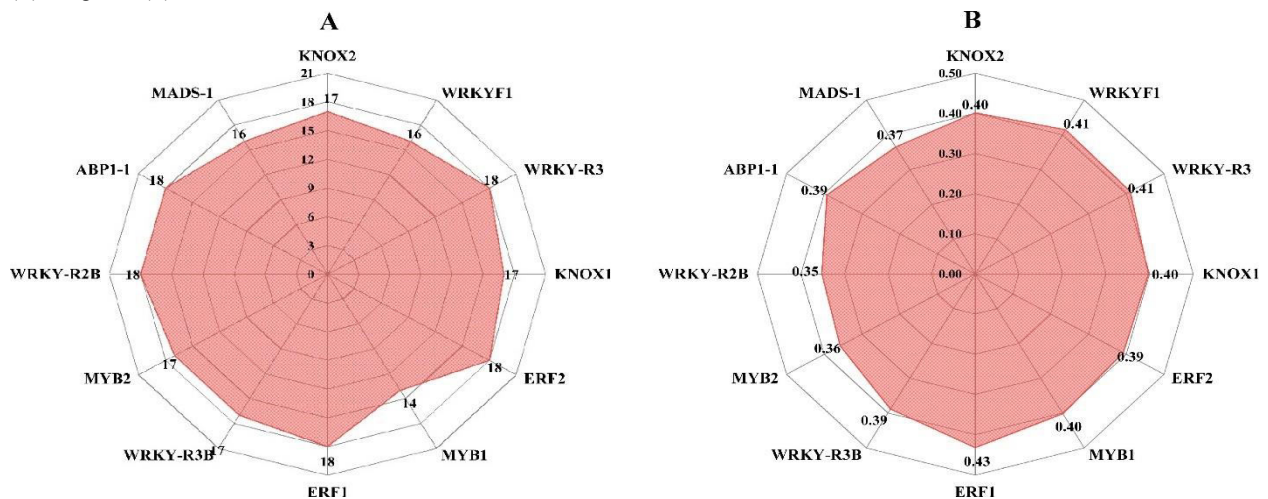


Figure 5. Number of polymorphic bands (A) and polymorphism information content (B) for various CDDP markers gathered from different species and genera of 63 mushroom samples.

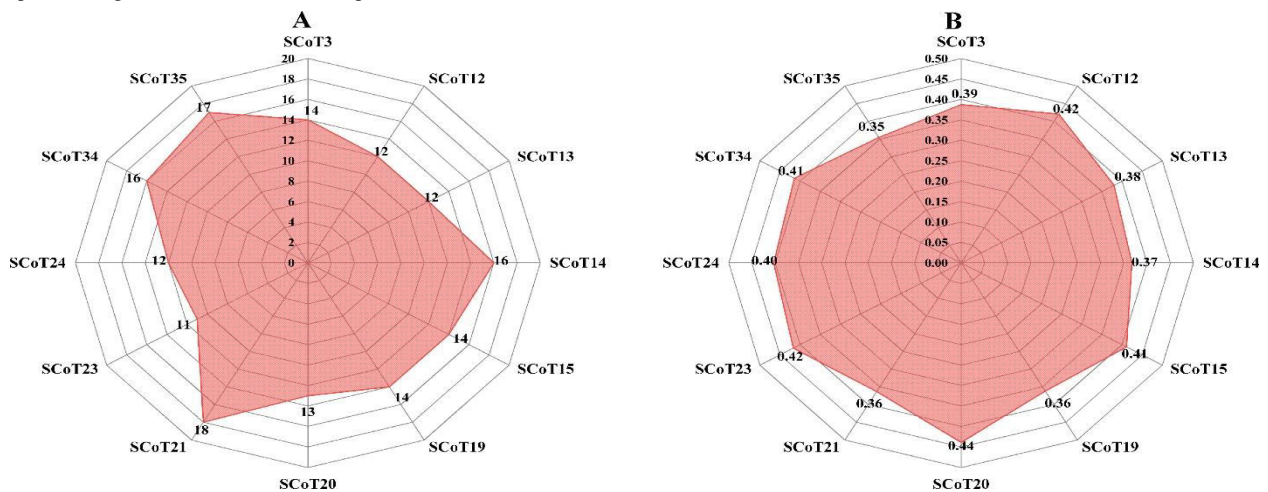


Figure 6. Polymorphic band number (A) and polymorphism information content (B) from various SCoT markers from 63 mushroom samples of different species and genera.

3.2. Marker information and genetic diversity of CDDP and SCoT methods acquired from mushroom samples

Using 12 polymorphic CDDP primers, the present investigation produced 204 markers from 63 samples, covering 11 species across 4 genera of mushrooms. From 14 (MYB1) to 18 (WRKY-R3, ERF2, MYB1, ERF1, WRKY-R2B, and ABP1-1) polymorphic fragments were found. The PIC had a mean of 0.39 and a range of 0.35 to 0.43 (WRKY-R2B to ERF1), as shown in figure 5. Concerning SCoT markers (Figure 6), the number of polymorphic bands varied from 11 (SCoT23) to 18 (SCoT21), while the PIC ranged from 0.35 (SCoT35) to 0.44 (SCoT20).

Species genetic diversity indicators, as determined by CDDP markers, are presented in table 1. The number of different alleles (Na), number of effective alleles (Ne), Shannon's information index (I), expected heterozygosity (He), and unbiased expected heterozygosity (uHe) varied from 1.01 (WRKY-R3) to 1.24 (ERF1), 1.23 (MADS-1) to 1.33 (MYB1), 0.21 (MADS-1) to 0.29 (MYB1), 0.14 (MADS-1) to 0.19 (MYB1 and KNOX1), and 0.15 (MADS-1 and KNOX1) to 0.22 (MYB1 and KNOX1), respectively. The Na, Ne, I, He, and uHe genetic diversity indices of the genera were as follows: 1.49 (WRKY-R3B) and 1.78 (KNOX1), 1.33 (WRKY-R2B) and 1.51 (ERF1), 0.33 (WRKY-R2B) and 0.44 (ERF1), 0.21 (WRKY-R2B) and 0.30 (ERF1), and 0.22 (WRKY-R2B) and 0.31 (ERF1), respectively. The genetic diversity indicators of

species and genera, as determined by SCoT markers, are summarized in table 1. Na, Ne, I, He, and uHe genetic diversity indices of the species differed from 0.98 (SCoT35) to 1.35 (SCoT15), 1.24 (SCoT35) to 1.35 (SCoT20), 0.22 (SCoT35) to 0.32 (SCoT15), 0.15 (SCoT35) to 0.21 (SCoT15 and SCoT 20), and 0.17 (SCoT35) to 0.24 (SCoT15), respectively. The Na, Ne, I, He, and uHe genetic diversity indices of the genera were 1.38 (SCoT24) and 1.77 (SCoT13), 1.31 (SCoT35) and 1.52 (SCoT20), 0.30 (SCoT35) and 0.46 (SCoT20), 0.19 (SCoT35) and 0.31 (SCoT20), and 0.20 (SCoT35) and 0.33 (SCoT20), respectively.

Table 1. Genetic diversity indices of several markers of CDDP and SCoT derived from different genera and species of 63 mushroom samples.

CDDP markers	Species					Genus				
	Na	Ne	I	He	uHe	Na	Ne	I	He	uHe
KNOX2	1.08	1.29	0.26	0.17	0.20	1.59	1.39	0.38	0.24	0.26
WRKYF1	1.15	1.30	0.27	0.18	0.20	1.50	1.37	0.35	0.23	0.24
WRKY-R3	1.01	1.24	0.22	0.14	0.16	1.54	1.39	0.37	0.24	0.25
KNOX1	1.21	1.31	0.28	0.19	0.22	1.78	1.40	0.40	0.25	0.27
ERF2	1.17	1.31	0.27	0.18	0.21	1.61	1.42	0.38	0.25	0.27
MYB1	1.23	1.33	0.29	0.19	0.22	1.63	1.48	0.41	0.28	0.29
ERF1	1.24	1.31	0.28	0.18	0.21	1.71	1.51	0.44	0.30	0.31
WRKY-R3B	1.10	1.26	0.23	0.15	0.17	1.49	1.40	0.36	0.24	0.25
MYB2	1.08	1.27	0.25	0.16	0.18	1.50	1.37	0.36	0.23	0.24
WRKY-R2B	1.10	1.26	0.24	0.16	0.17	1.53	1.33	0.33	0.21	0.22
ABP1-1	1.16	1.32	0.27	0.18	0.21	1.56	1.47	0.40	0.27	0.29
MADS-1	1.04	1.23	0.21	0.14	0.15	1.56	1.40	0.37	0.24	0.26
Mean	1.13	1.28	0.25	0.17	0.19	1.58	1.41	0.38	0.25	0.26
SCoT markers	Species					Genus				
	Na	Ne	I	He	uHe	Na	Ne	I	He	uHe
SCoT3	1.23	1.32	0.28	0.19	0.21	1.61	1.46	0.41	0.27	0.29
SCoT12	1.27	1.30	0.27	0.18	0.20	1.71	1.45	0.41	0.27	0.29
SCoT13	1.15	1.31	0.27	0.18	0.21	1.77	1.44	0.42	0.27	0.29
SCoT14	1.14	1.31	0.28	0.18	0.21	1.58	1.39	0.37	0.24	0.25
SCoT15	1.35	1.34	0.32	0.21	0.24	1.75	1.43	0.42	0.27	0.29
SCoT19	1.14	1.32	0.28	0.19	0.21	1.57	1.43	0.39	0.26	0.27
SCoT20	1.31	1.35	0.31	0.21	0.23	1.71	1.52	0.46	0.31	0.33
SCoT21	1.03	1.26	0.23	0.15	0.17	1.46	1.37	0.34	0.22	0.23
SCoT23	1.08	1.29	0.24	0.16	0.18	1.55	1.45	0.40	0.27	0.28
SCoT24	1.11	1.27	0.25	0.16	0.18	1.38	1.33	0.31	0.20	0.21
SCoT34	1.19	1.34	0.29	0.20	0.23	1.58	1.41	0.38	0.25	0.26
SCoT35	0.98	1.24	0.22	0.15	0.17	1.40	1.31	0.30	0.19	0.20
Mean	1.17	1.30	0.27	0.18	0.20	1.59	1.42	0.38	0.25	0.27

Na: number of observed alleles, Ne: effective number of alleles, He: expected heterozygosity or gene diversity, I: Shannon's information index, and uHe: unbiased expected heterozygosity

3.3. Genetic diversity parameters among different populations (species and genera) of mushroom strains

Gene diversity indicators at the population level (species and genera) exhibited variation among mushroom strains, as detailed in tables 2 and 3. The genetic diversity indices Na, Ne, I, He, and uHe for the species measured varied from 0.45 (*A. aegerita*) to 1.82 (*A. litoralis*), 1.04 (*A. aegerita*) to 1.47 (*A. vittadini*), 0.00 (*A. bisporus*) to 0.43 (*A. litoralis*), 0.00 (*A. bisporus*) to 0.28 (*A. litoralis*), and 0.00 (*A. bisporus*) to 0.30 (*A. litoralis*), respectively. The measured genera' genetic diversity indices were 1.40

(*Pleurotus*) to 1.89 (*Agaricus*), 1.29 (*Agrocybe*) to 1.49 (*Amanita*), 0.27 (*Agrocybe*) to 0.46 (*Amanita*), 0.17 (*Agrocybe*) to 0.30 (*Amanita*), and 0.19 (*Agrocybe*) to 0.31 (*Amanita*), respectively. According to CDDP data, the average proportion of polymorphic loci (PPL) was 45.23% across 11 species and 75.98% across 4 genera, with species ranging from 0.00% (*A. bisporus*) to 91.18% (*A. litoralis*) and genera ranging from 52.45% (*Agrocybe*) to 94.61% (*Agaricus*). The average number of private bands across all species was 0.36, ranging from 1 (*A. dura*) to 2 (*A. litoralis*), but the average number of private bands

across all genera was 2.75, with a range from 1 (*Agrocybe* and *Pleurotus*) to 5 (*Agaricus*).

Based on the SCoT data, the maximum Na, Ne, I, He, and uHe of 1.75, 1.43, 0.41, 0.27, and 0.28 were recorded with *A. litoralis*, respectively. The highest PPL of 87.57% was seen with *A. litoralis*. The average number of private

bands across all species was 0.36, ranging from 1 (*A. litoralis*) to 3 (*A. vittadinii*). At the genus level, the greatest values for Na, Ne, I, He, uHe, and PPL were 1.90, 1.48, 0.46, 0.30, 0.30, and 94.67% for the genus *Amanita*. A wide range of 2 to 5 specific bands appeared across all genera.

Table 2. Genetic diversity indices of various species and genera identified in a collection of 63 mushrooms based on CDDP data.

Species							
Species	Na	Ne	I	He	uHe	PPL	PB
<i>A. campestris</i>	1.01	1.27	0.23	0.16	0.19	42.16	0.00
<i>A. litoralis</i>	1.82	1.45	0.43	0.28	0.30	91.18	2.00
<i>A. pseudolutosus</i>	1.28	1.35	0.32	0.21	0.23	59.31	0.00
<i>A. aegerita</i>	0.45	1.04	0.03	0.02	0.03	5.39	0.00
<i>A. dura</i>	0.50	1.06	0.06	0.04	0.05	11.76	1.00
<i>A. crocea</i>	1.50	1.36	0.33	0.21	0.23	71.57	0.00
<i>A. lividopallescens</i>	1.32	1.38	0.33	0.22	0.24	62.25	1.00
<i>A. vittadinii</i>	1.67	1.47	0.42	0.27	0.29	82.35	0.00
<i>P. columbinus</i>	0.73	1.16	0.14	0.10	0.13	23.04	0.00
<i>P. eryngii</i>	1.13	1.31	0.27	0.18	0.22	48.53	0.00
<i>A. bisporus</i>	1.00	1.26	0.00	0.00	0.00	0.00	0.00
Mean	1.13	1.28	0.23	0.15	0.17	45.23	0.36
Genus							
Genus	Na	Ne	I	He	uHe	PPL	PB
<i>Agaricus</i>	1.89	1.46	0.44	0.29	0.29	94.61	5.00
<i>Agrocybe</i>	1.18	1.29	0.27	0.17	0.19	52.45	1.00
<i>Amanita</i>	1.85	1.49	0.46	0.30	0.31	92.65	4.00
<i>Pleurotus</i>	1.40	1.39	0.34	0.23	0.26	64.22	1.00
Mean	1.58	1.41	0.38	0.25	0.26	75.98	2.75

Na: number of observed alleles, Ne: effective number of alleles, He: expected heterozygosity or gene diversity, I: Shannon's information index, uHe: unbiased expected heterozygosity, PPL: percentage of polymorphic loci, and PB: number of private bands.

Table 3. Genetic diversity indicators of different species and genera observed in a collection of 63 determined by data from SCoT markers.

Species							
Species	Na	Ne	I	He	uHe	PPL	PB
<i>A. campestris</i>	1.10	1.31	0.27	0.18	0.22	46.75	0.00
<i>A. litoralis</i>	1.75	1.43	0.41	0.27	0.28	87.57	1.00
<i>A. pseudolutosus</i>	1.44	1.40	0.35	0.24	0.26	68.05	0.00
<i>A. aegerita</i>	0.69	1.15	0.13	0.09	0.12	21.30	0.00
<i>A. dura</i>	0.75	1.19	0.16	0.11	0.13	27.81	0.00
<i>A. crocea</i>	1.57	1.45	0.39	0.26	0.27	76.33	0.00
<i>A. lividopallescens</i>	1.43	1.37	0.34	0.22	0.24	68.05	0.00
<i>A. vittadinii</i>	1.64	1.45	0.40	0.27	0.28	81.07	3.00
<i>P. columbinus</i>	0.46	1.05	0.05	0.03	0.04	7.69	0.00
<i>P. eryngii</i>	0.98	1.27	0.24	0.16	0.19	42.01	0.00
<i>A. bisporus</i>	0.95	1.26	0.00	0.00	0.00	0.00	0.00
Mean	1.16	1.30	0.25	0.17	0.18	47.88	0.36
Genus							
Genus	Na	Ne	I	He	uHe	PPL	PB
<i>Agaricus</i>	1.87	1.46	0.44	0.29	0.29	93.49	2.00
<i>Agrocybe</i>	1.24	1.34	0.30	0.20	0.22	57.40	0.00
<i>Amanita</i>	1.90	1.48	0.46	0.30	0.30	94.67	5.00
<i>Pleurotus</i>	1.31	1.36	0.32	0.22	0.24	60.95	0.00
Mean	1.58	1.41	0.38	0.25	0.26	76.63	1.75

Na: number of observed alleles, Ne: effective number of alleles, He: expected heterozygosity or gene diversity, I: Shannon's information index, uHe: unbiased expected heterozygosity, PPL: percentage of polymorphic loci, and PB: number of private bands.

3.4. Molecular variation among different species and genera of mushroom strains

Analysis of molecular variance (AMOVA) was performed to evaluate the genetic variation among the species and genera identified from mushroom samples (Table 4). The variance analysis of CDDP and SCoT data indicated that 23.74% and 10.82% of the genetic variation

stemmed from interspecific variation, respectively, while 76.26% and 89.18% derived from intraspecific variation, respectively, based on the CDDP and SCoT data. The PhiPT genus and species values of CDDP data were 0.11 and 0.24, respectively, whereas the PhiPT SCoT data were 0.10 and 0.21 for genus and species, respectively.

Table 4. Analysis of molecular variance among several species and taxa based on CDDP and SCoT marker data

CDDP markers						
Source	Degree of freedom	Sum square	Mean square	Estimated variance	Percentage of variance	PhiPT
Among species	10.00	893.80	89.38	10.37	23.74	0.24
Within species	52.00	1732.93	33.33	33.33	76.26	
Total	62.00	2626.73		43.70	100.00	
Genus						
Among genus	3.00	308.29	102.76	4.77	10.82	0.11
Within genus	59.00	2318.44	39.30	39.30	89.18	
Total	62.00	2626.73		44.07	100.00	
SCoT markers						
Species						
Among species	10.00	679.31	67.93	7.38	20.81	0.21
Within species	52.00	1459.86	28.07	28.07	79.19	
Total	62.00	2139.17		35.45	100.00	
Genus						
Among genus	3.00	242.04	80.68	3.65	10.19	0.10
Within genus	59.00	1897.13	32.15	32.15	89.81	
Total	62.00	2139.17		35.80	100.00	

In relation to the CDDP data, Nei's genetic distances among species ranged from 0.07 (between *A. vittadinii* and *A. litoralis*) to 0.48 (between *A. lividopallescens* and *A. aegerita*) (Supplementary file, Table S3), whereas Nei's genetic dissimilarity among four genera varied from 0.03 (between *Amanita* and *Agaricus*) to 0.21 (between *Pleurotus* and *Agrocybe*). In terms of the species and genera, Nei's genetic distances derived from the SCoT data revealed the greatest distance between *P. columbinus* and *A. aegerita* (0.36), while the smallest distance was noted between *A. vittadinii* and *A. litoralis* (0.06). *Pleurotus* and *Agrocybe* exhibited the highest Nei's genetic dissimilarity at 0.16, while *Amanita* and *Agaricus* demonstrated the lowest at 0.04 (Supplementary file, Table S4).

3.5. Relationships and clustering of mushroom strains

The findings of the clustering and PCA plots generated from CDDP data indicated that the 63 strains were predominantly dispersed and categorized into seven principal groups (Figure 7A and B). The last group, designated as the seventh group and marked in a red circle, encompassed the majority of strains, totaling 24 strains. The clustering and PCA plots distinctly categorized 11 species into three clades at the species level. The PCA

results for mushroom species samples revealed that the first two principal components represented 46.60% and 25.80% of the variation, respectively, for a cumulative contribution rate of 72.40%. Clade I, indicated by a red line, has six species: *A. campestris*, *A. litoralis*, *A. pseudolutosus*, *A. crocea*, *A. lividopallescens*, and *A. vittadinii*. Clade II possessed three species: *P. columbinus*, *P. eryngii*, and *A. bisporus*. *A. aegerita* and *A. dura* constituted the third clade (Figure 7C, D, and E). The WRKYF1 marker displayed the most variance across the three clades, followed by the WRKY-R3, ERF2, and MADS-1 markers, while the MYB1 marker demonstrated the least variation among the three clades (Figure 7F). The clustering results, as shown in figure 8, led to the classification of the 63 strains into four categories, generating the four genera. The PCA results for mushroom genera samples indicated that the first two principal components accounted for 59.30% and 40.5% of the variation, respectively, generating a cumulative contribution rate of 99.80%. The initial group comprised two genera: *Amanita* and *Agaricus*. The second and third categories consisted of the *Pleurotus* and *Agrocybe* genera, respectively (Figure 8).

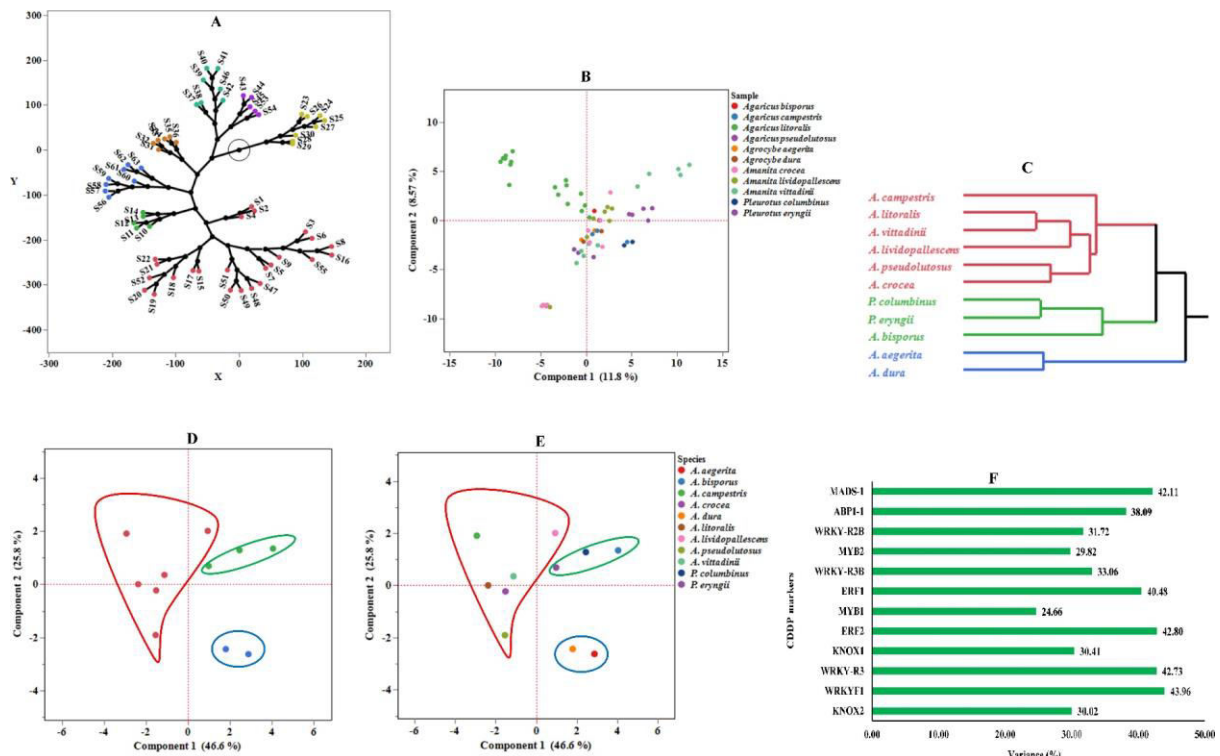


Figure 7. Clustering and interrelationships among 63 strains from various species and genera of mushrooms based on the CDDP data. A and B: Clustering and PCA plots illustrating the distribution and grouping of various strains; C, D, and E: grouping and PCA plots representing the classification of separate mushroom species; F: percentage of variation recorded by CDDP marker. S1-S63 represents the 63 mushroom strains.

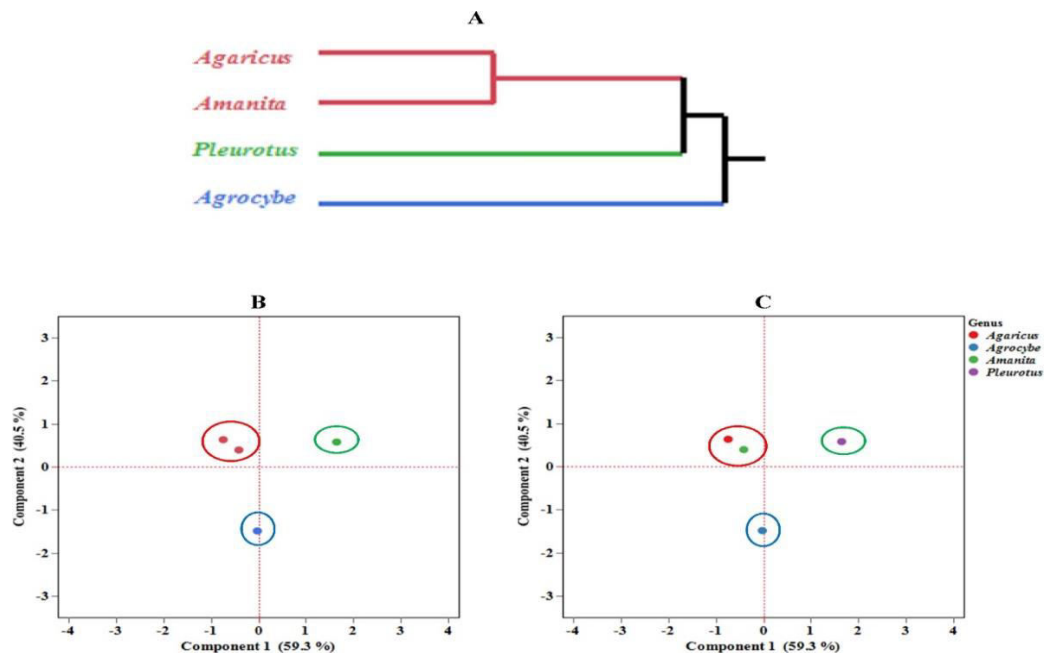


Figure 8. Clustering and interrelationships across four genera of mushrooms observed across 63 strains based on the CDDP data.

The results from the dendrogram and PCA plots derived from SCoT data revealed that the 63 strains were primarily distributed and classified into seven main categories (Figure 9A and B). The last group, identified as the seventh group and highlighted in red, included the majority of strains, reaching 19 strains. The clustering and PCA plots clearly divided 11 species into three clades at the species level. The PCA results for mushroom species samples revealed that the first two principal components represented 45.50% and 22.30% of the variation, respectively, for a cumulative contribution rate of 67.80%.

Clade I, denoted by a red line, comprises five species: *A. campestris*, *A. littoralis*, *A. crocea*, *A. lividopallescens*, and *A. vittadinii*. *A. pseudolutosus*, *A. aegerita*, and *A. dura* formed clade II, indicated by the green line. Clade III, indicated by a blue line, comprised three species: *P. columbinus*, *P. eryngii*, and *A. bisporus* (Figure 9C, D, and E). The SCoT13 marker exhibited the highest variance (38.26%) across the three clades, followed by the SCoT21 marker (37.20%), whilst the SCoT3 marker revealed the lowest variation (26.68%) among the three clades (Figure 9F). The four genera were established from the 63 strains

categorized into four classifications based on the results. The PCA results for mushroom genera samples indicated that the first two principal components accounted for 70.00% and 29.99% of the variation, respectively,

generating a cumulative contribution rate of 99.99%. The original group consisted of two genera: *Amanita* and *Agaricus*. The second and third categories comprised the *Pleurotus* and *Agrocybe* genera, respectively (Figure 10).

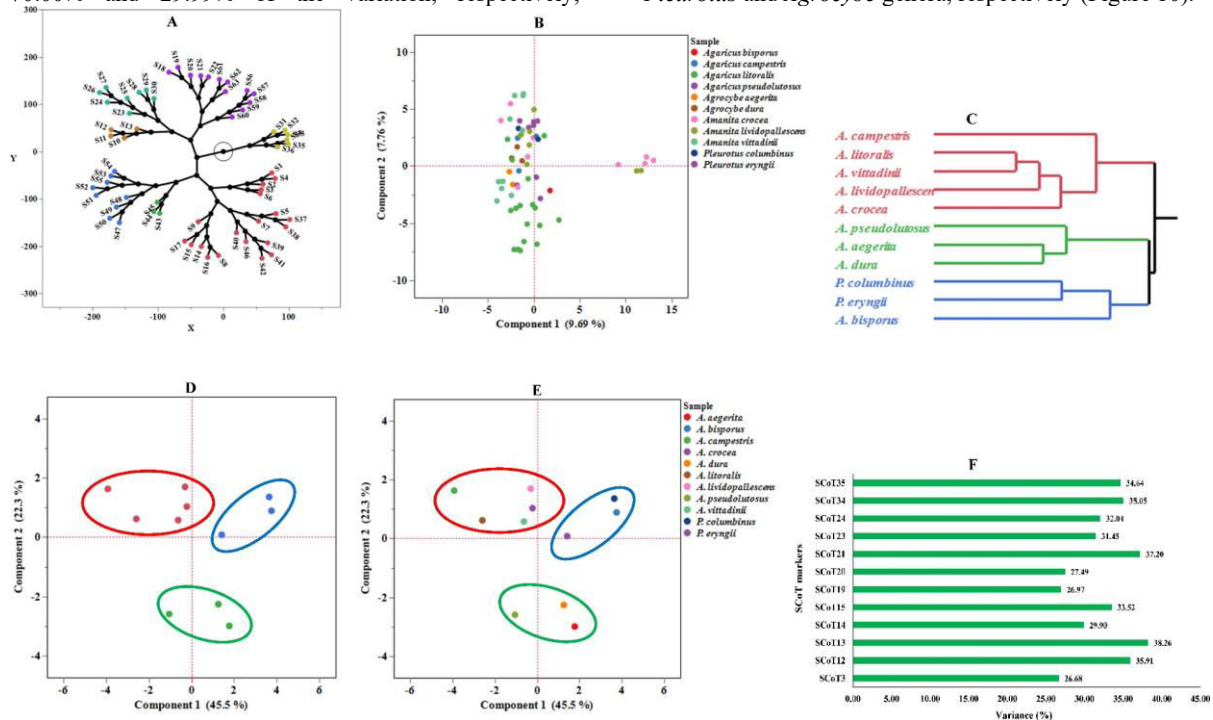


Figure 9. Clustering and relationships among 63 strains from several species and genera of mushrooms based on the SCoT data. A and B: Clustering and PCA plots depicting the dispersion and clustering of diverse strains; C, D, and E: clustering and PCA plots displaying the classification of distinct mushroom species; F: proportion of variation documented by SCoT markers. S1-S63 denote the 63 mushroom strains.

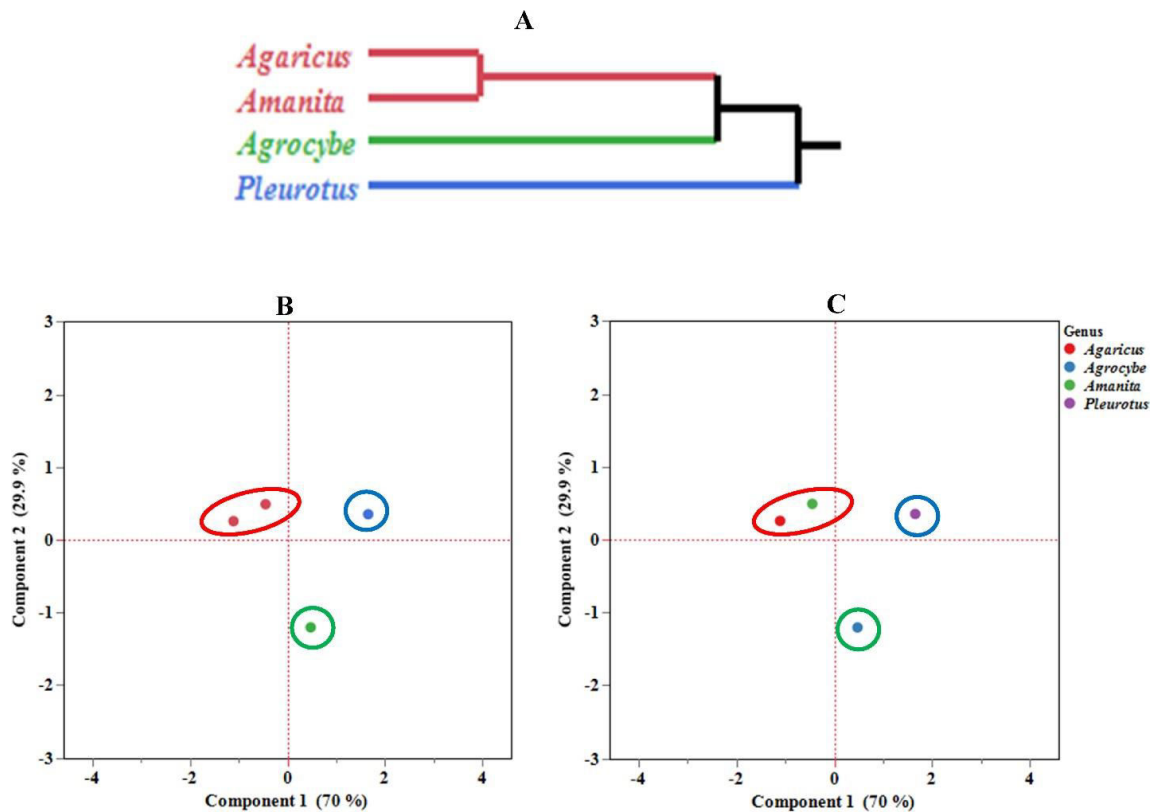


Figure 10. Grouping and relationships over four genera of mushrooms assessed 63 strains based on the SCoT data.

4. Discussion

Research on any kind of organism cannot proceed without first establishing an accurate taxonomy. Conventional mushroom identification relies on morphological characteristics such as fruit body size, shape, length, diameter, and any other distinguishing characteristics. The contradiction in macrofungi taxonomy at the species and genus levels has been corroborated by the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, encompassing ITS1 and ITS4. The current study involves the PCR product of mushrooms, measuring roughly 550-750 base pairs. Fujita *et al.* (2001) documented fragment sizes ranging from 350 to 880 bp for fungi using ITS1 and ITS4. Following PCR amplification using ITS1 and ITS4 primers, Adeniyi *et al.* (2018) reported a product of approximately 850 bp. Ab Rhaman *et al.* (2021) reported an amplicons size between 500 and 800 bp among mushroom species. The DNA gel electrophoresis of eight species with a molecular weight of 700–750 bp was presented in a study by Jalil *et al.* (2025).

Our study attempted to resolve the phylogenetic relationship of the 63 strains invested in the region of Iraqi Kurdistan using the ITS rDNA. Eleven species distributed in four genera were revealed by this study from the collected samples of wild mushrooms. The second group of this study included strains from the genera *Agaricus*, *Amanita*, and *Agrocybe*, whereas the *Pleurotus* strains were placed in the first group. The strains of species in the *Amanita* genus were stratified in the beginning subgroup of the second group. Subsequently, the species that were placed under the genera *Agaricus* and *Agrocybe* were clustered together with the same secondary subgroup. The second group also consists of the cultivated mushroom *A. bisporus*. The ITS region of the strains in the same clade group was, however, highly homogenous within the group, which is the reason for putting them grouped together (Stackebrandt *et al.*, 2002). Moreover, the bootstrap in this investigation was 9 and 100%. The BLASTn analysis of the ITS data showed that the dominant strains in all the identified mushroom samples corresponded to *A. litoralis*, followed by *A. vittadinii* and *A. crocea*, which indicates that the species adapt to different environments.

Understanding genetic diversity within and between species or genera is essential for creating efficient conservation management strategies for endangered and threatened species. There is a broad and varied genetic availability of wild mushroom strains in Iraqi Kurdistan. In order to find resources, find new genes, and breed new types, it is essential to study the genetic diversity of germplasms. Since critical mushroom species are still rarely bred, most cultivated strains are derived from tamed wild varieties. Problems like recurrent strains and unclear genetic links across varieties emerged as a consequence of the limitations in genetic diversity between parent strains brought about by isolating tissue culture and rebranding. Genomic research into the relationships between wild mushroom strains collected from various parts of Iraqi Kurdistan is crucial. Future breeding and use of germplasm also necessitate genetic diversity studies among strains (Gu *et al.*, 2024; Tahir *et al.*, 2015). Conventional molecular markers may not consistently elucidate phenotypic variations as effectively as genetic

diversity assessment using CDDP and SCoT. There has been no prior mention of applying CDDP and SCoT to create fingerprints of wild mushroom germplasm for the analysis of genetic associations (Gao *et al.*, 2018). Fungi typically exhibit significant levels of genetic variety in their wild populations when they are sexually reproducing, inhabiting diverse ecological niches, and/or found all over the world. This investigation used two different types of molecular markers to generate 373 bands. Comparatively, SCoT primers detected 14.08 polymorphic bands from various markers, but CDDP primers averaged 17 bands. Polymorphism in various parts of the mushroom's genome likely causes this variation, which is contributed by SCoT markers that specifically target the highly conserved region surrounding the start codon (ATG) in mushroom genes, while CDDP markers amplify portions of conserved functional genes (transcription factors). The high levels of polymorphism exhibited by various markers clearly indicated their applicability in genetic variability investigations in wild mushroom strains, and they could be used to identify plant materials that are related in different species and genera (Golian *et al.*, 2022; Khandayataray *et al.*, 2022).

Gene diversity determines the degree of genetic variety within breeding population accessions. The primary focus here is the evolutionary restrictions on alleles and the probable mutation rate for a certain locus. Genomic diversity reflects genetic diversity for haploid markers and provides an estimate of anticipated heterozygosity and genetic distance among population members (Salem and Sallam, 2016). Several researchers used the molecular markers and demonstrated their usefulness in evaluating the genetic diversity of the species (Khal *et al.*, 2023; Akhtar *et al.*, 2021; Shekhar *et al.*, 2014). Gene-targeted markers are especially useful for studies that seek to measure functional genetic diversity because they sample genomic regions that are important for traits or subject to selection more directly than anonymous markers, which mainly represent neutral variation. The purpose of gene-targeted markers like SCoT and CDDP is to amplify regions of DNA linked to coding or regulatory sequences. These areas are more likely to be linked to adaptive responses and phenotypic traits, which offer information on genetic diversity that is essential for selection and functionality (Platten *et al.*, 2019).

Our research indicates that CDDP and SCoT markers are effective for assessing genetic diversity in mushroom samples and validating the presence of significant genetic variation. In this investigation, the use of SCoT and CDDP markers with polymorphic variation possessed higher PIC parameters. This allowed for efficient selection of the most appropriate markers for the research on genetic divergence. A diversity index allows assigning strain members to different groups, enabling a quantitative measure of the diversity of the species or genus range. Variegated indices are used to indicate the differing degrees of diversity, where lower values indicate low diversity while higher values suggest high diversity (Oluyinka Christopher, 2020). There is a notable amount of variability in SCoT genome analysis between species. For instance, Shannon's information index mean value 'I', which is calculated from SCoT markers, is more than that which is computed from CDDP markers, suggesting that there exist much variation and ecological complexity

impacting the SCoT loci target area. Expected heterozygosity or gene diversity values were mostly low for both species and genus for most markers, supporting the evidence from AMOVA analysis of high diversity among various species. Average gene diversity values for 4 genera of mushrooms were higher than those of 11 species of mushrooms: 0.30 for both markers versus 0.17 and 0.18 for CDDP and SCoT, respectively. Results of AMOVA analysis demonstrated that the PhiPT value for CDDP data was higher than that of the SCoT marker, which means that CDDP markers are more informative than the SCoT approach for revealing the variation between different taxa. High variability within the species and genus of mushrooms, based on CDDP and SCoT data, in this study suggests that strains of the same species present significant variation, indicating the effects of environmental factors on the strains' genetic structure. Mushroom species require molecular markers for genetic characterization in order to better understand the variety of mushrooms available and develop strategies for their breeding (Lee *et al.*, 2019; Khatun *et al.*, 2017; Singh and Gupta, 2017). With CDDP data, our study found that the genus *Agaricus* had the largest number of unique bands (5), while the genus *Amanita* had only four. On the other hand, SCoT data identified four maximum specific bands linked to the *Amanita* genus. This study postulates that these unique bands associated with the *Agaricus* genus could potentially serve as biomarkers for differentiating strains of mushrooms across different taxa. In addition, these unique bands can be linked to phenotypic and other enhancements and to the construction of a new marker that evaluates genetic diversity (Jiao *et al.*, 2024; Liu *et al.*, 2022). Cluster analyses are important in aiding interspecies mapping investigations because they assist in understanding genetic diversity (Eltaher *et al.*, 2018). The dendrogram produced confirms a large diversity of mushroom strains and taxa. The diversity among strains, species, and genera points to an abundance of genetic material and an elevated amount of genetic variation. The CDDP markers had a wider spectrum of dissimilarities than the SCoT markers, indicating a high degree of structural variability in transcription factor genes. It was considered more effective to discriminate between the strains of different taxa and genera using markers CDDP and SCoT. The *A. litoralis* isolates located within the Basneh region were determined from the CDDP plot (group 1; olive circle), while those found in the Penjwen region belonged to group 5 (mint circle). All *A. pseudolutosus* strains ranged within the same group (group 3; teal circle). The isolates of *A. vittadinii* were positioned in two groups: group 2 (purple circle) and group 5 (blue circle). The different SCoT markers used in the clustering plot depicted the allocation of strains of different species and genera into different clades; for example, the *A. litoralis* strains collected from different areas of the regions were arranged in clades 3 and 4. The differences existing among strains of a particular mushroom species collected from different regions can be largely accounted for by the different environmental conditions of those regions. These environmental factors have an influence on not only the growth of mushrooms but also their productivity as well as the genetic diversity and adaptability of the fungi (Vieira Junior *et al.*, 2022; Bellettini *et al.*, 2019). This diversity in strains distribution

denotes a considerable level of genetic variation existing among members of the same taxa.

One of the reasons for the discrepancy between the CDDP and SCoT clustering, as well as with the ITS method, is that different techniques increase the genomic variation of the different strains of the same taxon and genus. Gap spacer DNA that is amplified by the universal barcode sequence (ITS) separates the large subunit rRNA from the small subunit rRNA genes on the chromosome. SCoT markers focus on the highly conserved area not far from the ATG in the fungal genes, while CDDP markers focus on the regions of conserved functional genes, which are transcription factors (Aziz and Tahir, 2023; Khal *et al.*, 2023; Tahir *et al.*, 2023).

5. Conclusions

In this study, the molecular phylogenetic analysis was found to be useful for tracing the relationships of the 63 strains of mushrooms from the Iraqi Kurdistan region. According to the clustering analysis, there is a high level of divergence in both the species and genus levels. The species of mushrooms displayed the presence of AT content in greater quantities compared to the GC content. The genetic relationships of mushroom samples at the strain, species, and genus levels are highly facilitated by the CDDP and SCoT markers, which are very efficient in polymorphism detection. The results also verified the possibility of markers CDDP and SCoT being able to differentiate strains of the same species from different regions. In Iraq, there are still many wild types of mushrooms that are yet to be studied, and there is a need to carry out research that aims to discover new species in this area. The study of mushroom strains should involve studying not just the rDNA ITS region and some molecular markers but also the morphological and chemical profile of the mushroom samples to elucidate evolutionary relations among the strains, species, and genera.

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Mushroom accession numbers

The profiles of strain DNA sequences scrutinized in this study can be found in the databases of the National Center for Biotechnology Information.

Contribution statement

Nawroz A. Tahir: Supervision, Resources, Project administration, Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Conceptualization, and Validation. Kazhan M. Sleman: Data curation, Methodology, and Investigation. Writing – review & editing, Visualization.

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Declaration of competing interest

The authors affirm that they have no known opposing financial interests or personal relations that could have seemed to affect the data described in this article.

Footnote

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Supplementary data

**Figure S1.** Wild and cultivated mushrooms species.**Table S1.** Name, strains, accessions number, and altitudes of different mushroom samples collected from different locations.

Location of sampling	Number of samples	Code	Identified sample	Strain	Average of altitude (m)	Accession number
Qaladiza	8	S1	<i>Agrocybe aegerita</i>	AgA-QAL-1	586	PQ679358
Qaladiza		S2	<i>Agrocybe dura</i>	AgD-QAL-2		PQ680084
Qaladiza		S3	<i>Agrocybe dura</i>	AgD-QAL-3		PQ680083
Qaladiza		S4	<i>Agrocybe aegerita</i>	AgA-QAL-4		PQ680085
Qaladiza		S5	<i>Agaricus campestris</i>	AgC-QAL-5		PQ679044
Qaladiza		S6	<i>Agrocybe dura</i>	AgD-QAL-6		PQ679043
Qaladiza		S7	<i>Agaricus campestris</i>	AgaC-QAL-7		PQ679045
Qaladiza		S8	<i>Agaricus campestris</i>	AgaC-QAL-8		PQ679046
Penjwen	8	S9	<i>Agaricus litoralis</i>	AgaL-PEN-9	1272	PQ680647
Penjwen		S10	<i>Agaricus litoralis</i>	AgaL-PEN-10		PQ680646
Penjwen		S11	<i>Agaricus litoralis</i>	AgaL-PEN-11		PQ680649
Penjwen		S12	<i>Agaricus litoralis</i>	AgaL-PEN-12		PQ680655
Penjwen		S13	<i>Agaricus litoralis</i>	AgaL-PEN-13		PQ680656
Penjwen		S14	<i>Agaricus litoralis</i>	AgaL-PEN-15		PQ680668
Dukan	8	S15	<i>Amanita crocea</i>	AmC-DUK-17	798	PQ686237
Dukan		S16	<i>Amanita crocea</i>	AmC-DUK-18		PQ686016
Dukan		S17	<i>Amanita lividopallescens</i>	AmL-DUK-19		PQ685979
Dukan		S18	<i>Amanita lividopallescens</i>	AmL-DUK-20		PQ681289
Dukan		S19	<i>Amanita crocea</i>	AmC-DUK-21		PQ681290
Dukan		S20	<i>Pleurotus eryngii</i>	PIE-DUK-22		PQ681292
Dukan		S21	<i>Pleurotus eryngii</i>	PIE-DUK-23		PQ685981

Dukan		S22	<i>Pleurotus eryngii</i>	PIE-DUK-24		PQ681296
Basneh		S23	<i>Agaricus litoralis</i>	AgaL-BAS-25		PQ682616
Basneh		S24	<i>Agaricus litoralis</i>	AgaL-BAS-26		PQ682630
Basneh		S25	<i>Agaricus litoralis</i>	AgaL-BAS-27		PQ682658
Basneh	8	S26	<i>Agaricus litoralis</i>	AgaL-BAS-28	1573	PQ682661
Basneh		S27	<i>Agaricus litoralis</i>	AgaL-BAS-29		PQ682665
Basneh		S28	<i>Agaricus litoralis</i>	AgaL-BAS-30		PQ682666
Basneh		S29	<i>Agaricus litoralis</i>	AgaL-BAS-31		PQ683183
Basneh		S30	<i>Agaricus litoralis</i>	AgaL-BAS-32		PQ685808
Zewe		S31	<i>Amanita crocea</i>	AmC-ZEW-33		PQ686266
Zewe		S32	<i>Amanita lividopallescens</i>	AmL-ZEW-34		PQ686317
Zewe		S33	<i>Amanita lividopallescens</i>	AmL-ZEW-35		PQ686318
Zewe	8	S34	<i>Amanita crocea</i>	AmC-ZEW-36	1583	PQ686633
Zewe		S35	<i>Amanita crocea</i>	AmC-ZEW-37		PQ686958
Zewe		S36	<i>Amanita crocea</i>	AmC-ZEW-38		PQ686964
Zewe		S37	<i>Pleurotus columbinus</i>	PIC-ZEW-39		PQ686965
Zewe		S38	<i>Pleurotus columbinus</i>	PIC-ZEW-40		PQ687023
Ranya		S39	<i>Agaricus pseudolutosus</i>	AgaP-RAN-41		PQ720781
Ranya		S40	<i>Agaricus pseudolutosus</i>	AgaP-RAN-42		PQ720782
Ranya		S41	<i>Agaricus pseudolutosus</i>	AgaP-RAN-43		PQ722530
Ranya	8	S42	<i>Agaricus pseudolutosus</i>	AgaP-RAN-44	882	PQ722533
Ranya		S43	<i>Amanita vittadinii</i>	AmV-RAN-45		PQ720786
Ranya		S44	<i>Amanita vittadinii</i>	AmV-RAN-46		PQ720789
Ranya		S45	<i>Amanita vittadinii</i>	AmV-RAN-47		PQ720788
Ranya		S46	<i>Agaricus pseudolutosus</i>	AgaP-RAN-48		PQ722534
Qaradagh		S47	<i>Amanita crocea</i>	AmC-QAR-49		PQ720780
Qaradagh		S48	<i>Amanita lividopallescens</i>	AmL-QAR-50		PQ720981
Qaradagh		S49	<i>Amanita lividopallescens</i>	AmL-QAR-51		PQ721015
Qaradagh		S50	<i>Amanita lividopallescens</i>	AmL-QAR-52		PQ721021
Qaradagh	8	S51	<i>Amanita lividopallescens</i>	AmL-QAR-53	1708	PQ721023
Qaradagh		S52	<i>Amanita crocea</i>	AmC-QAR-54		PQ721027
Qaradagh		S53	<i>Amanita vittadinii</i>	AmV-QAR-55		PQ722535
Qaradagh		S54	<i>Amanita vittadinii</i>	AmV-QAR-56		PQ721036
Qaradagh		S55	<i>Agaricus litoralis</i>	AgaL-QAR-57		PQ721038
Halabja		S56	<i>Amanita vittadinii</i>	AmV-HAL-58		PQ721310
Halabja		S57	<i>Amanita vittadinii</i>	AmV-HAL-59		PQ721312
Halabja		S58	<i>Amanita vittadinii</i>	AmV-HAL-60		PQ721315
Halabja	8	S59	<i>Amanita vittadinii</i>	AmV-HAL-61	900	PQ721317
Halabja		S60	<i>Amanita vittadinii</i>	AmV-HAL-62		PQ721318
Halabja		S61	<i>Agaricus litoralis</i>	AgaL-HAL-63		PQ721319
Halabja		S62	<i>Agaricus litoralis</i>	AgaL-HAL-64		PQ721320
Sulaimani Center	1	S63	<i>Agaricus bisporus</i>	AgaB-SC-65	882	PQ895553

Table S2 Sequences and annealing temperatures of ITS gene, CDDP and SCoT markers.

Primers	Sequence (5' to 3')	Annealing temperature (°C)
ITS gene		
ITS 1	TCCGTAGGTGAACCTGCGG	55
ITS 4	TCCTCCGCTTA TTGATATGC	55
CDDP markers		
KNOX2	CACTGGTGGGAGCTSCAC	50
WRKYF1	TGGCGSAAGTACGGCCAG	50
WRKY-R3	GCASGTGTGCTCGCC	50
KNOX1	AAGGSAAGCTSCCSAAG	50
ERF2	GCSGAGATCCGSGACCC	50
MYB1	GGCAAGGGCTGCCGC	50
ERF1	CACTACCCCGSCTSCG	50
WRKY-R3B	CCGCTCGTGTGSACG	50
MYB2	GGCAAGGGCTGCCGG	50
WRKY-R2B	TGSTGSATGCTCCCG	50
ABP1-1	ACSCCSATCCACCGC	50
MADS-1	ATGGGCCGSGGCAAGGTGC	50
SCoT markers		
SCoT3	CAACAATGGCTACCACCG	51.27
SCoT12	ACGACATGGCGACCAACG	55.93
SCoT13	ACGACATGGCGACCATCG	55.39
SCoT14	ACGACATGGCGACCACGC	58.6
SCoT15	ACGACATGGCGACCGCGA	59.9
SCoT19	ACCATGGCTACCACCGGC	57.1
SCoT20	ACCATGGCTACCACCGCG	57.5
SCoT21	ACGACATGGCGACCCACA	56.7
SCoT23	CACCATGGCTACCACCAG	52.43
SCoT24	CACCATGGCTACCACCAT	51.6
SCoT34	ACCATGGCTACCACCGCA	56.3
SCoT35	CATGGCTACCACCGGCC	57.9

Table S3. Nei's genetic distance among different species (A) and genera (B) of mushroom samples based on CDDP data.

A

Species	<i>A. campestris</i>	<i>A. litoralis</i>	<i>A. pseudolutosus</i>	<i>A. aegerita</i>	<i>A. dura</i>	<i>A. crocea</i>	<i>A. lividopallescens</i>	<i>A. vittadinii</i>	<i>P. columbinus</i>	<i>P. eryngii</i>	<i>A. bisporus</i>
<i>A. campestris</i>	0.00										
<i>A. litoralis</i>	0.15	0.00									
<i>A. pseudolutosus</i>	0.20	0.10	0.00								
<i>A. aegerita</i>	0.43	0.30	0.16	0.00							
<i>A. dura</i>	0.35	0.24	0.16	0.09	0.00						
<i>A. crocea</i>	0.23	0.12	0.11	0.30	0.28	0.00					
<i>A. lividopallescens</i>	0.27	0.14	0.23	0.48	0.40	0.21	0.00				
<i>A. vittadinii</i>	0.16	0.07	0.13	0.31	0.27	0.16	0.14	0.00			
<i>P. columbinus</i>	0.28	0.25	0.26	0.42	0.38	0.25	0.34	0.24	0.00		
<i>P. eryngii</i>	0.23	0.16	0.20	0.35	0.32	0.20	0.26	0.16	0.08	0.00	
<i>A. bisporus</i>	0.32	0.26	0.30	0.40	0.35	0.30	0.32	0.27	0.28	0.17	0.00

B

Genus	<i>Agaricus</i>	<i>Agrocybe</i>	<i>Amanita</i>	<i>Pleurotus</i>
<i>Agaricus</i>	0.00			
<i>Agrocybe</i>	0.11	0.00		
<i>Amanita</i>	0.03	0.15	0.00	
<i>Pleurotus</i>	0.12	0.21	0.12	0.00

Table S4. Nei's genetic distance among various species (A) and genera (B) of mushroom samples derived from the SCoT data.

A

Species	<i>A. campestris</i>	<i>A. litoralis</i>	<i>A. pseudolutosus</i>	<i>A. aegerita</i>	<i>A. dura</i>	<i>A. crocea</i>	<i>A. lividopallescens</i>	<i>A. vittadinii</i>	<i>P. columbinus</i>	<i>P. eryngii</i>	<i>A. bisporus</i>
<i>A. campestris</i>	0.00										
<i>A. litoralis</i>	0.14	0.00									
<i>A. pseudolutosus</i>	0.20	0.11	0.00								
<i>A. aegerita</i>	0.31	0.22	0.15	0.00							
<i>A. dura</i>	0.29	0.18	0.16	0.11	0.00						
<i>A. crocea</i>	0.24	0.11	0.17	0.29	0.23	0.00					
<i>A. lividopallescens</i>	0.21	0.09	0.21	0.33	0.28	0.14	0.00				
<i>A. vittadinii</i>	0.20	0.06	0.14	0.26	0.22	0.14	0.10	0.00			
<i>P. columbinus</i>	0.29	0.27	0.29	0.36	0.30	0.32	0.27	0.23	0.00		
<i>P. eryngii</i>	0.27	0.15	0.16	0.25	0.22	0.24	0.21	0.15	0.16	0.00	
<i>A. bisporus</i>	0.30	0.23	0.25	0.33	0.27	0.28	0.32	0.25	0.35	0.17	0.00

B

Genus	<i>Agaricus</i>	<i>Agrocybe</i>	<i>Amanita</i>	<i>Pleurotus</i>
<i>Agaricus</i>	0.00			
<i>Agrocybe</i>	0.10	0.00		
<i>Amanita</i>	0.04	0.11	0.00	
<i>Pleurotus</i>	0.11	0.16	0.11	0.00

Computational Design and Validation of a Loop-Mediated Isothermal Amplification (LAMP) Assay for the S450W *rpoB* Mutation in *Mycobacterium tuberculosis*

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Abstract

Tuberculosis (TB) in Indonesia is exacerbated by rising resistance to first-line drugs like rifampicin, often linked to point mutations in the *rpoB* gene, such as S450W. While Loop-Mediated Isothermal Amplification (LAMP) is used for resistance detection, its application for specific point mutations requires further optimization. This study aimed to design and validated a LAMP assay specifically for the S450W *rpoB* mutation using computational and in vitro methods. Computational analysis first confirmed the S450W mutation as a viable biomarker. Subsequently, in vitro validation demonstrated high analytical sensitivity and specificity. The real-time LAMP assay achieved a lower limit of detection (LOD) of 581.4 copies per reaction, demonstrating a tenfold increase in sensitivity than the colorimetric assay, which had an LOD of 58,140 copies per reaction. The designed primer set also showed high specificity, successfully differentiating the S450W mutant sequence from the wild-type and non-template controls. These results establish our LAMP assay as a sensitive and specific tool for the rapid detection of the S450W rifampicin resistance mutation, offering significant potential for clinical diagnostics in high-burden settings.

Keywords: Loop Mediated Isothermal Amplification, *Mycobacterium tuberculosis*, point mutation, rifampicin resistant, S450W.

1. Introduction

Indonesia is the second country with the highest rate of tuberculosis cases in the world after India (Kaaffah et al., 2023). According to Indonesia's tuberculosis control report (Indonesia Ministry of Health, 2023), tuberculosis cases in Indonesia reached at least 638.646 in 2023, with a total estimated number of known cases of MDR-TB/RR-TB is 24.637. Yogyakarta Province has a high achievement of confirming rifampicin resistance tests (222 cases), along with 3 other provinces, specifically South Sumatra (442 cases), Bali (422 cases) and Papua (222).

Multidrug-resistant tuberculosis is a growing public health concern in many parts of the world, especially in low-income countries where most cases occur. However, current conventional drug susceptibility testing methods are either time-consuming, such as the proportion method on solid media, or costly, such as the BACTEC 960 System (Abdul-Imam Almazini, 2013). MDR-TB potentially detected using rifampicin resistant marker, as 90% of rifampicin-resistant isolates are also found to be resistant to isoniazid (Raoot & Dev, 2015).

The *rpoB* gene encodes the RNA polymerase β Sub-unit (RNAP) protein which plays a role in the synthesis and transcription of bacterial oligo-ribonucleotides (Campbell et al., 2001). Rifampicin works by blocking and inhibiting the transcription process (Wulandari et al., 2024). Mutations in *rpoB* are known to inhibit the interaction between rifampicin (RIF) and RNAP (Li et al., 2021).

A recent study from Filipenko et al. (2019) showed S450L *rpoB* mutation can be used as Allele-Specific LAMP biomarker. Although the resistance profile of S450L *rpoB* gene is already known, another promising mutant variant, especially S450W as LAMP biomarker, needs to be further investigated.

Loop Mediated Isothermal Amplification (LAMP) is one of the pathogen detection methods based on nucleic acid amplification (Chaouch, 2021; Kashir & Yaqinuddin, 2020). LAMP is known to be able to accurately detect pathogens so that it can be used as a Point-of-Care (POC) diagnostic test (Augustine et al., 2020; Notomi et al., 2000). The results of colorimetric LAMP detection can be visually observed (Wong et al., 2018). The LAMP amplification process takes less than 65 minutes, has a high level of accuracy, low cost (about \$2.5 or

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\$40,139.75/test), and requires simple instruments (Jaroenram et al., 2020). LAMP has potential to be used in limited sources area due to the minimum instrument needed (Jekloh et al., 2022; Sreedeeep et al., 2020).

Identification of resistance profiles is usually carried out using in vitro study at the laboratory, specifically with the drug susceptibility test (DST) or minimum inhibitory concentration (MIC) method (Seid et al., 2024). However, characterization using conventional method tends to take a long time to analyze (Geethalakshmi et al., 2024). In addition, areas of mutation and changes in protein stability cannot be analyzed directly. Sanger sequencing and whole-genome sequencing followed by machine learning-based computational analysis have great potential for predicting tuberculosis drug resistance (Ayaş et al., 2024; Paredes-Gutierrez et al., 2025). The use of sanger sequencing is still considered quite promising in determining the location of mutations in clinical DNA samples.

This study focused on identified S450W in *rpoB* gene of MTB as rifampicin resistant biomarker. Computational analysis using molecular docking is expected to provide more in-depth information about the mutation effects occurring at the molecular level. Mutation sequence is used as template in primer set design for colorimetric and real-time LAMP reaction.

2. Materials and Methods

2.1. DNA Sample Collection

Total of three DNA clinical samples were collected from sputum specimen of a tuberculosis patient during 2024 until 2025 and one H37Rv as reference sample was collected from culture media (Table 1). Sample collection was carried out at M. Djamil Hospital, Padang Pariaman, West Sumatra, and Microbiology Laboratory (Biosafety level 2+), Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, Yogyakarta, Indonesia. Study have been approved by Medical and Health Research Ethics Committee (MHREC) of Universitas Gadjah Mada (Ref: KE/FK/1288/EC/2024). DNA isolation process of the sample using Sputum DNA Isolation Kit (Slurry)TM (Norgen Biotek Corp, Canada). Samples that confirmed positive on GeneXpert-RIF test and drug susceptibility test were labelled as presumptive antibiotic resistant.

Samples were included in this study if they met the following criteria simultaneously: (1) The sample was identified as presumptively Rifampicin-resistant based on initial testing using either GeneXpert MTB/RIF or the MGIT Drug Susceptibility Testing (DST) system; and (2) The isolated DNA from the sample exhibited adequate concentration and purity, defined as an A260/280 ratio within the range of 1.8–2.0, to ensure robust LAMP amplification. Samples were excluded from the study if: (1) The extracted DNA showed poor quality, specifically defined as having an A260/280 ratio outside the acceptable range of 1.8–2.0, which suggests potential contamination or the presence of PCR/LAMP inhibitors

Table 1. Sample identity

Code	Presumptive Profile Assay	Resistance profile	Concentration (ng/μL)	Purity (260/280)	Geo Location
Y6	MGIT DST+	RIF	28.0	1.98	Indonesia: Yogyakarta
S1h	GeneXpert+	RIF	28.4	2.00	Indonesia: Padang
R1	GeneXpert+	RIF	30.5	1.94	Indonesia: Padang
H37Rv	wild type	-	29.3	1.83	Indonesia: Bandung

2.2. PCR Primer Design

The *rpoB* gene was amplified using the Polymerase Chain Reaction (PCR) primer design based on previous reference with target modification (Vo et al., 2024). PCR primer design was made based on the *Mycobacterium tuberculosis* H37Rv reference sequence template (NC_000962.3). Primer designed to amplify RRDR (rifampicin resistant determining region) in *rpoB* gene NC_000962.3 (759807...763325). PCR primer created using SnapGene, was examined using BLAST (blast.ncbi.nlm.nih.gov) and quality was checked using OligonucleotideAnalysis (geneinfinity.org).

The quality control criteria were strictly maintained to ensure optimal amplification efficiency, specifically by confirming that: (1) Self-annealing was negligible; (2) Hairpin formation was avoided; and (3) The melting temperatures (T_m) of the Forward and Reverse primers were closely matched (ideally within ±3 °C) to guarantee simultaneous and efficient binding during the isothermal reaction. Self-customized primer pair used in this study can be seen in Table 2.

Table 2. PCR primer set

Antibiotic	Gene	Primer Design (5' to 3')	Amplicon (bp)	T _m (°C)	T _a (°C)
Rifampicin (RIF)	<i>rpoB</i>	F:CAATTCATGGA CCAGAACAA R: GCATCACAGTGA TGTAATCG	673	59.2 61.3	59.2

2.3. PCR Reaction

The PCR reaction using high-fidelity mastermix (Colman et al., 2015). PCR mixture was prepared using Nuclease-Free Water (Himedia, India), Phusion Plus 2X High-Fidelity Mastermix (Thermo Scientific, USA), a working primer solution (10 μM), and DNA samples. The final reaction volume was 10 μL, composed of 3 μL of Nuclease-Free Water, 5 μL of Mastermix, 1 μL of primer solution, and 1 μL of DNA sample. PCR program includes pre-denaturation in 98°C (30 seconds), denaturation in 98°C (10 seconds), annealing temperature 59.2°C (30 seconds), extension of 72°C (15 seconds) with a cycle repeat 30 times, and the extension step in 72°C (10 minutes). The entire PCR process was carried out with a Biometra Thermal Cycler (Jena Analytics, Germany). This was followed by electrophoresis, which was conducted for 30 minutes at 100 V using the Mupid-eXu® Submarine (Advance, Japan). For gel preparation, 0.4 g of agarose was dissolved in 40 mL of TAE buffer (Himedia, India), heated, and then supplemented with 2.5 μL of RedSafeTM

DNA staining (South Korea) to achieve a final concentration of 1% agarose. The electrophoresis results were visualized using the AlphaImager™ Mini Gel Documentation System (ProteinSimple, USA).

2.4. DNA Purification

Purification was necessary to increase the concentration and purity of DNA from clinical samples collected in Yogyakarta. This process was performed using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). DNA purification process was based on previous protocol (Arunachalam & Sreeja, 2025).

2.5. DNA Sequencing

Positive samples from the PCR step were used for DNA sequencing. Each sample consisted of 35 µL of unpurified PCR product with a minimum concentration of 100 ng/µL. Single-pass DNA sequencing was performed by Apical Scientific (Malaysia) using an Applied Biosystems Genetic Analyzer (Thermo Fisher Scientific, USA) and a BigDye® Terminator V3.1 sequencing kit (Thermo Fisher Scientific, USA). For quality control, the resulting electropherograms were visualized using FinchTV and Geneious Prime. Sequences with a low-quality electropherogram (confidence score lower than 50%) were excluded. High-quality forward and reverse sequences were then used to generate a consensus sequence after a pairwise alignment process in BioEdit. Multiple alignment of the consensus sequences was performed with the MTB H37Rv reference sequence using SnapGene. Sequence identity was confirmed with BLAST, and the multiple sequence alignment was visualized using MSA viewer.

2.6. In Silico Analysis: Protein and Ligand Preparation

This preparation method was adopted from Maladan et al., (2023); Singh et al., (2021) with some modifications. In silico mutagenesis was carried out with substitution of amino acid residues in mutant amino acid sequence manually using notepad and visualized using SnapGene translation tool. The 3D structure of native proteins was obtained from homology modelling using SWISS MODEL (swissmodel.expasy.org) based on protein template database. The protein conformations with identity value close to 100%, GMQE closes to 1, and the best ramachandran plot are selected as the receptor. Meanwhile, the crystal structure of rifampicin as ligand was collected from protein-ligand complex separation with PDB ID: 5UHB in RSCB PDB (rcsb.org). Protein and ligand preparation were performed by eliminating water molecules and irrelevant protein chain structures using Biovia Discovery Studio (3ds.com/products/biovia/discovery-studio). Hydrogen atoms were added using PyMol (pymol.org) and energy minimization using Swiss PDB Viewer software (spdbv.unil.ch/disclaim.html).

2.7. Molecular Docking

Molecular docking process using CB-Dock2 program (cadd.labshare.cn/cb-dock2/index.php) by entering protein receptor (PDB format) and ligand (SDF format) on the website. The best protein-ligand conformations are selected based on the suitability of amino acid residue bonds and docking scores. The structure of protein-ligand complexes in wild type and mutant isolate were then

compared. Docking results are visualized in 2D form using Biovia Discovery Studio.

2.8. LAMP Primer Design

Primer set was designed using web-based program PrimerExplorer V5 (primerexplorer.eiken.co.jp/e/). LAMP primer design method used in this study is based on some previous references (Aulia et al., 2023; Varona & Anderson, 2021) with several modifications. Mutant sequence being input as template in PrimerExplorer. This primer design strategy specifically placed the mutation S450W (C1349G) in 3' end of F2 primer (Table 2), without any probe and nucleotide base mismatch included. Primer set with low GC rate, similar Tm, minimum dimer, and low self-annealing based on oligonucleotide analysis is preferable as final primer design. This design set does not include LF primer in LAMP reaction to minimize missamplification issue (Khumwan et al., 2022).

2.9. Colorimetric LAMP Reaction

LAMP reaction was carried out using WarmStart Colorimetric 2X Mastermix (#M1800, Biolabs, UK) using previous research design method with several modification (Liu et al., 2012). LAMP mixing using single 02 mL PCR tube containing 6,5 µL mastermix, 1,25 µL LAMP primer 10X stock (Table 2) (concentration each primer: 8 µM FIP, 8 µM BIP, 2 µM F3, 2 µM B3, 4 µM LB), 4,5 µL nuclease free water, and 0,5 µL purified DNA template (total 12,5 µL mixing solution). The primer concentration was decided based on optimization result to minimize missamplification. LAMP temperature and time optimization was carried out using Biometra® (Analytik Jena, Germany) that suitable for temperature gradient purposes. Temperature used for LAMP reaction is 68,5°C for 35 minutes. Limit of detection depends on last detectable color changes by visual and electrophoresis using five serial dilutions of DNA template control.

Table 3. LAMP primer set

Mutant	Primer code	Sequence (5' to 3')	Tm (°C)	GC rate
<i>rpoB</i> S450W	RM2_F3	CGCTGTCGGGGTTGACC	61.87	0.71
	RM2_B3	TGACCCGCGCGTACAC	61.01	0.69
	RM2_FIP	GGTGCACGTCGCGGACCT-ACAAGCGCCGACTGTG	66.84	0.63
			58.73	0.72
	RM2_BIP	GTCGCACTACGGCCGGATGT-GACAGCGAGCCGATCAGA	66.55	0.61
			60.66	0.65
	RM2_LB	CCGATCGAAACCCCTGAGG	61.09	0.61

2.10. Real-Time LAMP Reaction

Procedure used for this step is adopted from related references (Alhamid et al., 2023; Khumwan et al., 2022) with simple modification. Real-time reaction mix using 6,25 µL WarmStart Colorimetric 2X Mastermix (#M1800, Biolabs, UK), 2,5 µL nuclease free water (Himedia, India), 2 µL Sensifast™SYBR-No Rox (Meridian bioscience, USA), 1,25 µL LAMP primer stock 10X, and 0,5 µL DNA template. Real-time LAMP was performed using Quant Studio™ 5-Real Time PCR System (ThermoFisher Scientific, USA). Real-time program setting used 12,5 µL reaction mix, temperature 68,5°C, quantification time every 1 minute for 50 cycles, in duplicate. Limit of detection (LOD) was based on last positive detectable

amplification curve with cut off C_q value ≤ 35 . LOD calculation using formula from Ma et al., (2019), number of copies/test = $(M \times 6.02 \times 10^{23} \times 10^{-9}) / (n \times 660)$ with M is amount of DNA in nanogram, n is length of *M. tuberculosis* genome (4.411.529 bp), 660 is average weight of single basepair (Da), and 6.02×10^{23} is Avogadro's constant.

2.11. Statistical analysis

Statistical analysis of the qLAMP data was focused on analytical validation (Bates et al., 2025). The Limit of Detection (LOD) determination was performed in duplicate (n=2) for each concentration in the serial dilution. All quantitative data, including the Real-Time LAMP Amplification Bar (Figure 8B), are presented as the Mean $\pm$ Range Standard Deviation (SD) to demonstrate the

precision and reproducibility of the assay at different target concentrations. All graphical and descriptive statistical analyses were performed using GraphPad Prism Software, Inc. (USA).

3. Results and Discussions

3.1. The *rpoB* Gene Amplification

PCR primer set are designed to amplify 673 bp of *rpoB* gene (1294...1966) containing common mutation at RRDR (Figure 1). RRDR site located in 81 bp long sequence from 1276 to 1356 bp of *rpoB* gene based on MTB numbering system. PCR primer located in 50-57% GC content region to increase amplification efficiency.

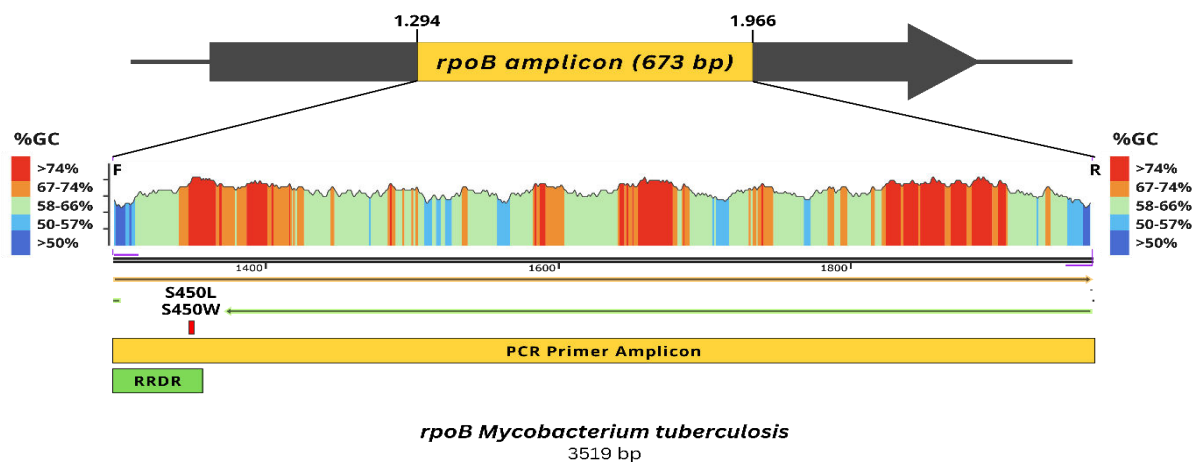


Figure 1. *rpoB* PCR primer map shows amplification area in Rifampicin Resistant Determining Region (RRDR). S450L and S450W found as point mutation in this study, annotated with red-color line. GC content represented in color gradient. Open reading frame (ORF) of coding strand visualized as orange arrow, meanwhile the template strand visualized as green dashed arrow (illustration edited using SnapGene software and canva.com).

The three of sputum DNA samples, when amplified using the PCR primer set, produced a single band at 673 bp on agarose gel electrophoresis (Figure 2). Meanwhile, the reference sequence H37Rv was considered as a negative result. This single, confident band indicates that

the primers successfully and specifically amplified the RRDR, which includes the S450 codon, a known high-prevalence mutation site. The single band also suggests that the primers did not form dimers.

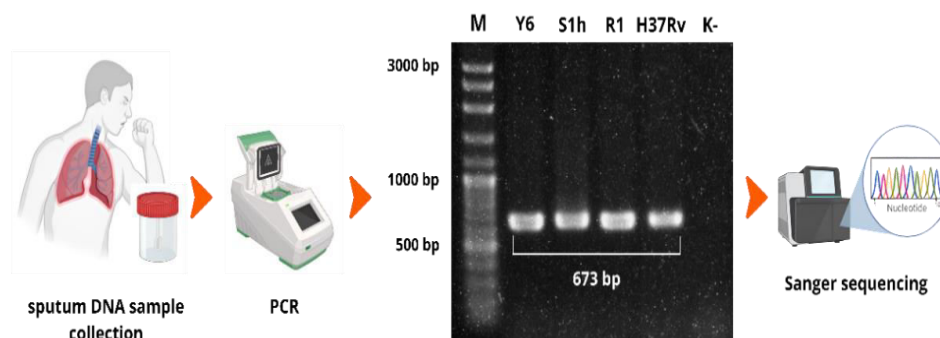


Figure 2. Unpurified PCR product used for DNA sequencing. Visualization of control samples electrophoresis result (icon assets and illustration editing using biorender.com and canva.com, PCR visualization using research documentation).

3.2. Mutation Profiling

DNA sequencing is used to determine sample mutation profile, along with multiple alignments for each sample sequence with reference sequence (H37Rv) to locate the mutation position. Samples used for DNA sequencing

included one Yogyakarta sample (Y6), and two Padang samples (S1h and R1). All samples have single point mutation at codon 450 in RRDR (Figure 1,3). Conkle-Gutierrez et al. (2022) stated that rifampicin resistance is generally caused by mutations in the RRDR of the *rpoB*

gene. Based on multiple alignments, Y6 showed single point mutation in S450W, changing the nucleotide base from TCG (Serine) into TGG (Tryptophan) (Figure 3). Meanwhile, the S1e, S1h, S1m and R1 showed different mutations, specifically S450L that changed nucleotide base from TCG (Serine) into TTG (Leucine) (Figure 3). Electropherogram quality control using GeneiousPrime showed confident curve peak appear with phred score (Q20, Q30, Q40) at 100% and HQ% score per sequence higher than 90% indicate low error reading rate in mutation identification.

The S450W mutation in the *rpoB* gene is an uncommon mutation but is known to cause significant drug resistance. Although its frequency is low compared to the more common S450L mutation, it is considered a highly resistant mutant. The frequency and type of mutations in MTB can vary by geographical location. The effect of this mutation on drug resistance was investigated using molecular docking to analyze changes in protein conformation at the drug binding site.



Figure 3. Multiple sequence alignment result. Location of mutation in *rpoB* gene based on alignment with H37Rv as reference sequence. Electropherogram comparison between mutant and non-mutant variant showed high phred score, indicates good quality of *rpoB* sequences.

3.3. Resistance Prediction Based on Molecular Docking

Molecular docking analysis revealed significant conformational changes in mutant proteins compared to the wild type. The binding affinity between the protein and the rifampicin ligand changed drastically. In the wild-type protein, the RNA polymerase β subunit binds directly to rifampicin through several amino acid residues, including S450 (serine). This finding is consistent with research by Li et al. (2021), which confirmed that S450 directly binds to rifampicin via a hydrogen bond in its native state.

In this study, the docking scores directly correlate with binding affinity, with more negative values indicating a stronger bond. The wild-type protein showed the strongest binding affinity with rifampicin at -8.8 kJ/mol (Figure 4A), supported by five conventional hydrogen bonds. The S450L mutant variant had a reduced binding affinity of -7.4 kJ/mol (Figure 4C), forming only two hydrogen bonds. In contrast, the S450W mutant showed the lowest binding affinity at -4.7 kJ/mol (Figure 4B), with only a single hydrogen bond. These results indicate that the S450W

mutation most effectively disrupts the binding of rifampicin.

The analysis revealed a significant difference in binding affinity. The S450W mutation resulted in a much larger change in binding affinity (4.1 kJ/mol difference) compared to the S450L mutation (1.4 kJ/mol difference), indicating that S450W more effectively disrupts the protein-drug interaction. In the S450L mutant, the substituted leucine (L) residue still forms a direct alkyl bond with rifampicin, allowing for a degree of binding. Conversely, in the S450W mutant, the new threonine (W) residue does not form a direct chemical bond with rifampicin. Although another residue, arginine (R), maintains a hydrogen bond, this is not enough to preserve the original binding strength. This disruption in binding and changes in protein conformation collectively suggest that S450W is more resistant to rifampicin. The molecular docking results, binding affinity, and resistance analysis prediction are summarized in Table 4.

Table 4. Samples mutation profile

Code	Mutation	Annotation	Binding Affinity (kJ/mol)	Resistance associated	Reference
Y6	TCG > TGG	S450W	-4.7 (low)	+	(World Health Organization, 2023)
S1h	TCG > TTG	S450L	-7.4 (low)	+	
R1	TCG > TTG	S450L	-7.4 (low)	+	
H37Rv	No mutation	S450	-8.8 (high)	-	

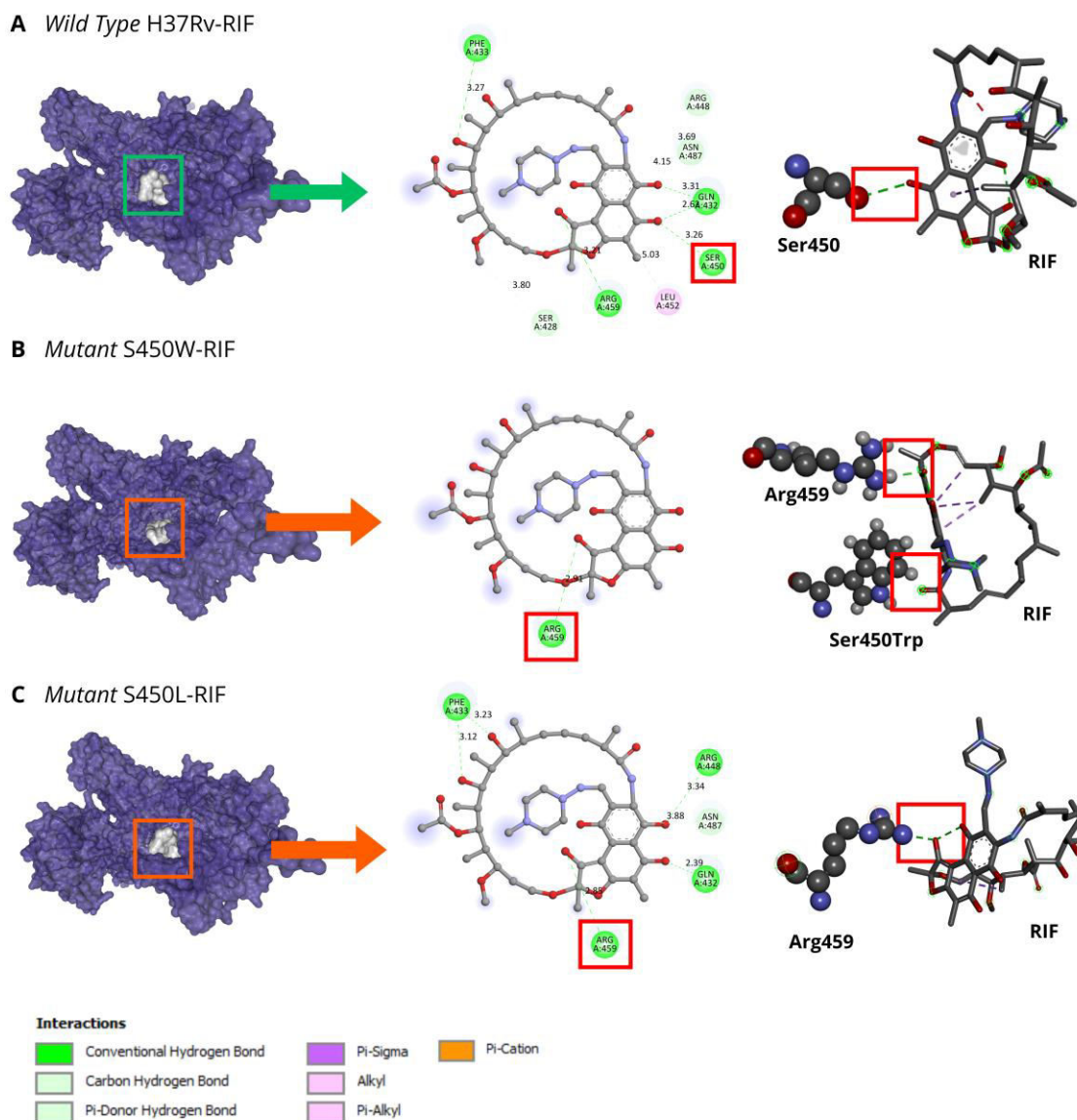


Figure 4. Molecular docking result. (A) Wild type-RIF showed Serine (S450) as residue that binding directly with rifampicin, indicates its location in RNA Polymerase β Sub-unit binding pocket. (B) S450W-RIF showed that the mutant variant no longer binding directly with rifampicin. (C) S450L-RIF still has ability to binding with rifampicin but with different type of molecular interaction.

3.4. LAMP Primer Optimization

The RM2 LAMP primer set was designed to amplify the S450W mutation in the *rpoB* gene (Figure 5). The F2 primer was specifically engineered to target the single point mutation site (TGG) in the S450W mutant. This mutation is strategically located at the 3' end of the F2 primer, a critical position for ensuring specificity. As noted in a previous study, a mutation in this position, particularly in the F2 region of the FIP primer, can differentiate between mutant and wild-type isolates (Khumwan et al., 2022). This is because the mutant nucleotide base in the F2 primer prevents it from annealing to the wild-type sequence, leading to a failure in the amplification initiation. While computational analysis confirmed the high quality of this primer set, further optimization is still necessary to screen for potential amplification issues that

can arise from the long structure of the FIP and BIP primers.

The colorimetric LAMP reaction with phenol red dye will change color from red to yellow as pH changes (Peltzer et al., 2021; Selva Sharma & Lee, 2024). The buildup of protons at the end of the reaction will cause the pH to drop so that the positive sample will turn yellow, while the negative sample will remain pink (Tanner et al., 2015). Visual observation result showed that the optimum temperature for colorimetric LAMP reaction is 65°C and 70°C (Figure 6A). LAMP reaction in 65°C and 70°C also showed highly distinctive ladder-like band as unique pattern of LAMP reaction product in electrophoresis visualization (Figure 6D). The significance of color change in positive control samples compared with NTC was calculated using G/R ratio-temperature curve analysis in Microsoft Excel (Figure 6B).

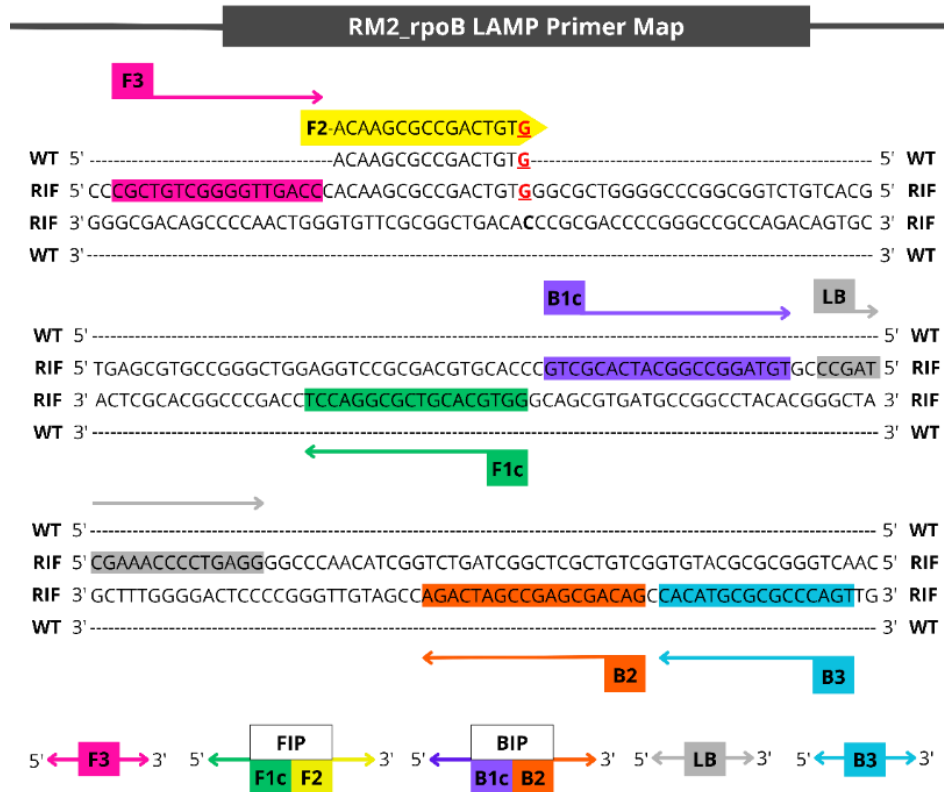


Figure 5. RM2 LAMP primer sets with color coded sequence. Mutation site located in 3' end of F2 primer to enhance the annealing specificity. F2 and F1c primer formation known as FIP, while the BIP primer contains B1c and B2.

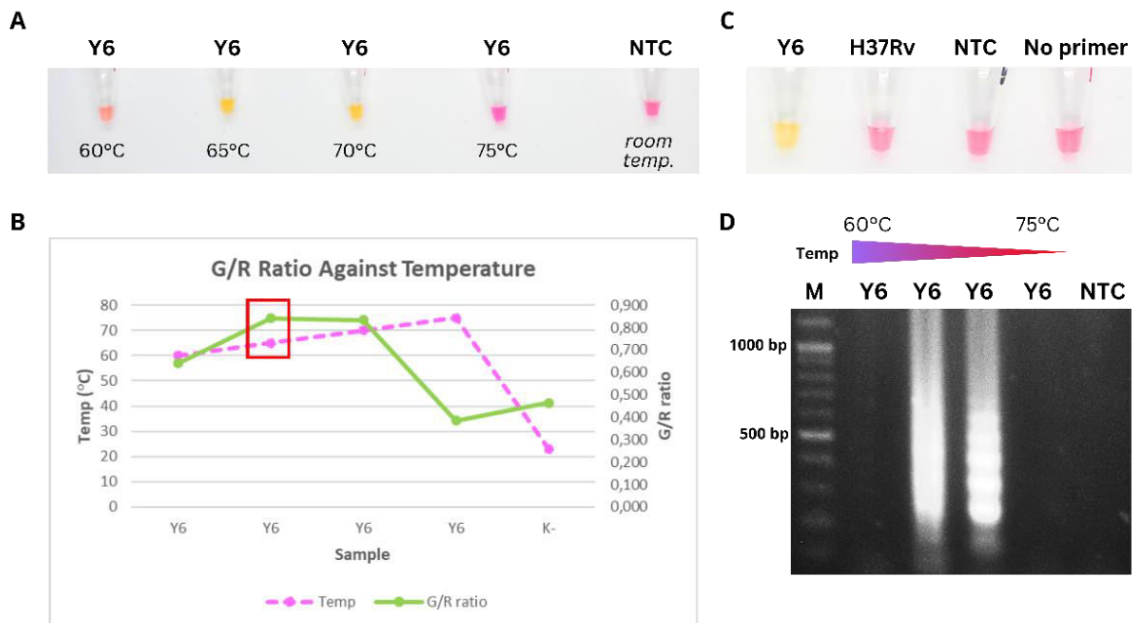


Figure 6. LAMP primer optimization. (A) Temperature optimization based on colorimetric LAMP visualization. (B) colorimetric LAMP G/R ratio curve optimization using 5°C gradient temperature increase. (C) colorimetric LAMP comparison with optimum temperature based on G/R Ratio-Temperature curve. (D) Colorimetric LAMP electrophoresis showed ladder-like band in temperature range at 65°C to 70°C. All the optimization running time is 35 minutes.

The analysis of the LAMP reaction at different temperatures revealed that the optimal amplification for the S450W mutant occurred at 68.5°C. The highest difference in the Green/Red (G/R) ratio curve between the mutant sample and the non-template control (NTC) was observed at 65°C. This finding was consistent with both the colorimetric observation and the electrophoresis results. Similarly, the LAMP reaction at 70°C also showed

a high G/R ratio difference, though it was slightly lower than at 65°C. Based on the curve analysis, a G/R ratio threshold of ≥ 0.7 was effective in distinguishing between negative and positive samples. However, further optimization determined that 68.5°C was the ideal temperature for the amplification process. This temperature was found to consistently maintain clear

colorimetric differentiation between the S450W mutant (Y6) and the wild-type (H37Rv) samples.

3.5. Limit of Detection (LOD) Determination

Real-time LAMP amplification settings were based on the optimal conditions determined from the colorimetric LAMP reaction. The assay was performed at 68.5°C for 50 minutes using a five-step serial dilution. The Y6 (S450W) sample was used as a positive control to determine the limit of detection (LOD). The S1h and R1 (S450L) samples served as mutant (MT) controls, while H37Rv was the wild-type (WT) control. The colorimetric LAMP assay had a detection limit at the second dilution, with a concentration of 2.8×10^{-2} ng/μL, equivalent to 58,140 copies/test, as confirmed by electrophoresis (Figure 7).

However, a clear visual color change from pink to yellow was only observed at a much higher concentration of 28 ng/μL (5,814,000 copies/test). At the next two dilutions (Y6.1 and Y6.2), the color remained orange, indicating that the amplification had not reached its endpoint. Further optimization showed that extending the incubation time to 40 minutes was feasible to achieve maximum color change. However, extending the reaction beyond 50 minutes is known to increase the risk of false positives due to mispriming and the accumulation of non-target products. Therefore, a 35-minute cut-off value was chosen for this test, a duration adopted from a previous study by Ma et al., (2019).

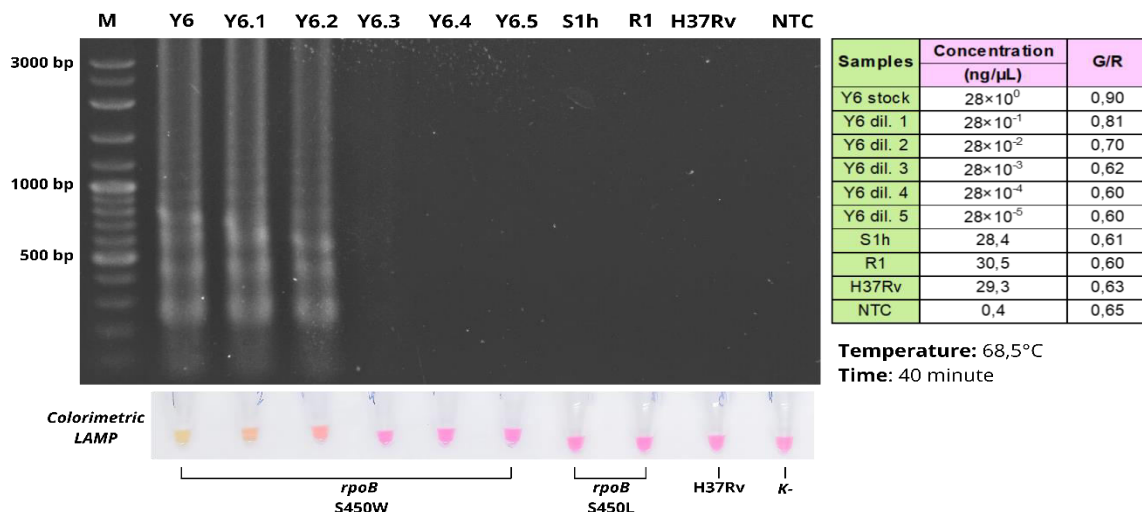


Figure 7. Colorimetric LAMP confirmation using electrophoresis. Limit of detection (LOD) determination using serial dilution compared with wild type H37Rv and S450L mutant sample as reaction control showed detectable band in second dilution.

The real-time LAMP assay in this study achieved a detection limit of 2.8×10^{-4} ng/μL, equivalent to 581.4 copies/test (Figure 8A). The amplification curve for the fifth dilution showed inconsistency, which is attributed to its low DNA concentration and high error rate, as indicated by the standard deviation bars (Figure 8B). The negative control (NTC) showed no amplification, even when the reaction was extended to 50 minutes. The detection limit achieved in this study is comparable to other real-time methods. For instance, Takarada et al., (2020) reported a detection limit of 200 fg/μL (41.53 copies/test) using a LAMP-specific allele system to detect mutations in the

RRDR of *Mycobacterium tuberculosis*. In another study, J. Kim et al., (2021) used AS-RPA/SYBR to detect mutations in the *rpoB* and *katG* genes, reporting a detection limit of 5 ng/test (1,038,270 copies/test). While the mutation control sample (R1) showed late amplification after 45 minutes, this late detection suggests that the assay can distinguish between the S450W and S450L mutations. This is because a ΔC_t value ≥ 20 cycles and a cut-off time of 35 minutes can effectively differentiate the two mutants. This specificity is crucial for identifying different resistance types.

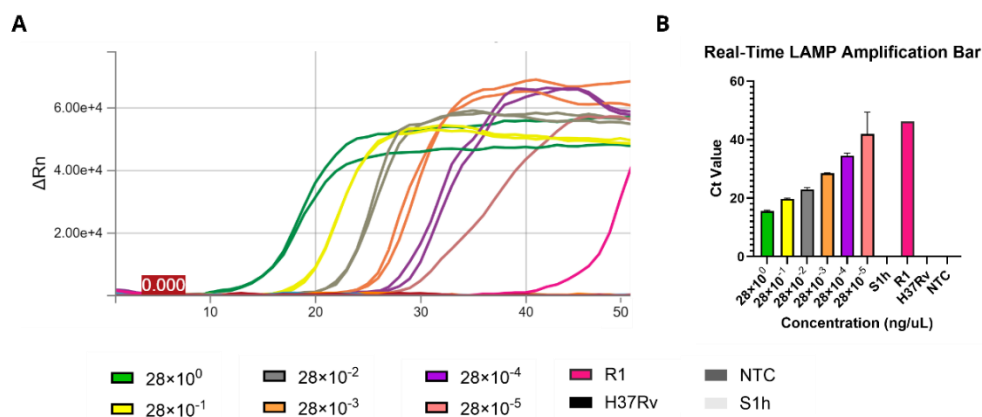


Figure 8. Real-time LAMP reaction results comparison. (A) LOD analysis based on real-time LAMP result. (B) Real-time LAMP amplification deviation standard with error bar. Curve and bar using the same color coded showed below the data.

The colorimetric LAMP assay in this study had a higher limit of detection (LOD) than the one reported by Takarada et al. (2020), suggesting that the primer set's sensitivity still needs improvement. In contrast, the real-time LAMP method in this study showed a lower LOD than the AS-RPA/SYBR method (Kim et al., 2023). The higher sensitivity of the Takarada et al. (2020) study is likely due to its use of multiple mutation sites, whereas this study focused on a single site. Further research using multiple mutations or mismatches, as suggested by (Khumwan et al., 2022), could increase the sensitivity of the LAMP primer sets.

This study introduces a new LAMP assay to detect the S450W mutation in MTB, a method not previously reported for clinical isolates from Indonesia. While the S450W mutation is less common than S450L, our computational analysis using molecular docking suggests it confers a higher resistance profile (Rando-Segura et al., 2021). This finding highlights the value of computational tools for rapidly predicting resistance mechanisms and identifying new biomarkers for rifampicin-resistant TB.

This study provides insights into specific LAMP primer set design strategies for amplifying the S450W *rpoB* mutation. This was achieved by strategically placing mutated bases at the 3' end of the FIP primer and reducing the length of the LF primer to minimize non-specific amplification. The colorimetric LAMP assay successfully differentiated the S450W mutation from the wild type at 68.5°C within 35 minutes. The developed primer set also demonstrated the ability to distinguish S450W from S450L mutations at the same codon using a specific cut-off time, which highlights its high discriminatory power. For future development, it is recommended that additional triple mismatches be incorporated into the F2 primer to further enhance the assay's specificity, as demonstrated in another research.

Colorimetric LAMP is a promising tool for use in resource-limited areas due to its effective turnaround time and low cost. The primer set in this study was able to detect the S450W *rpoB* mutation in less than 40 minutes. The estimated time from sputum sample preparation to colorimetric LAMP visualization is less than 2 hours. Based on cost estimates for MTB tests in Indonesia, the LAMP colorimetric method costs less than Rp. 200,000 (US\$12.32) per test. This is significantly more affordable than other screening methods, such as GeneXpert, which costs around Rp. 850,000 (US\$52.41), or the gold-standard DST-MGIT culture test, which costs around Rp. 900,000 (US\$55.50) per reaction. A study by Sohn et al. (2019) in Vietnam also confirmed the lower cost of TB-LAMP (US\$14.17) compared to GeneXpert (US\$40.62) in a medium-volume laboratory.

The affordability of the LAMP method makes it a promising screening tool, especially in countries like Indonesia with a high incidence of tuberculosis. Because it is more cost-effective than other methods, such as GeneXpert and DST-MGIT culture, LAMP can be used to screen a higher volume of samples in a single run. The real-time LAMP assay developed in this study demonstrated a superior limit of detection (LOD) of approximately 2.8×10^{-4} ng/ μ L (581.4 copies/test). This is significantly more sensitive than our colorimetric LAMP assay (2.8×10^{-2} ng/ μ L, or 58,140 copies/test) and other reported methods like the one from Kim et al. (2023)

1,038,270 copies/test. Although real-time platforms require more sophisticated instrumentation, their enhanced sensitivity provides a more robust detection capability, which is crucial for rapid point-of-care screening to guide initial treatment, early diagnosis and for detecting low bacterial loads.

The limitations of this study are twofold: first, the sample size was restricted to four double-validated clinical isolates (n=4), serving only for technical proof-of-concept; and second, the assay's specificity is strictly limited to the *rpoB* S450W mutation, meaning it cannot detect resistance conferred by mutations located outside the Rifampicin Resistance Determining Region (RRDR).

As this is an early development of the S450W biomarker, further in-depth exploration through a clinical trial is warranted. A deeper analysis should specifically focus on validating the primer set's sensitivity and specificity using a larger sample size. Additionally, cross-testing against non-tuberculosis bacteria is crucial to definitively confirm the primer set's ability to differentiate between *Mycobacterium tuberculosis* and other bacterial species.

4. Conclusion

This study successfully developed a specific, fast, and sensitive real-time LAMP assay for detecting S450W point mutations in the *Mycobacterium tuberculosis rpoB* gene. We have incorporated a colorimetric assay that exhibits high sensitivity (a low Limit of Detection - LOD). This method is advantageous for practical applications due to its rapidity (results in <40 minutes), cost-effectiveness, and independence from complex laboratory equipment. The assay also showed a strong ability to distinguish S450W mutations from both the wild-type and S450L mutations on the same codon using a cut-off time of 35 minutes. Overall, the LAMP assay developed in this study has great potential as an affordable and efficient molecular diagnostic tool for the rapid detection of rifampicin resistance, which can significantly support TB control efforts in Indonesia.

Conflict of Interest

The authors declare no conflict of interests.

Author Contribution

Dian R. Setyorini contributed to the data collection, analysis, and manuscript writing. Selin F. Satiya and Jessie S. Liusarany contributed to sample collection and sample preparation. Irvan Faizal, Kiki Kurniawan, and Tri Wibawa conducted major supervision, material preparation, and study design. Review and corrections for the previous version of the manuscript conducted by Irvan Faizal and Tri Wibawa. All authors read and approved the final manuscript.

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Antifungal and anti-mycotoxigenic Potential of Resveratrol on *Aspergillus Niger* Isolated from Iraqi Maize

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Abstract

Ochratoxins (OA), a nephrotoxic and carcinogenic mycotoxin synthesized by *Aspergillus* species, exhibits a considerable risk to food safety and human health. Natural bioactive resveratrol, a plant-derived polyphenol, has been studied for their antifungal and anti-mycotoxigenic activities. Twenty-five local maize (*Zea mays*) samples were examined for fungal contamination morphological and microscopically, whereas molecular confirmation was attained through ITS1/ITS4 rDNA sequencing. Quantitative [RT-PCR] was utilized to evaluate *AOPKS* production following resveratrol treatments as (3, 6, and 9 µg/ml), utilizing 18S rRNA as a housekeeping control. This study shows unprecedented investigation about the efficacy of resveratrol on SMA2.

The SMA2 isolate was identified as *A. niger* via ITS1&4 sequencing and phylogenetic analysis. A reduction in SMA2 conidial growth was observed with increasing resveratrol concentrations relative to the control. Gene expression study indicated that *AOPKS* transcription was downregulated in response to resveratrol, with fold change reductions of 0.104 ± 0.120 , 0.194 ± 0.241 , and 0.277 ± 0.307 at proportion of 3, 6 & 9 [µg/ml] in sequence, compared to the control (1.0 ± 0.000). These findings highlight resveratrol's potential as a natural antifungal agent to reduce ochratoxin contamination in food systems

Key words: *Aspergillus niger*, Resveratrol, Ochratoxin, Gene expression

1. Introduction

Maize is regarded as one of the crops most vulnerable to mycotoxins globally. *Aspergillus niger* frequently grows on maize and other cereals and is capable of colonizing kernels both pre- and post-harvest, resulting in quality degradation, diminished marketability, and heightened post-harvest waste, also recognized generator of ochratoxin (Soares *et al.*, 2013, Anjorin *et al.*, 2021).

Ochratoxin is a prominent secondary metabolite of fungi, recognized by its high toxicity, prevalent occurrence, and substantial contamination of agricultural products.

The fungi that produce OT belong to the genus *Aspergillus* and *Penicillium* (Perrone *et al.*, 2007).

Ochratoxin A is a heat-resistant mycotoxin exhibiting extensive toxicological effects. Nephrotoxic, teratogenic, immunotoxic and oxidize stress regards ochratoxin directly related to accumulative chronic dietary exposing. Ochratoxins are categorized by IARC as probably carcinogenic to humans (Jha, 2022, Yang *et al.*, 2024).

Resveratrol (3,5,4', trihydroxy_trans stilbene) is a natural polyphenolic molecule from the (stilbene) family, primarily located in grapes (*Vitis vinifera*), red wine, peanuts, berries, and specific medicinal plants like *Polygonum cuspidatum* (Baur and Sinclair, 2006). Resveratrol, a secondary plant metabolite, is synthesized in response to stressors, including mechanical damages,

infections, physical or chemical effectors. Resveratrol presents a heterogenous biological effectivity, including anti-oxidant, anti-inflammatory, anti-bacterial, and anti-cancer effects (Frémont, 2000). Given OTA's resistance to specific processing methods and its bioaccumulation in food chains, the optimal option for risk mitigation is to minimize fungal infection and toxin generation during both pre- and post-harvest stages. This study aimed to evaluate the effect of resveratrol on the expression of the ochratoxin biosynthetic gene (*AOPKS*) in *Aspergillus niger* isolated from maize, using quantitative PCR analysis.

2. Materials and Methods

2.1. Isolation of fungi, cultivation and morphological identifications.

Twenty-five grain maize specimens were collected from local sources and examined fungal contamination. Each sample underwent surface sterilization using 1% sodium hypochlorite, was rinsed with sterile distilled water, and subsequently inoculated onto plates with PDA; [HiMedia, India]. The Plates incubated at $[27 \pm 2 \text{ }^{\circ}\text{C}]$ during over 5-7 days in darkness. Fungal colonies exhibiting macroscopic characteristics akin to *Aspergillus* were purified through successive subculturing on PDA to get single-spore isolates. Initial identification was conducted based on macroscopic colony traits (color, texture, conidial organization) and microscopic

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morphology (conidiophore structure, vesicle shape, phialides, and conidia). Identification adhered to the conventional taxonomic key (Kirk *et al.*, 2008). Isolate exhibiting morphological characteristics indicative of *Aspergillus niger* was chosen for molecular validation.

2.2. Genomic DNA extraction and PCR Amplification

DNA was isolated from newly cultivated mycelium according to the protocol of Wizard Genomic DNA Purification Kit, Promega/ USA. The Quantus Fluorometer was employed to ascertain the concentration of extracted DNA to evaluate the quality of samples for subsequent applications. 200 µl of diluted Quantifluor Dye was combined with 1 µl of DNA. Following a 5-minute incubation at ambient temperature, DNA concentration values were measured. The Internal Transcribed Spacer (ITS) rRNA gene cluster was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'); these primers were supplied by Macrogen Company in a lyophilized form. PCR reactions were conducted in a 25 µL volume comprising 22µl of Master mix (12.5 Master Mix) + (1 µM Forward primer) + (1 µM reverse primer) + (7.5 Nuclease Free Water) per tube and add 3µl of Template. The amplification conditions were as follows: The procedure begins with an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles including denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute, concluding with a final extension at 72 °C for 7 minutes. The PCR product was dispatched for Sanger sequencing utilizing the ABI3730XL automated DNA sequencer by Macrogen Corporation in Korea. The results were obtained by email and subsequently analyzed using Geneious software. Agarose gel (1.5%) stained with ethidium bromide was utilized for the detection of amplicons via electrophoresis.

2.3. phylogenetic studies & NCBI isolate submission

Analysis of sequences and validation of microorganism homogeneity data utilizing the rRNA database (NCBI) following the amplification of fungal RNA ribosomal sequences. All procedures encompassed fungal genomic DNA extraction, polymerase chain reaction amplification, sequencing, and assembly. For fungi, analysis of the ITS region (18S prior to update), length exceeding 500 bp, is assured.

2.4. Detection of ochratoxin biosynthetic gene (AOPKS)

The AOPKS gene, a polyketide synthase gene linked to ochratoxin production, was assessed by polymerase chain reaction (PCR). Primers specific to AOPKS were developed utilizing previously available sequences. The sequence of primers was as following:

Aopks-F '5-CAG ACC ATC GAC ACT GCA TGC- '3, Aopks-R '5- CTG GCG TTC CAG TAC CAT GAG- '3 (Yassein *et al.*, 2021).

The amplification of PCR was performed in a total reaction volume [20 µL including 14.5µl of Master mix per tube and add 4.5µl of Template (10x mastermix, 0.5µM forward primer +0.5 µM reverse primer+4.5 nuclease free water +4.5ng/ µl of

genomic DNA template).

The thermal cycling settings included an initial denaturation at 95 °C for 4 minutes, followed by 35 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension [72 °C: 45 sec, concluding with a final extension [72 °C:7 minutes]. The PCR products analyzed on ethidium bromide dyed agarose gels [1.5%], and observed using UV transillumination.

2.5. Screening the effects of resveratrol on *A. niger*

Aspergillus niger isolate was cultured on Yeast Extract Sucrose (YES) agar to determine colony-forming units (CFUs) per milliliter by using a hemocytometer for fungi. Yeast Extract Sucrose (YES) agar is a widely used growth medium for *Aspergillus* species, especially for cultivating mycotoxin-producing strains. This media consists of (20 g/L yeast- extract), (150 g/L- sucrose), and (15 g/L- agar) all dissolved in one liter of distilled water then sterilized. Isolate was cultured then incubated for 3-7 days at 30°C. Prepare a diluted fungal suspension, count the number of viable cells in a known volume on the hemocytometer, then apply the formula:

$$\text{CFU/mL} = (\text{Number of cells counted}) \times (\text{Dilution Factor}) \times 10^4.$$

The "10⁴" factor accounts for the volume of the hemocytometer grid, converting the count from the grid volume to a per-milliliter basis (Wang, 2021 and Maugeri, 2007).

2.5.1. 2.5.1 . Resveratrol effects on Mycelioid colony growth

Aspergillus niger was cultivated on YES (HIMEDIA) agar which contains resveratrol (resveratrol distributed by NutriVita Shop Irvine CA92618) at different concentrations (3, 6, and 9 µg/mL) was used according to Wang *et al.* 2015 to determine its effects on mycelioid colony growth. Aqueous mixture containing 1.6×10^5 conidia was injected onto solid media congaing petri dish then incubated at 30 °C in the absence of light for three-days. Conidia were inoculated, and the amount was quantified by means of a hemocytometer (Hoffman, 2006).

2.5.2. Resveratrol effects on Ochratoxin expressing gene (AOPKS)

For gene expression, no.8 tube containing 15ml of YES broth was mixed with resveratrol at different concentration (3, 6, and 9 µg/mL). It was administered with $[5 \times 10^5]$ of freshly produced *A. niger* conidia separately. On other hand, no.8 tube containing 15ml of YES broth was inoculated as control. The inoculation flasks have been kept at 30 °C with constant agitation at one hundred sixty rpm for either 3 or 7 days. All this was done in triplicate.

2.5.3. Analysis of AOPKS expression (qPCR analysis)

One step RT- PCR was used to Analysis and Calculation of gene expression levels of AOPKS gene rely on RNA concentration after to its conversion to cDNA. All procedures were encompassing total RNA purification, qPCR amplification, and data analysis. The expression levels of AOPKS were standardized against the housekeeping gene 18S rRNA - F '5TCGACCCCGGAGAAGGA -'3, 18S rRNA R '5GCCTGCTGCCTACCTTGA -'3 to account for

variations in RNA quantity and cDNA synthesis efficiency between samples. RNA was extracted from the sample following the TRIzol™ Reagent procedure. Initially, sample (includes three replicate for each treatment) lysis was conducted on a fungal solution to generate a cell pellet. A volume of 1.4 mL of fungal cells was precipitated via centrifugation (2 minutes at 13,000 rpm), after which the supernatant was discarded, and 0.75 mL of TRIzol™ Reagent was added to the pellet. The lysate was homogenized through repeated pipetting. RNA precipitation was achieved by mixing 0.5 mL isopropanol to hydrous phase, followed by a 10-minute incubation and subsequent centrifugation at 12,000 rpm for 10 minutes. The pellet of RNA resulted after ethanol treated and centrifugation was subsequently aspirated with ethanol and air-dried. The Quantus Fluorometer was employed to ascertain the concentration of extracted RNA to evaluate the quality of samples for subsequent applications. One Step RT-PCR analysis applied using GoTaq® 1-Step RT-qPCR System, MgCL2, Nuclease Free Water, Quantifluor RNA System (Promega, USA), total reaction volume as 9µl of Master mix per tube and add 1µl of Template. The thermal cycling real time PCR protocol was: 37 °C for 15 min /one cycle for RT enzyme activation and 95°C for 5min / one cycle for initial denaturation, succeeded 40 cycles of [95 °C : 20 sec], [60 °C for 20 sec], and 72 °C:20 sec]. Analysis of melt curve was carried out to confirm primer specificity.

2.6. statistical analysis

The data was analyzed statistically utilizing R_ software {version 4.4.0} Foundation of (R) -Vienna-Austria). Data visualizations were executed with the ggplot2 tool (version 3.5.0), while information computation testing was conducted via the dplyr, rstatix, and ggpubr packages.

3. Results

3.1. phylogenic study, molecular identification of *Aspergillus niger* isolate using ITS1&ITS4 markers

All observable *Aspergillus niger* is characterized by the presence of black conidial heads, biserial phialides, globose vesicles, and its rapid proliferation on standard media (PDA).

Macroscopic and microscopic characteristics are compared with the conventional taxonomic key (Figure 1).



Figure 1: Macroscopic and microscopic features of *Aspergillus niger* isolate [SMA2] from local maize

The PCR amplification of genomic DNA (Concentration range 10-25ng/µl) isolated from the *Aspergillus niger* isolate, utilizing the universal ITS1 and ITS4 primers, yielded a clear and unique amplicon. The anticipated fragment size (~550–600 bp) was detected via agarose gel electrophoresis (Figure 2). No nonspecific bands or primer-dimer formations were seen, confirming the primers' specificity for the targeted ITS region. The generated PCR products were sequenced, and BLAST analysis verified the isolate's identity as *Aspergillus niger*, demonstrating over 99% similarity with reference of [NCBI] database.

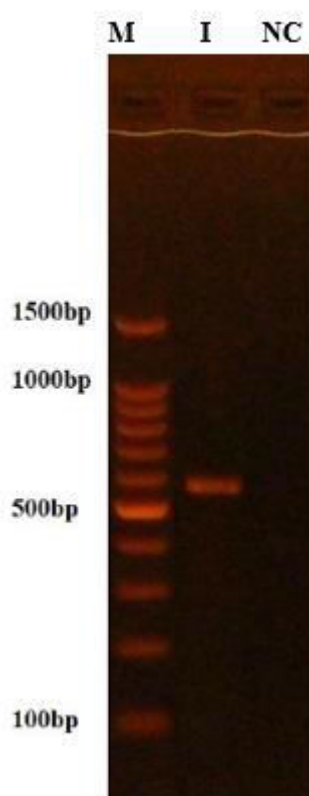


Figure 2: PCR amplification of the ITS gene of an unidentified fungus species was subjected to fractionation on gel electrophoresis colored with ethidium bromide. M: 100 bp. SMA2 with ~500bp .NC is negative control.

The ITS1 & 4 sequences derived from the *Aspergillus niger* isolate were successfully submitted to the GenBank database under accession number [Accession No.]. The isolate was classified as *Aspergillus niger* strain [SMA2]. BLASTn analysis verified a substantial similarity (>99%) with documented *A. niger* sequences.

The phylogenetic tree indicated that the isolate was firmly situated within the *Aspergillus niger* clade, demonstrating a close evolutionary affinity with reference strains which include the actual GenBank accession number [PX219647.1, <https://www.ncbi.nlm.nih.gov/nuccore/PX219647.1>]. Bootstrap values corroborated the robust clustering of our isolate with *A. niger*, affirming its taxonomic classification (Figure 3).

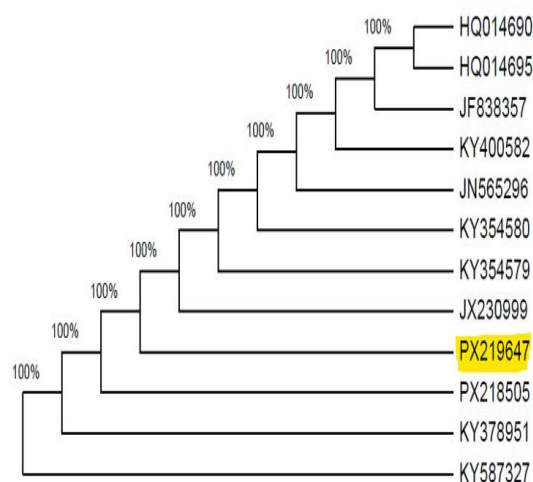


Figure 3: Maximum likelihood technique for evolutionary analysis, actual GenBank accession number [PX219647.1, <https://www.ncbi.nlm.nih.gov/nuccore/PX219647.1>]

Phylogenetic tree was interpreted utilizing maximum likelihood approach by Tamura-Nei (1993) nucleotide substitution system, resulting in a peak log likelihood tree (-787.19) presented. The tree is illustrated with branch lengths, indicated alongside the branches, calculated through the maximum probability technique (Nei *et al.*, 2000) and quantified by the number of substitutions per site. The primary tree for the rule of thumb evaluation could be established through selecting the tree with a greatest possibility from the NJ tree (Saitou *et al.*, 1987) and MP tree. NJ was constructed utilizing a matrix of paired distances calculated based on the Tamura 1993 concept. MP demonstrated the minimum within 10 MP tree evaluations. The beginning structure of a tree is random preexisting structures. The proportion of locations with at least one identifiable base in at least one sequence for each descendant clade is displayed next to each interior node in the tree. The analytical method entailed encoding 12 sequences of nucleotide utilizing first, second, third, and non-coding places, resulting in a final dataset of 574 locations. Evolutionary studies were performed in MEGA12 (Kumar *et al.*, 2024), employing several parallel computing threads.

3.2. Detection of ochratoxins biosynthesis gene

The amplification results of the Aopks- target region from *Aspergillus niger* were separated using on agarose gel electrophoresis (1.5%). Lanes SMA2 exhibit 454 bp PCR products (Figure 4).

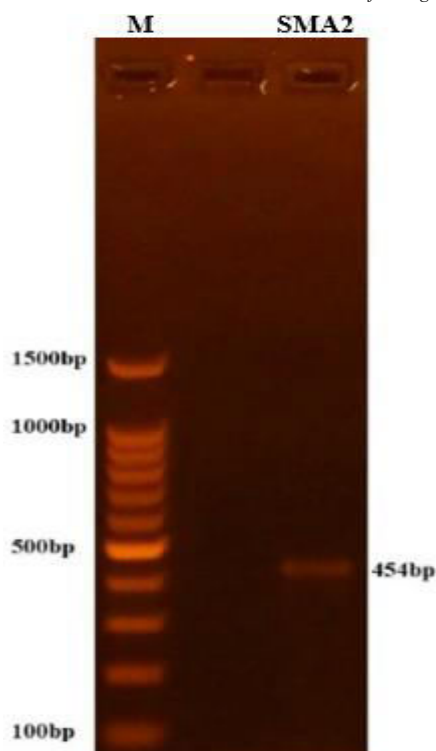


Figure 4: AOPKS amplification of *aspergillus niger* were separated agarose gel electrophoresis, Eth.Br staining. Ladder M: 100bp. PCR of SMA2 resemble 454 bp.

3.3. Impact of resveratrol on growing *Aspergillus niger*

Colony-forming unit (CFU) assay demonstrated notable heterogeneity in the development of conidial biomass of *Aspergillus niger* (SMA2) between resveratrol-treated and untreated solid fungal development cultures. Fungal mycelial progress was altered (Figure 5). Significant alteration in the growth features was observed following graduated treatments in solid YES. The somatic properties of *A. niger* varied among [YES] and [YES-Res] growth which exhibit reduced size. Resveratrol can influence the rate of sprouting of conidia effecting mycelial propagation. Findings demonstrated resveratrol influences the development of fungal mycelia (Figure 6).

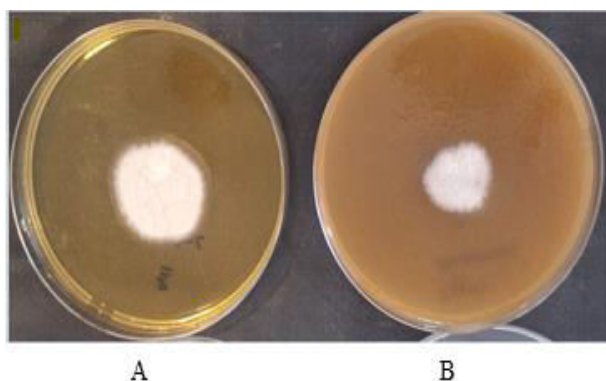


Figure 5: Mycelioid colony of *Aspergillus niger* cultivated on YES agar for 1 days. A- YES(Control), B-YES-3µg/mL Res (Treatment).

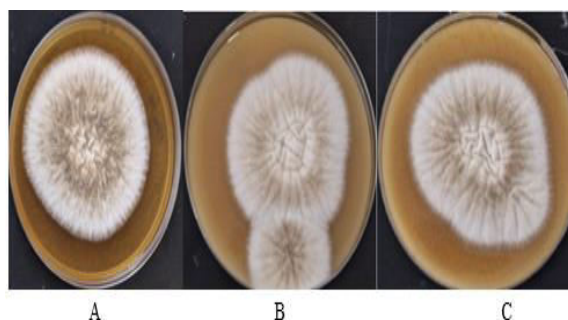


Figure 6: Hyphal like-colony of *A. niger* propagate on YES for 3 days. A- control YES, B- YES-6µg/mL Res (Treatment), C-YES-9µg/mL Res (Treatment).

Resveratrol suppressive effect on the conidial expansion of *Aspergillus niger* was reported, which is dose-dependent. Treatment with (3µg/mL) of resveratrol led to a moderate decrease in colony-forming units (CFUs), reflecting an inhibition of [42.44%] relative to the untreated control which counted as of [1232*10⁶] CFU/ml. Elevating the concentration to (6µg/mL) resulted in a more significant inhibitory impact, diminishing development by [55.16%]. The most significant suppression occurred at (9µg/mL), where conidial development was substantially impeded, achieving [77.31%] inhibition compared to the control (Figure 7).

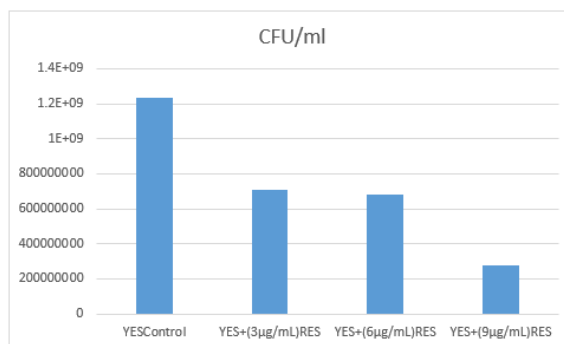


Figure 7: Colony Forming Unit (CFU) Variability of *Aspergillus niger* under Different Resveratrol Concentration. [X= treatments (control + resveratrol concentrations), Y= CFU/ml variability]

3.4. Expression analysis of the (AOPKS metabolism regards resveratrol reaction

Quantitative PCR (q-PCR) was shown that resveratrol induced reduction of the AOPKS (putative ochratoxin polyketide synthase) expression in the *Aspergillus niger* [[SMA2] derived from local maize. Expression levels were standardized to the housekeeping gene 18S rRNA and computed utilizing the 2^{-ΔΔCt} methodology. AOPKS expression did not decrease progressively with increasing resveratrol concentration.

Treatment with 3 µg/ml resveratrol diminished AOPKS expression to 0.104-fold (p=0.14), 89.6% compared with control (untreated). At 6 µg/ml, transcript levels decreased to 0.194 -fold (p=0.14; 80.6% reduction), whereas the maximum concentration of 9 µg/ml shows minimum suppression (0.277, p=0.163; 72.3% reduction) compared with other concentrations and control (Figure 8, Table 1).

Normalization against 18S rRNA verified that the diminished inhibitory impact at elevated doses was not attributable to technical discrepancies in RNA quality or quantity. Statistical analysis indicated that the most

significant suppression occurred at the lowest dosage examined (3 µg/ml), although elevated values exhibited a partial restoration of AOPKS expression.

Table 1: Fold change, AOPKS expression variability under resveratrol treatments

Treatments	Sample	18srRNA	Aopks	DCT	DDCT	Folding	Ave. Folding± SD
3 µg/ml Resveratrol	SMA2	17.85	26.45	8.60	4.76	0.037	0.104± 0.120
	SMA2	18.13	26.58	8.45	2.04	0.243	
	SMA2	18.05	25.52	7.47	4.93	0.033	
6 µg/ml Resveratrol	SMA2	16.12	24.29	8.17	4.33	0.050	0.194± 0.241
	SMA2	16.39	23.88	7.49	1.08	0.472	
	SMA2	16.94	23.51	6.57	4.03	0.061	
9 µg/ml Resveratrol	SMA2	17.04	23.38	6.34	2.50	0.18	0.277± 0.307
	SMA2	16.26	23.35	7.10	0.69	0.62	
	SMA2	16.03	23.67	7.64	5.10	0.03	
Control	SMA2	21.53	25.38	3.84	0.00	1.00	1.00± 0.000
	SMA2	23.24	29.65	6.41	0.00	1.00	
	SMA2	24.03	26.57	2.54	0.00	1.00	

The figure below shows the data processing and that analyzed statistically (Figure 8, Figure 9).

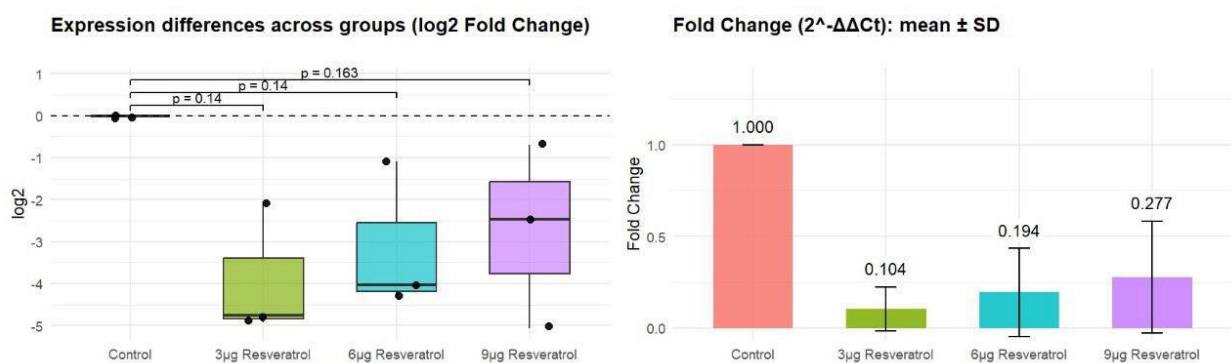


Figure 8: Expression differences across groups (log2), fold change.

The trend demonstrates that resveratrol administration markedly reduces AOPKS expression, albeit the inhibition diminishes with higher concentrations (Figure 9).

Dose–response on log2(Fold Change) with 95% CI

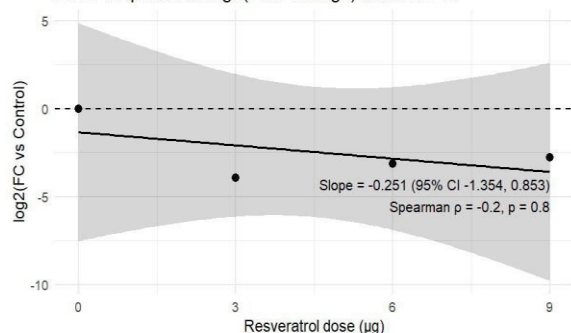


Figure 9: Dose-response on log2(fold change) with 95% CI

4. Discussion

Significant advancements have been made in mitigating ochratoxin contamination in agricultural products (Carbas *et al.*, 2022). Unlike previous studies that focused on aflatoxin-producing *Aspergillus flavus*, this work

demonstrates AOPKS suppression in *A. niger* from Iraqi maize, highlighting species- and strain-specific responses (Wang *et al.*, 2015). This follows the revelation that ochratoxin is highly harmful for living organisms (van der Merwe *et al.*, 1965). We should give significant and serious consideration to improving plant stress tolerance and defense mechanisms in response to biotic stressors.

Numerous inhibitors have been shown to impede ochratoxin production in *A. niger*. Plant-derived metabolites are particularly noteworthy (Zhang, *et al.*, 2025).

Ochratoxins are harmful secondary metabolites predominantly generated by *Aspergillus* and *Penicillium* species, presenting considerable threats to food safety and human health. Traditional management methods frequently depend on chemical fungicides, potentially resulting in resistance and unwanted residues. Recently, secondary plant metabolites, including polyphenols, flavonoids, and stilbenes, have garnered interest as environmentally sustainable alternatives for mycotoxin management. These bioactive substances demonstrate antifungal and anti-mycotoxigenic activities by disrupting fungal proliferation, modulating oxidative stress, and influencing transcriptional pathways associated with mycotoxin

production (Aldred *et al.*, 2008, Hui *et al.*, 2023, Gao *et al.*, 2025).

Numerous studies have shown that resveratrol, quercetin, and essential oil components can suppress ochratoxin synthesis by targeting critical biosynthetic genes, including *AOPKS* (polyketide synthase) and regulatory transcription factors (e.g., CreA). The utilization of these compounds not only diminishes fungal colonization but also inhibits toxin buildup, indicating a dual function in plant defense and food preservation. Comprehending the molecular basis of this inhibition will aid in formulating natural solutions to reduce ochratoxin contamination in agricultural goods (Wei *et al.*, 2022).

In this study, we found impressive *AOPKS* downregulation along with *A. niger* cultivated with resveratrol.

OTA stimulates ROS creation in cells, comprising (O_2^- , H_2O_2 , $\bullet OH$). Protein and lipid impairment is directly related to the presence of [ROS] hence leading to cytotoxicity. OTA cause antioxidant dysfunction like glutathione peroxidase [GPx] and others, hence intensifying oxidative damage (Więckowska *et al.*, 2024).

Resveratrol inhibited ochratoxin biosynthesis through its biological actions are mainly ascribed to be antioxidant, anti-inflammatory, and anticancer attributes. Resveratrol activates pathway of nuclear transcription factor 2, increasing the production of enzymes which act as antioxidant and thereby diminishing oxidative stress in cells (Sorrenti *et al.*, 2013, El Khoury *et al.*, 2016).

Also, our experimental data suggest that resveratrol may affect the mycelial development and conidial formation of *A. niger* (Figures 3-5, 3-6, and 3-7). Wang *et al.* (2015) demonstrate that the majority of genes in the aflatoxin biosynthetic pathway (*aflA* and *aflB*) were strongly inhibited by resveratrol administration, leading to an inadequate supply of the precursor hexanoate for aflatoxin production. Moreover, resveratrol markedly enhanced the antioxidative enzymes effectivity for radicals' neutralization, resulting in reduced mycotoxins formation. Additionally, the genes associated with fungal reproductive units' development were suppressed, disrupting the ordered differentiation of cells and inhibiting conidia production and mycelial development (Wang *et al.*, 2015). Resveratrol is a stilbene polyphenol known for its established antifungal properties. The technique involves compromising cell membrane integrity, regulating oxidative stress, and obstructing fungal signaling pathways, which can decelerate or impede mycelial growth. The lipophilic properties of resveratrol enable it to engage with fungal cell membranes, compromising permeability and obstructing nutrient absorption essential for hyphal growth (Okamoto *et al.*, 2010).

5. Conclusions

Resveratrol treatment of *A. niger* [SMA2] reduced ochratoxin production and conidia formation, while also inducing aberrant mycelial growth. Initially, resveratrol directly suppressed the expression of the ochratoxin biosynthetic gene *AOPKS*. Low transcriptional levels in *A. niger* treated with 3 $\mu g/ml$ resveratrol may lead to a considerable minimization in ochratoxin synthesis.

Moreover, it influences the development of mycelia (colony). Modified morphology of mycelia and mycelioid colonies may stem from lowered gene family production. Finally, resveratrol significantly reduced conidia generation in *A. niger*, likely due to the inhibited transcription of genes associated with conidial development.

Acknowledgments

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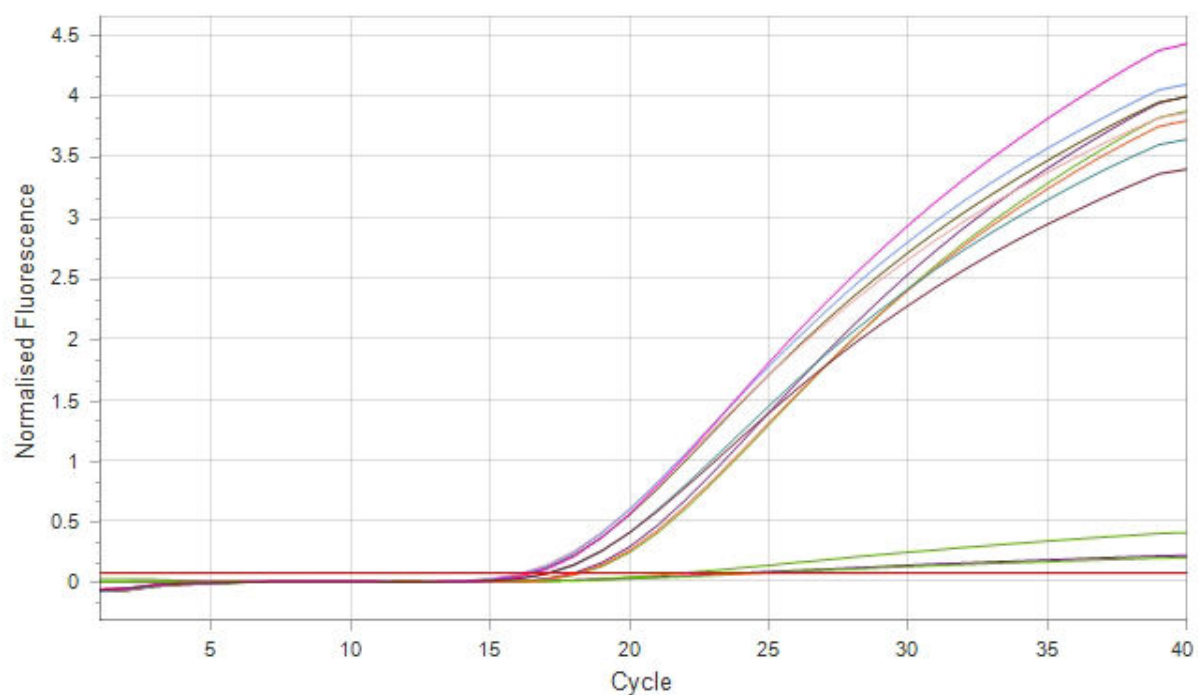
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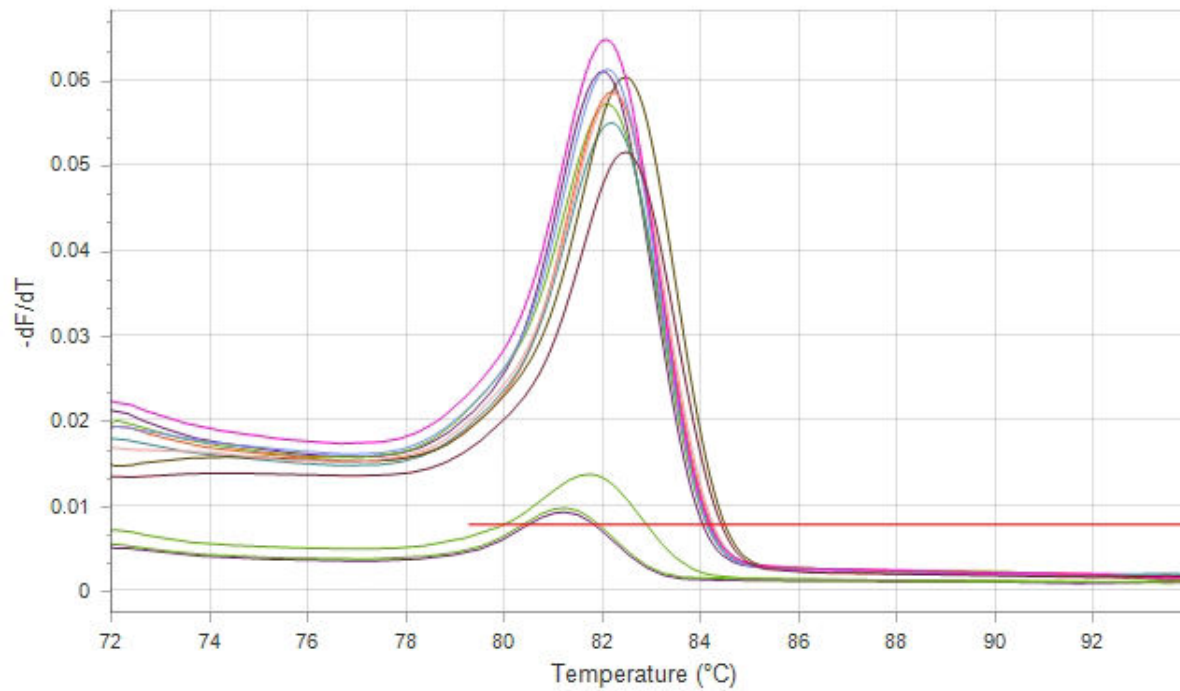
Appendix

Cycling: 18srRNA



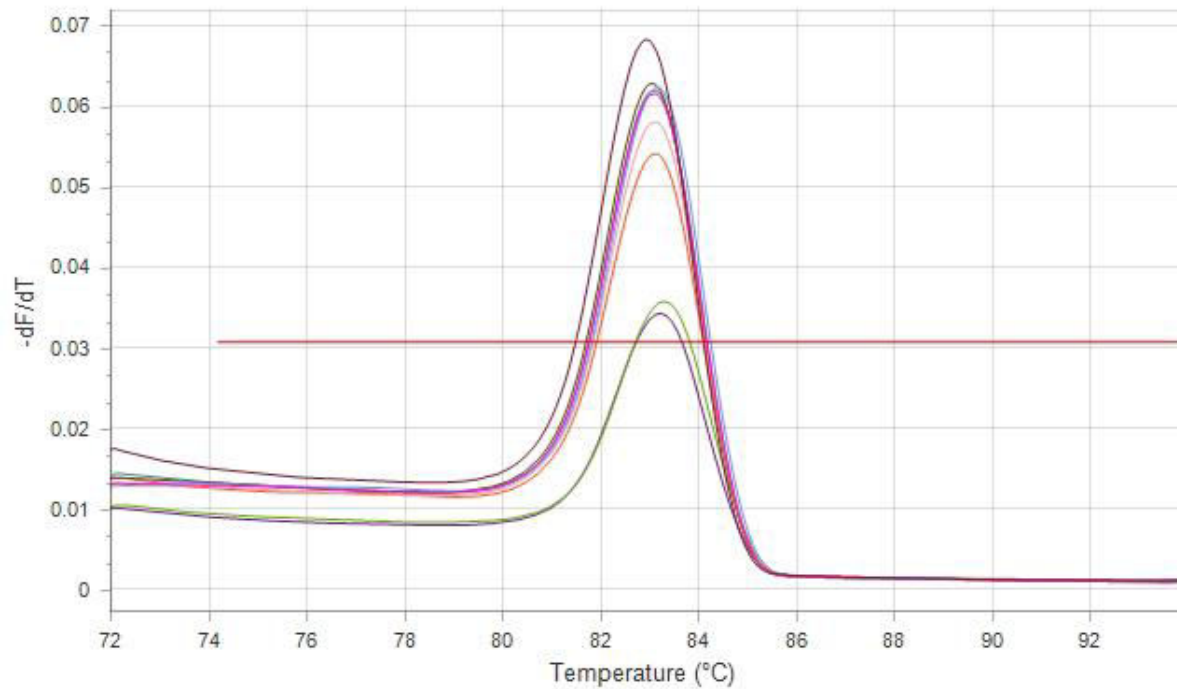
Well	Colour	Sample Name	Cq	Efficiency	Efficiency R ²	Result
1[B]	■	2.3.1	18.03	1.90	0.97944	
1[D]	■	C2.2	24.11	0.20	0.92930	
2[B]	■	2.3.2	18.25	1.70	0.98919	
2[C]	■	C2	22.02	0.39	0.96043	
2[D]	■	C2.3	24.83	0.19	0.90980	
3[B]	■	2.3.3	18.17	1.92	0.97804	
4[B]	■	2.9.1	17.16	1.57	0.99217	
5[B]	■	2.9.2	16.40	1.51	0.99692	
6[B]	■	2.9.3	16.15	1.49	0.99377	
7[B]	■	2.6.1	16.25	1.51	0.99541	
8[B]	■	2.6.2	16.54	1.68	0.99568	
9[B]	■	2.6.3	17.09	1.52	0.99285	

Melt: 18srRNA



Well	Colour	Sample Name	Tm (°C)
1[B]	■	2.3.1	81.99
1[D]	■	C2.2	81.18
2[B]	■	2.3.2	82.07
2[C]	■	C2	81.70
2[D]	■	C2.3	81.18
3[B]	■	2.3.3	82.17
4[B]	■	2.9.1	82.15
5[B]	■	2.9.2	82.44
6[B]	■	2.9.3	82.22
7[B]	■	2.6.1	82.08
8[B]	■	2.6.2	82.05
9[B]	■	2.6.3	82.44

Melt: Aopks



Sample 2

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Aspergillus niger isolate 7 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tr	1061	1217	100%	0.0	100.00%	KY318469.1
☐	Aspergillus niger strain T4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tr	1061	1061	100%	0.0	100.00%	MG833314.1
☐	Aspergillus niger strain C.S-5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and intern	1061	1167	100%	0.0	100.00%	MH511143.1
☐	Aspergillus niger strain WM10.74 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tran	1061	1061	100%	0.0	100.00%	HQ014696.1
☐	Aspergillus tubingensis isolate JH01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	1061	1061	100%	0.0	100.00%	HQ728255.1
☐	Aspergillus niger strain CIRM-BRFM.1439 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, c	1061	1061	100%	0.0	100.00%	PV106399.1
☐	Aspergillus luchuensis isolate FSpS06 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, ar	1061	1061	100%	0.0	100.00%	QPB10884.1
☐	Aspergillus tubingensis strain IHEM.17168 isolate ISHAM-ITS_ID MITS334 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5	1061	1061	100%	0.0	100.00%	KP131624.1
☐	Aspergillus niger isolate Asp.nig small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inte	1061	1061	100%	0.0	100.00%	MW585603.1
☐	Fungal sp. strain N160C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequenc	1061	1061	100%	0.0	100.00%	KU837366.1
☐	Aspergillus niger voucher HQJ.AB4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	1061	1061	100%	0.0	100.00%	MK640609.1
☐	Aspergillus niger haplotype ZmH27 18S small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, i	1061	1061	100%	0.0	100.00%	MG228419.1
☐	Aspergillus tubingensis strain ZIF7.2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	1061	1061	100%	0.0	100.00%	MN911298.1
☐	Aspergillus sp. isolate 1B internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequen	1061	1061	100%	0.0	100.00%	KY318479.1
☐	Aspergillus niger strain 91716 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcri	1061	1061	100%	0.0	100.00%	JN565296.1
☐	Aspergillus niger 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	1061	1061	100%	0.0	100.00%	KF879927.1
☐	Aspergillus niger strain An/MMA 2018 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, an	1061	1061	100%	0.0	100.00%	MK028957.1

Cyto-Genotoxic and Cell Cycle Arrest Assessment of a Synthetic Antioxidant Butylated Hydroxyanisole (BHA)

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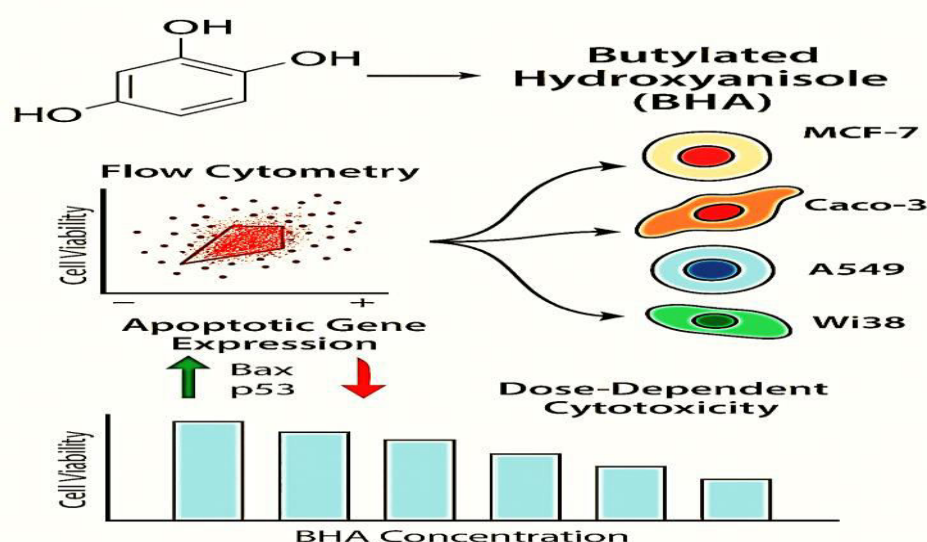
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Abstract

In order to stop oxidative deterioration, butylated hydroxyanisole (BHA), a synthetic antioxidant, is widely used in the food, pharmaceutical, and cosmetic industries. Recent cellular and molecular research indicates that BHA may have cytotoxic and genotoxic effects, especially when used at higher concentrations or for longer periods of time, even though it is generally recognized as safe (GRAS) at low quantities. Using the MTT assay, this study examined the effects of different BHA doses (100, 50, 25, 12.5, and 6.25 µg/ml) on the viability of multiple human cell lines, including Wi38 (normal lung fibroblasts), A549 (lung carcinoma), Caco-2 (colon carcinoma), and MCF-7 (breast carcinoma). Propidium iodide (PI) staining labeling was used in flow cytometry to further assess cell cycle. The findings showed that BHA had a cytotoxic effect on every type of cell that was studied. For both malignant and non-malignant cells, the IC₅₀ values were determined to be 58.55 µg/ml for MCF-7, 60.04 µg/ml for Caco-2, 93.61 µg/ml for A549, and 94.01 µg/ml for Wi38. These findings showed a concentration-dependent decrease in cell viability. Additional flow cytometric analysis showed that BHA treatment induced G2/M phase arrest and induced significant G2/M cell cycle arrest and altered the expression of apoptosis-related genes. Furthermore, it was discovered that increased BHA dosages had an impact on the expression of apoptosis-related genes. In particular, the anti-apoptotic gene *Bcl-2* was downregulated in both Caco-2 and ABC-1 cells, whereas the pro-apoptotic markers *Bax* and *p53* were upregulated. In conclusion, the results demonstrate that BHA can change apoptotic gene expression and promote programmed cell death in both healthy and malignant human cells, hence inducing cytotoxic and genotoxic reactions in a dose-dependent manner.

Keywords: Butylated hydroxyanisole (BHA); Cytotoxicity; Genotoxicity; Human cell lines; MTT assay; Apoptosis; Cell cycle arrest; Flow cytometry; Gene expression *Bax*; *p53*; *Bcl-2*.

Graphical Abstract



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1. Introduction

A common synthetic antioxidant used to stop oxidative rancidity in food items, cosmetics, and medications is butylated hydroxyanisole (BHA). 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole are its two isomeric constituents. BHA is frequently added to formulations that contain fat and oil in order to extend their shelf life because of its lipophilic qualities.

The toxicological profile of BHA has been extensively investigated through numerous in vitro studies employing both human and animal cell lines. HepG2 (human liver cancer), Caco-2 (human colon carcinoma), and A549 (human lung carcinoma) are cell lines that are often studied. Research indicates that exposure to BHA reduces cell viability, interferes with mitochondrial activity, and causes apoptosis (Lee et al 2012; Okamura et al 2023).

Characterizing BHA-induced apoptosis and changes in cell cycle progression has been made possible thanks in large part to flow cytometry. BHA treatment dramatically raised the sub-G1 population and Annexin V/PI staining in A549 cells, two markers of apoptosis. Additionally, treatment to BHA caused G0/G1 phase arrest, indicating that cell cycle progression was inhibited (Lee et al 2012; Tan et al 2022).

The application of quantitative real-time PCR (qRT-PCR) has been used to investigate the underlying molecular pathways. According to studies, BHA downregulates the anti-apoptotic gene *Bcl-2* while upregulating pro-apoptotic genes including *Bax* and *Caspase-3*. Furthermore, exposure to BHA has been shown to modulate the expression of several genes associated with oxidative stress and DNA repair pathways, including *p53*, *GADD45*, and *XRCC1* (Demiray et al. 2021; Farhadi et al. 2024). Food additives' genotoxic and apoptotic effects on human cell lines were investigated by Rashad et al. (2018), with particular attention on how they affected genes linked to apoptosis and cell cycle regulation. Additional research by Rashad et al. (2019, 2021) showed that these substances hindered the growth of human cells, both malignant and non-cancerous, and were cytotoxic to *Saccharomyces cerevisiae*. The comet test was used to assess DNA damage and identify the ideal amounts of these food-related compounds in a variety of yeast knockout strains.

According to a different study by Rashad et al. (2022), gold nanorods (AuNRs) cytotoxicity affected HepG2, Caco-2, A549, and CDD-19Lu cell lines, mostly by inducing cell cycle arrest in the G2/M phase. This implies that AuNRs have harmful effects on both healthy and cancerous cells. BHA has also been demonstrated to lower cell viability in a number of human cell lines. In a different study, Ali et al. (2024) discovered that doxorubicin caused PC3 prostate cancer cells to undergo apoptosis and G2/M arrest, along with increased *p53* and *Caspase-3* expression and decreased *Bcl-2* at higher dosages.

Tamarix nilotica ethyl acetate extracts were tested for their anticancer effects on A549 lung carcinoma cells by Abdel-Tawab et al. (2019). Normal Wi38 fibroblasts were largely unaffected by the extracts, whereas cancer cells showed notable cytotoxicity. The specific toxicity for cancer cells was validated by the Neutral Red assay.

El-Attar et al. (2019) used the MTT test to examine the cytotoxic activity of extracts from *Salvadora palaestina* and *Ziziphus album*. HepG2 cells treated with these plant extracts experienced G2/M phase arrest, according to flow cytometry. Additionally, qRT-PCR analysis revealed that, in comparison to untreated controls, the cell cycle regulators Cyclin B1 and CDK1 were downregulated.

According to the results of the MTT assay, SF6 significantly increased the cytotoxicity and induced apoptosis in HepG2, MCF-7, and HeLa cancer cells, while no toxicity was seen in normal lymphocytes. The results of qRT-PCR showed that SF6 upregulated the apoptosis regulating genes *Bax* and *p53* in cancer cells (Parveen and Varalakshmi 2023).

While single-nucleotide polymorphisms (SNPs) had negligible toxicity to normal fibroblast cells, they dramatically boosted calprotectin's toxicity against A549, MCF7, and PANC-1 cells at 0.75 µg/ml. It was also clear that co-incubation of SNPs and camptothecin successfully disrupted the pathways crucial for cancer cell survival because SNPs reduced the production of vascular endothelial growth factor (VEGF) and altered the profile of inflammatory markers and antioxidant enzymes (Alqaraleh et al. 2025). The chosen therapeutic leech species, the protein dosage or content of the leech saliva, and the applied cell line may all have different and variable impacts on the cell survival, migration, and death of different cell lines (Ünal et al., 2025). NTBF was identified as a novel inducer of cell death due to its cytotoxic activity, ability to arrest the cell cycle, and potent induction of apoptosis, primarily through activation of the caspase-dependent apoptotic pathway rather than the NRF2/GCLM antioxidant signaling pathway. Furthermore, NTBF strain ATCC-23745 might be a secret probiotic that is employed in future research on the therapy of colorectal cancer (Loniakan et al., 2023).

Using HepG2, A549, and Wi38 cell lines, Mousa et al. (2022a, 2022b) investigated the cytotoxic effects of different heavy metal concentrations. Significant DNA damage was found by the comet assay, especially in yeast strains, suggesting a high potential for genotoxicity.

The purpose of the study is to determine the underlying apoptotic mechanisms by assessing the cytotoxic and genotoxic effects of the synthetic antioxidant Butylated Hydroxyanisole (BHA) on human cell lines using cellular and molecular assays, such as RT-PCR and flow cytometry.

2. Materials and Methods

2.1. Materials

2.1.1. Butylated hydroxyanisole (BHA)

- Properties

The industrial antioxidants butylated hydroxyanisole (BHA) comes in two isomeric varieties: 2-tert-butyl-4-hydroxyanisole (2-BHA) and 3-tert-butyl-4-hydroxyanisole (3-BHA). BHA compositions must have at least 85% of the 3-BHA isomer, according to requirements set by the European Union and JECFA (EFSA, 2011).

- Concentrations

100, 50, 25, 12.5, and 6.25 µg/ml of BHA were utilized.

2.1.2. Cell lines

2.1.2.1. Mammalian Cell Lines

The American Type Culture Collection (ATCC, Rockville, MD, USA) provided the cell lines used in this study, including MCF-7 (human breast carcinoma), Caco-2 (human colon carcinoma), A549 (human lung carcinoma), ABC-1 (human lung adenocarcinoma), and Wi38 (normal human lung fibroblasts). Based on the cytotoxicity results, Caco-2 and ABC-1 cell lines were selected for further mechanistic investigations, including cell cycle analysis by flow cytometry and quantitative real-time PCR (qRT-PCR), to evaluate the molecular events associated with BHA-induced cytotoxicity and apoptosis.

2.1.2.2. Culture Media and Reagents

Lonza (Belgium) provided the necessary cell culture reagents, which included fetal bovine serum (FBS), RPMI-1640 medium, HEPES buffer, L-glutamine, gentamycin, and 0.25% Trypsin-EDTA.

2.1.2.3. Cell Line Maintenance

The cells were grown in RPMI-1640 media with 50 µg/ml of gentamycin and 10% heat-inactivated fetal bovine serum added. For the purpose of maintaining ideal growth circumstances, sub-culturing was carried out two or three times every week.

2.2. Methods

2.2.1. Cytotoxicity evaluation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess cytotoxicity. Following a 24-hour incubation period, tumor cell lines were seeded into Corning 96-well plates at a density of 5×10^4 cells per well for antitumor testing. Following incubation, the 96-well plates were treated in triplicate with different doses of BHA. The control was a 0.5% solution of dimethyl sulfoxide (DMSO). According to Capone et al. (2014), the MTT test was used to assess cell viability after a 24-hour treatment period.

2.2.2. Cell cycle analysis by propidium iodide (PI) assay using flow cytometry

Warmed trypsin-EDTA combined with warm phosphate-buffered saline (PBS) with 0.25% ethylenediaminetetraacetic acid (EDTA) (500 µl trypsin + 500 µl PBS) were added to cell lines and maintained for 10 minutes at 37°C. The process adhered to Rashad et al.'s technique (2018, 2022). Attune flow cytometry was used to examine the labeled cell lines (Applied Biosystems, USA).

2.2.3. Cell Cycle Analysis by PI assay using flow cytometry

2.2.3.1. Cell Collection and Staining

The supernatant was removed following centrifugation at $1-5 \times 10^5$ cells. Following two rounds of washing in warm PBS solution, the recovered cells were once again suspended in 500 µl of 1X binding buffer. Following the

protocol described by Vermes et al. (1995) and Ali et al. (2024), 5 µl of Propidium Iodide (PI) was added, and the cells were left to stand for 5 minutes at low temperatures in dark conditions.

2.2.3.2. Flow Cytometry Analysis

PI-stained cells was analyzed using an Attune Flow Cytometer (Applied Biosystems, USA).

2.2.4. Quantitative RT-PCR Analysis of *Bax*, *Bcl-2*, and *p53* Gene Expression Following BHA Treatment

Total RNA was extracted from treated and untreated cell lines using the GeneJET RNA Purification Kit (Thermo Scientific, #K0731, USA) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically. A total of 5 µg of RNA was reverse transcribed into complementary DNA (cDNA) using the RevertAid H Minus Reverse Transcriptase Kit (Thermo Scientific, #EP0451, USA). Quantitative real-time PCR (qRT-PCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) to evaluate the expression levels of the apoptosis-related genes *Bax*, *Bcl-2*, and *p53* described by Rashad et al. (2026). *β-actin* was used as the housekeeping gene for normalization. Each PCR reaction was carried out in a total volume of 25 µL containing 12.5 µL of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, #K0221, USA), 2 µL of cDNA, 1 µL of each forward and reverse primer, and 8.5 µL of nuclease-free water. Thermal cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. A melt curve analysis was performed from 63°C to 95°C to verify amplification specificity (Table 1 & Table 2). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

Table 1. The thermal cyclers condition used during real time PCR.

Initial denaturation	Denaturation	Annealing	Extension	Cycles
95°C/ 10 min	95°C/ 15 sec	60°C/ 30 sec	72°C/ 30 sec	40

β-actin was used as the housekeeping gene for normalization of target gene expression levels. Relative gene expression was quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method described by Livak and Schmittgen (2001). The threshold cycle (Ct) values of the target genes were normalized to the Ct value of the reference gene (*β-actin*) to obtain ΔCt values. The untreated control group served as the calibrator, whereas the treated groups were considered the test samples. Relative fold changes in gene expression were calculated using the following equations:

$$\Delta Ct (\text{test}) = Ct (\text{target gene in treated group}) - Ct (\beta\text{-actin in treated group})$$

$$\Delta Ct (\text{calibrator}) = Ct (\text{target gene in control group}) - Ct (\beta\text{-actin in control group})$$

$$\Delta\Delta Ct = \Delta Ct (\text{test}) - \Delta Ct (\text{calibrator})$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Table 2. Forward and reverse primer sequences for *Bax*, *Bcl-2*, *p53*, and β -actin genes.

Gene	Forward primer (5'→3')	Reverse primer (3'→5')
<i>Bax</i>	CCTGTGCACCAAGGTGCCGGAAC	CCACCCTGGTCTTGGATCCAGCCC
<i>Bcl-2</i>	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
<i>p53</i>	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC
β -actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG

2.2.5. Statistical analysis

Data are presented as mean $\pm$ standard error (SEM) of three independent experiments. With SPSS 18.0 software (2011), one-way analysis of variance (ANOVA) was used to establish statistical significance. The formula $IC_{50} = (0.5 - b) / a$, where b is the intercept and a is the slope factor, was used to determine the half-maximal inhibitory concentration (IC_{50}), which shows how effective a substance is at blocking a particular biological or biochemical activity.

3. Results

3.1. Cytotoxicity evaluation

The MTT test was used to measure cytotoxicity. Cell viability gradually decreased as the BHA concentration rose, as Table 3 illustrates. Higher doses of BHA increased

its cytotoxicity and toxicity, which reduced the viability of all cell lines. Table 3 and Figure 1 show the dose-response curves for cell viability and the concentration that causes 50% growth inhibition (IC_{50}) for MCF-7, Caco-2, A549 and Wi38 cell lines.

The IC_{50} levels of tetrazolium, which served as a positive control in this investigation, were also examined in response to BHA dosages. The findings showed that all cancer cell lines had mild cytotoxicity when exposed to BHA concentrations. In particular, the IC_{50} values for human lung cancer (A549), human colon carcinoma (Caco-2), and human breast carcinoma (MCF-7) were 93.61 μ g/ml, 60.04 μ g/ml, and 58.55 μ g/ml, respectively. With an IC_{50} of 94.01 μ g/ml, BHA also demonstrated cytotoxic effects on human lung normal cells (Wi38) (Table 3 and Figure 1). These results demonstrate that BHA decreased the viability of both cancerous and healthy cells.

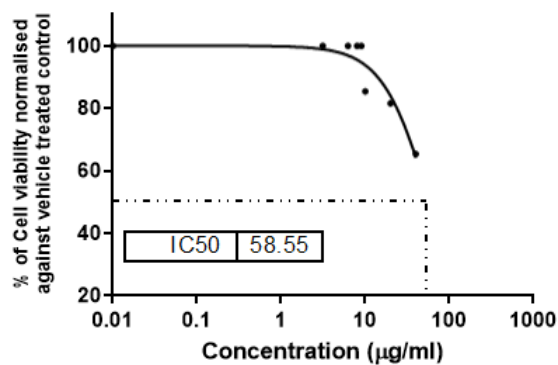
Table 3. Cytotoxic effects and IC_{50} values of BHA on MCF-7, Caco-2, A549, and Wi38 cell lines.

IC_{50}	Conc. μ g/ml	SD	Viability %	Toxicity %	ID
58.55	6.25	0	100	0	MCF-7
	12.5	0	100	0	[Breast cell line]
	25	0.02	65.39	34.61	
	50	0.022	81.62	18.38	
	100	0.012	85.47	14.53	
60.04	6.25	0	100	0	Caco-2
	12.5	0	100	0	[Human Colon cancer cell line]
	25	0.01	85	15	
	50	0.013	82	18	
	100	0.012	66	34	
93.61	6.25	0	100	0	A549
	12.5	0.01	100	0	[Human lung cancer cell line]
	25	0.009	86.3	13.7	
	50	0.205	81	19	
	100	0.004	76.11	23.89	
94.01	6.25	0	100	0	Wi38
	12.5	0.07	100	0	[Human lung normal cell line]
	25	0.065	86	14	
	50	0.004	81	19	
	100	0.061	76.1	23.9	

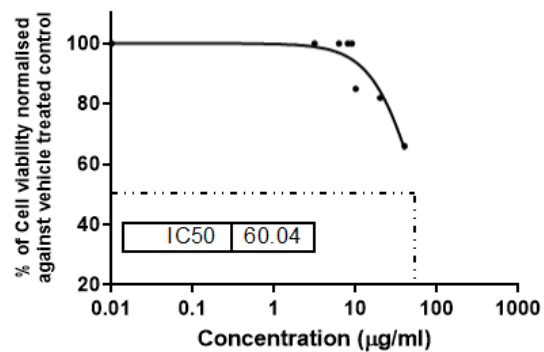
SD= standard deviation

IC_{50} =The half-maximal inhibitory concentration

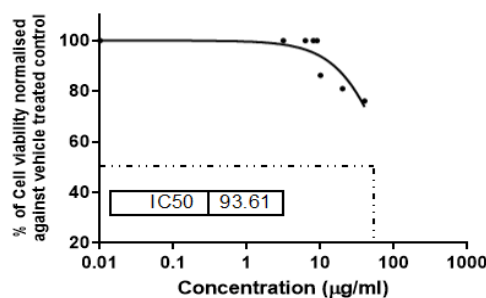
MCF-7 [Breast carcinoma cell line]



Caco-2 [Colon carcinoma cell line]



A549 [Lung carcinoma cell line]



Wi38 [Lung normal cell line]

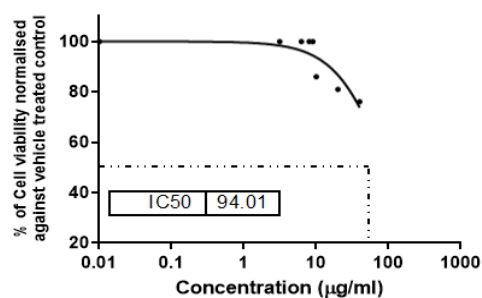


Figure 1. The non-malignant human's lung cell line Wi38 (used as a control) and the malignant human cell lines MCF-7, Caco-2, and A549 were utilized to assess the inhibitory effect of BHA on cell growth. The MTT assay was used to evaluate cell viability. In comparison to the untreated control group, statistical significance was established at $*p < 0.05$. The data are shown as mean values ($n = 3$). For every one of the four cell lines, the half-maximal inhibitory concentration (IC50) was determined.

3.2. Cell Cycle Analysis by PI Assay Using Flow Cytometry

The arrangement of DNA content in Caco-2 and ABC-1 cell lines was affected by the 50 µg/ml BHA exposure. After receiving BHA treatment, the average DNA content in the G0/G1 phase dropped from $60\% \pm 2.24$ (Caco-2 control) and $62\% \pm 2.36$ (ABC-1 control) to $15\% \pm 2.67$ and $48\% \pm 2.27$, respectively. Following treatment, the DNA content decreased from $24\% \pm 1.28$ (Caco-2) and $21\% \pm 1.28$ (ABC-1) to $20\% \pm 1.11$ and $17\% \pm 1.50$ in the S phase, showing a similar pattern.

On the other hand, after BHA treatment, the G2/M phase revealed a substantial increase in DNA content. Tables 4 and 5 show that G2/M DNA content averages for Caco-2 and ABC-1 cells treated with BHA were $65\% \pm 2.90$ and $35\% \pm 2.32$, respectively, while the untreated controls had G2/M DNA content averages of $16\% \pm 1.00$ and $17\% \pm 1.47$.

Overall, both cell lines' normal cell cycle progression was disturbed by treatment with BHA at 50 µg/ml, which resulted in a significant increase in the number of cells halted in the G2/M phase. According to these findings, BHA causes cell cycle arrest at the G2/M checkpoint, as shown in Figure 2, where both Caco-2 and ABC-1 cells exhibit a significant buildup of DNA. These alterations indicate that BHA induces cell cycle arrest at the G2/M phase, contributing to its cytotoxic effects.

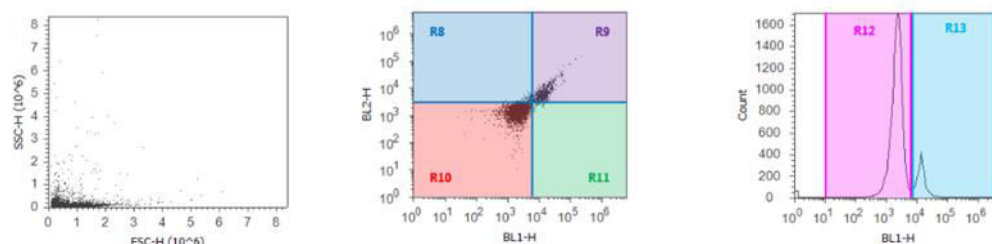
Table 4. shows the percentage of DNA content in unprocessed Caco-2 cells (control) during various cell cycle phases in comparison to Caco-2 cells that received 50 µg/ml of BHA.

Groups	Average of DNA content % of cells in each cell cycle phase		
	G0/G1 phase (Mean± SEM)	S phase (Mean± SEM)	G2/M phase (Mean± SEM)
Caco-2-Control	$60\% \pm 2.24^{a1}$	$24\% \pm 1.28^{a2}$	$16\% \pm 1.00^{c3}$
Caco-2-Treated with BHA	$15\% \pm 2.67^{c3}$	$20\% \pm 1.11^{a2}$	$65\% \pm 2.90^{a1}$

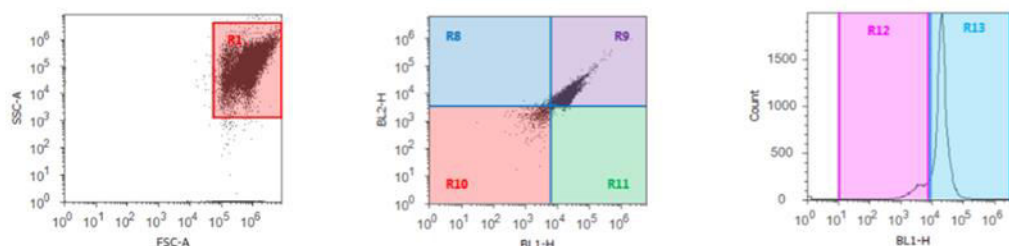
Table 5 shows the variance of DNA content percentages in both handled (control) and stimulated (50 µg/ml BHA) ABC-1 cells across cell cycle cycles.

Groups	Average of DNA content % of cells in each cell cycle phase		
	G0/G1 phase (Mean± SEM)	S phase (Mean± SEM)	G2/M phase (Mean± SEM)
ABC-1 Control	$62\% \pm 2.36^{a1}$	$21\% \pm 1.28^{a2}$	$17\% \pm 1.47^{c2}$
ABC-1 Treated with BHA	$48\% \pm 2.27^{b1}$	$17\% \pm 1.5^{a3}$	$35\% \pm 2.32^{a2}$

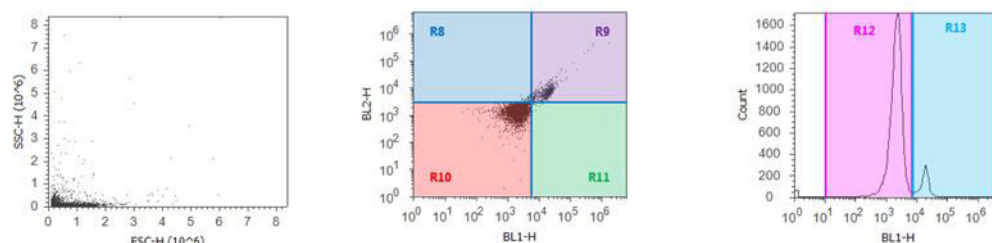
Untreated Caco-2 cells



Caco-2 cells treated by BHA



Untreated ABC-1 cells



ABC-1 cells treated by BHA

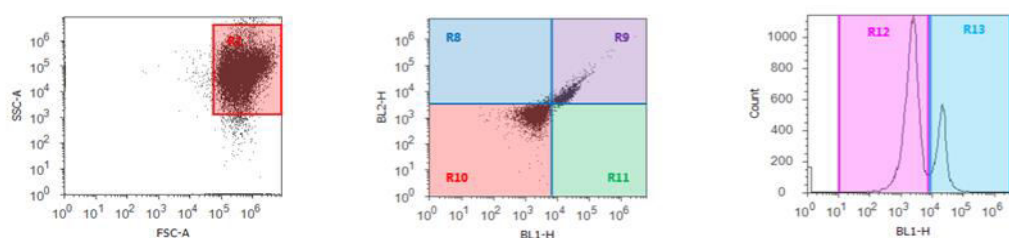


Figure 2. When comparing controls without treatment with cells treated with 50 µg/ml BHA, flow cytometric examination of the cell cycle distribution in the colon and lung cancer cell lines Caco-2 and ABC-1 reveals accumulation during the G2/M phase.

The findings of this investigation showed that BHA causes G2/M phase arrest in both Caco-2 and ABC-1 cell lines, hence causing cytotoxic effects. Flow cytometry confirmed an increase in the G2/M population after BHA treatment, and this arrest was seen across a dose range of 6.25 to 100 µg/ml. At doses of 6.25, 12.5, 25, 50, and 100 µg/ml, which correspond to physiologically significant exposure levels, a dose-dependent cytotoxic reaction was visible. The activation of *Bax* and the mitochondrial (intrinsic) route are two of the several routes that control the induction of apoptosis. Using cell culture models, the effect of BHA on cell death was further evaluated at a dose of 50 µg/ml. Figure 2 shows that BHA induced marked G2/M cell cycle arrest in both Caco-2 and ABC-1 cells.

3.3. Evaluation of BHA-Induced Genotoxicity by Quantitative RT-PCR Gene Expression Analysis of *Bax*, *Bcl-2*, and *p53* in Caco-2 Cell Lines

Using quantitative real-time PCR (RT-PCR), the expression of important apoptosis-regulating genes, such as *Bax*, *p53*, and *Bcl-2*, was examined in order to assess the possible genotoxic effects of BHA on Caco-2 cell lines.

Pro-apoptotic genes were significantly upregulated after BHA therapy. In particular, compared to untreated controls, *p53* increased by 3.58 and *Bax* expression increased by 8.51-fold (Table 6). On the other hand, the expression of the anti-apoptotic gene *Bcl-2* dropped to 0.08 times that of the control group, indicating a considerable downregulation (Table 6). Figure 3 further demonstrates these results by confirming that the treated cells had increased expression of *p53* and *Bax*. The data presented reflect the relative gene expression levels

calculated according to the $2^{-\Delta\Delta Ct}$ method, where β -actin was used as the housekeeping gene for normalization. For each sample, the ΔCt value was obtained by subtracting the Ct value of β -actin (reference gene) from that of the target gene ($\Delta Ct = Ct_{\text{target}} - Ct_{\text{reference}}$). The $\Delta\Delta Ct$ value was calculated by comparing the ΔCt of the treated group with the ΔCt of the control group ($\Delta\Delta Ct = \Delta Ct_{\text{treated}} - \Delta Ct_{\text{control}}$). The relative expression (fold change) was then determined

using the equation $2^{-\Delta\Delta Ct}$, where values greater than 1 represent gene upregulation, while values below 1 indicate downregulation.

According to the research, BHA mainly causes apoptosis in Caco-2 cell lines by downregulating survival signals mediated by *Bcl-2* and upregulating pro-apoptotic pathways (such as *p53* and *Bax*).

Table 6. RT-qPCR measurements of the relative mRNA expression levels of the *Bax*, *p53*, and *Bcl-2* genes in Caco-2 cell lines after BHA treatment.

Sample data	Groups TE& TE	<i>Ct</i> (<i>Bax</i>) values	β -actin ΔCt -HC	$\Delta\Delta Ct$ (treated vs control)	Fold change ($2^{-\Delta\Delta Ct}$)	SEM
Sample code (<i>Bax</i>)	Caco-2 control	29.63	1.75	0.00	1.00	0.06
	Caco-2 treated	24.74	-1.34	-3.09	8.51	0.29
Sample data	Groups TE& TE	<i>Ct</i> <i>p53</i> values	β -actin ΔCt -HC	$\Delta\Delta Ct$ (treated vs control)	Fold change ($2^{-\Delta\Delta Ct}$)	SEM
Sample code (<i>p53</i>)	Caco-2 control	28.13	0.25	0.00	1.00	0.07
	Caco-2 treated	25.49	-1.59	-1.84	3.58	0.21
Sample data	Groups TE& TE	<i>Ct</i> <i>Bcl-2</i> alues	β -actin ΔCt -HC	$\Delta\Delta Ct$ (treated vs control)	Fold change ($2^{-\Delta\Delta Ct}$)	SEM
Sample code (<i>Bcl-2</i>)	Caco-2 control	28.96	1.08	0.00	1.00	0.06
	Caco-2 treated	32.64	4.66	3.58	0.08	0.01

Legend: Ct = cycle threshold for the target gene (*Bax*). $\Delta\Delta Ct = (\Delta Ct_{\text{treated}} - \Delta Ct_{\text{control}})$, where $\Delta Ct = Ct_{\text{target}} - Ct_{\text{reference}}$ (reference = β -actin). Fold change = $2^{-\Delta\Delta Ct}$. Values are shown as mean fold change $\pm$ SEM. Upregulation is indicated by fold change >1 .

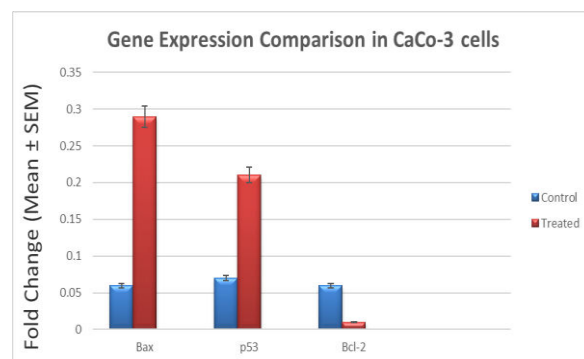


Figure 3: Comparative RT-PCR analysis of the expression levels of apoptosis-related genes (*p53*, *Bax*, and *Bcl-2*) in response to 50 μ g/ml BHA treatment. *P less than 0.05 in relation to the untreated control.

3.4. Quantitative RT-PCR Analysis of BHA-Induced Genotoxicity on Genes Associated with Apoptosis in ABC-1 Cell Lines

The expression levels of important apoptosis-regulatory genes, including *Bax*, *p53*, and *Bcl-2*, were analyzed using quantitative real-time PCR (qRT-PCR) in order to

ascertain the genotoxic effects of BHA on ABC-1 lung cancer cells. The pro-apoptotic genes' expression significantly increased after BHA treatment. In particular, *p53* and *Bax* expression were 4.20 and 3.56 times higher, respectively, than in the medicated control group (Table 7). On the other hand, the anti-apoptotic gene *Bcl-2*'s expression was drastically decreased, falling to 0.05 times that of control levels. Figure 4 depicts these alterations, emphasizing the increased expression of *p53* and *Bax* following therapy.

According to these findings, BHA mainly induces apoptosis in ABC-1 cells by upregulating the expression of genes linked to apoptosis and downregulating the expression of genes linked to survival, such as *Bcl-2*.

Table 7. qRT-PCR analysis of the relative expression levels of the genes *Bax*, *p53*, and *Bcl-2* in ABC-1 cell lines following BHA therapy.

Sample data	Groups TE& TE	Ct (<i>Bax</i>) values	β -actin Δ CT-HC	$\Delta\Delta$ Ct (treated vs control)	Fold change ($2^{-\Delta\Delta$ Ct)	SEM
Sample code (<i>Bax</i>)	ABC-1 control	27.53	-0.35	0.00	1.00	0.04
	ABC-1 treated	25.9	-2.18	-1.83	3.56	0.24
Sample data	Groups TE& TE	Ct <i>p53</i> values	β -actin Δ CT-HC	$\Delta\Delta$ Ct (treated vs control)	Fold change ($2^{-\Delta\Delta$ Ct)	SEM
Sample code (<i>p53</i>)	ABC-1 control	31.27	2.39	0.00	1.00	0.04
	ABC-1 treated	25.4	0.32	-2.07	4.20	0.25
Sample data	Groups TE& TE	Ct <i>Bcl-2</i> values	β -actin Δ CT-HC	$\Delta\Delta$ Ct (treated vs control)	Fold change ($2^{-\Delta\Delta$ Ct)	SEM
Sample code (<i>Bcl-2</i>)	ABC-1 control	26.78	0.90	0.00	1.00	0.06
	ABC-1 treated	32.33	5.25	4.35	0.05	0.01

Legend: Ct = cycle threshold for the target gene (*Bax*). $\Delta\Delta$ Ct = (Δ Ct_{treated} – Δ Ct_{control}), where Δ Ct = Ct_{target} – Ct_{reference} (reference = β -actin). Fold change = $2^{-\Delta\Delta$ Ct. Values are shown as mean fold change $\pm$ SEM. Upregulation is indicated by fold change >1.

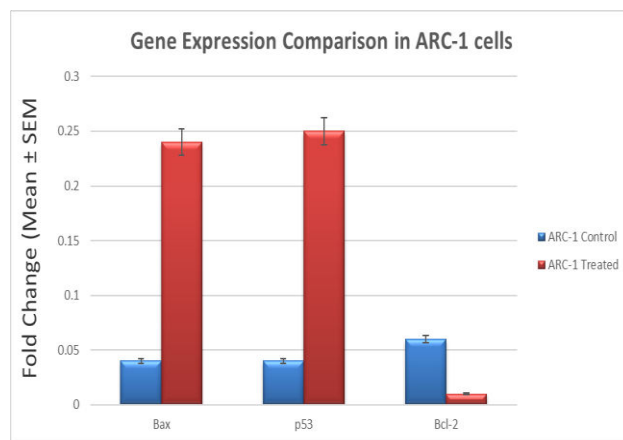


Figure 4: Quantitative RT-PCR was used to evaluate the effects of 50 μ g/ml BHA on apoptotic-related genes, including mRNA expression of *Bax*, *Bcl-2*, and *p53*, with *P < 0.05 when compared to the control group.

In comparison to the untreated control group, the results showed a considerable elevation of *Bax* gene expression in both Caco-2 and ABC-1 cells after BHA treatment. Following exposure to the BHA chemical This increase in expression was clearly evident in both treated cell lines, as Tables 6 and 7 and Figures 3 and 4 demonstrate.

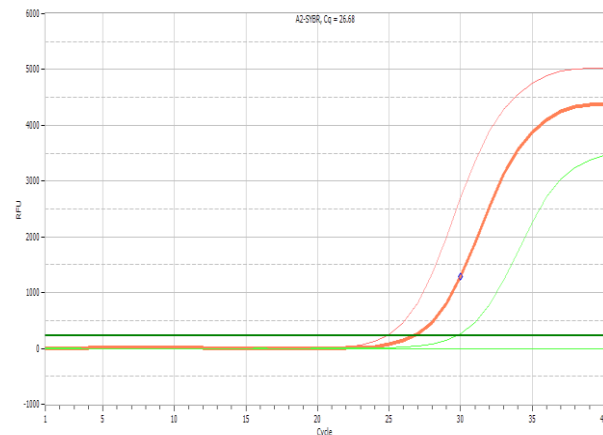


Figure 5 shows the cycle threshold (Ct) measurements for the expression of the *Bax* gene as linear (top) and logarithmic (bottom) amplified curves.

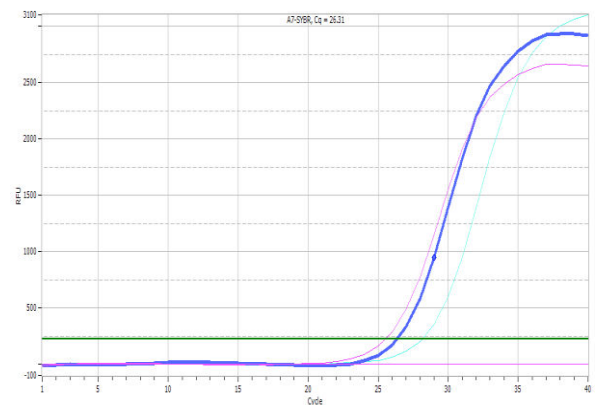


Figure 6. The quadratic (upper) and log (lower) amplification curves in illustrate the *p53* gene's frequency of transcription.

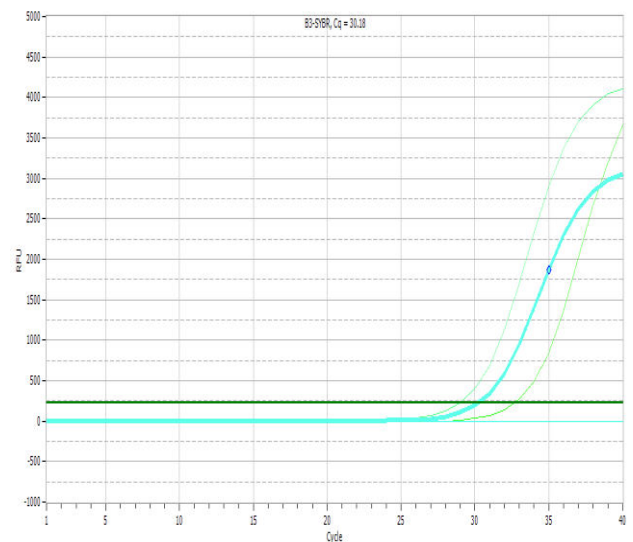


Figure 7 The log (lower) and logarithmic (upper) multiplying curves that match the Ct values for the *Bcl-2* gene are displayed.

4. Discussion

Butylated Hydroxyanisole's (BHA) cytotoxic and genotoxic impacts on human carcinoma cell lines, such as Caco-2 (colon cancer) and ABC-1 (lung cancer) cells, were the focus of this investigation. Using MTT tests, flow cytometry, and quantitative RT-PCR, we investigated the effects of BHA on cell viability, cell cycle progression, and the expression of genes linked to apoptosis (*Bax*, *Bcl-2*, and *p53*).

5. Cell viability and cytotoxicity

After BHA treatment, the MTT assay showed a dose-dependent decrease in cell viability. The MCF-7, Caco-2, A549, and Wi38 cell lines had IC₅₀ values of 58.55 µg/ml, 60.04 µg/ml, 93.61 µg/ml, and 94.01 µg/ml, respectively, as indicated in Table 3. These findings imply that BHA has cytotoxic effects on cell lines that are malignant as well as those that are not. These results are in line with earlier research that documented BHA's cytotoxic effects on many cancers cell types (Capone et al 2014). To maximize its therapeutic potential for different cancer types, more research is necessary, as evidenced by the comparatively high amounts of BHA needed to produce noticeable cytotoxicity.

In a number of human cell lines, including MCF-7 (breast carcinoma), Caco-2 (colon carcinoma), A549 (lung carcinoma), and Wi38 (normal lung cells, used as a control), studies showed an important buildup and cytotoxic effect of different BHA concentrations. When compared to the other lines, BHA showed the strongest effect on Wi38 cell viability, suggesting greater sensitivity. MCF-7 cells, on the other hand, responded the least, showing just slight alterations in viability. These results are in line with research by Rashad et al. (2019, 2022), who found that different dietary additives had distinct effects on the morphology and survival of human cancer cell lines Caco-2, MCF-7, A549, and normal Wi38 cells. Their findings supported worries about these drugs' potential to cause cancer by highlighting notable differences between the treated and control groups. The employing of these models to evaluate cytotoxic potential was further validated by Mousa et al. (2022), who found that exposure to heavy metals significantly decreased the growth of HepG2, A549, and Wi38 cell lines as measured by the MTT assay.

6. Analysis of the Cell Cycle

Both Caco-2 and ABC-1 cell lines experienced G2/M phase arrest when exposed to BHA, according to flow cytometric monitoring of the cell cycle. The percentage of cells in the G2/M phase significantly increased, as seen in Figures 2, 3, and 5, suggesting that BHA impedes the progression of the cell cycle. These results are consistent with earlier research indicating that a number of cytotoxic drugs, such as BHA, induce G2/M arrest by interfering with the regular cell cycle and causing checkpoint activation or DNA damage (Rashad et al. 2022 & Rashad et al. 2025). A reduction in the G0/G1 and S phases and an increase in cells in the G2/M phase indicate that BHA impedes cell cycle progression, which in turn prevents cell division and proliferation.

ZnSO₄ substantially suppressed cell growth by causing G2/M cell cycle arrest, as demonstrated by Mousa et al. (2022a), who reported a considerable buildup of HepG2 cells in the G2/M phase. According to in vitro research, human cell lines exposed to this substance showed decreased viability, elevated cytotoxicity, and the start of apoptosis. Rashad et al. (2018) confirmed the inhibitory effects on cancer cell development by investigating gene expression in Caco-2 cells after apoptosis and cell cycle arrest. These results were corroborated by flow cytometric analysis, which revealed that HepG2 liver cancer cells had reduced viability. In a separate investigation, Rashad et al. (2022) confirmed the possible harmful mechanism through cell cycle arrest by observing G2/M phase accumulation in HepG2 cells exposed to dietary additives.

Furthermore, using flow cytometric analysis, Ali et al. (2024) showed that doxorubicin markedly caused apoptosis and G2/M phase cell cycle arrest in PC3 cells. These outcomes are consistent with what we have discovered thus far.

Cadmium chloride was demonstrated to cause apoptosis in HepG2 cells, with a progressive rise in annexin-positive cells in comparison to untreated controls, in line with these previous investigations (EL-Attar et al. 2019; Mousa et al 2022b). The current study's use of flow cytometry further demonstrated that BHA caused both cell cycle arrest and apoptosis. BHA treatment specifically increased the sub-G1 population and annexin V/PI staining in A549 cells, demonstrating the pro-apoptotic impact. Furthermore, BHA treatment hindered normal cell cycle progression by causing arrest at the G0/G1 phase (Abdel-Tawab et al 2019; Tan et al 2022).

These results are mostly in line with the research of Mizobuchi et al. (2022), who found that BHA-sensitive and BHA-resistant cell types responded differently to genotoxic stress. Their research demonstrated BHA's capacity to regulate cellular stress responses and apoptotic pathways by connecting these differences to differences in reactive oxygen species (ROS) formation, DNA damage, and apoptosis levels after UV exposure.

7. Gene Expression and Apoptosis

The molecular processes behind BHA-induced cell death were clarified by the RT-PCR investigation. The pro-apoptotic gene *Bax* and the tumor suppressor gene *p53* were significantly upregulated in both Caco-2 and ABC-1 cell lines following BHA treatment, as indicated in Table 7 and Figures 3 and 4. In Caco-2 and ABC-1 cells, for example, *Bax* expression increased 8.51 and 3.56 times, respectively, but *p53* expression increased 3.58 and 4.20 times. These results imply that BHA triggers apoptosis by triggering signaling pathways that promote apoptosis, such as the mitochondrial pathway. Moreover, the anti-apoptotic gene *Bcl-2* was significantly downregulated upon BHA treatment, with expression levels falling to 0.08-fold in Caco-2 cells and 0.05-fold in ABC-1 cells. The idea that BHA encourages apoptosis by blocking survival signals that shield cells from programmed cell death is further supported by this downregulation of *Bcl-2* (Esazadeh et al 2024).

The downregulation of *Bcl-2* and the overexpression of *p53* and *Bax* suggest that BHA mainly triggers apoptosis in cancer cells via activating pro-apoptotic pathways.

These findings are consistent with earlier research showing that BHA can induce mitochondrial-dependent apoptosis through altering the expression of important proteins that regulate apoptosis (Luan and Honma, 2022). Moreover, the substantial increase of *p53* implies that BHA might trigger DNA damage reactions, which would then trigger apoptotic signaling. The current study's findings are consistent with other investigations showing that cadmium chloride causes HepG2 cells to undergo apoptosis. Cadmium chloride-treated samples showed a progressive rise in annexin-positive cells relative to untreated controls, indicating apoptotic progression. Furthermore, Mousa et al. (2022a) found that differing degrees of DNA damage and apoptosis indicated that malignant and normal cell lines were differently sensitive to doxorubicin-induced genotoxic stress. At 96 hpf, a greater number of apoptotic cells was seen in larvae exposed to BHA. Oxidative stress was indicated by a decrease in the activity of glutathione peroxidase (GPx), glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) (Veshaal et al. 2025).

According to recent research compiled in this description, high gastrointestinal tract consumption of synthetic antioxidants such TBHQ, BHA, and PG can have cytotoxic, genotoxic, and perhaps carcinogenic consequences. According to thermodynamic analyses, these chemicals' binding to albumin is mostly mediated by hydrophobic interactions. Moreover, molecular modeling demonstrates that the predominant binding site for TBHQ, BHA, and PG is subdomain IIA of albumin. Together, these findings lend credence to the idea that, in order to lower any health concerns, dietary consumption of TBHQ, BHA, and PG from food additives should be kept to a minimum (Esazadeh et al 2024). Elevated levels of 8-OHdG have been linked to exposure to BHA and TBHQ, suggesting oxidative DNA damage. The evidence points to a possible function for BHA and/or chemicals with numerous hydroxyl groups in inducing apoptosis through enhanced formation of reactive oxygen species (ROS) (Baran et al 2020).

8. Conclusions

The results of this investigation show that butylated hydroxyanisole (BHA) has dose-dependent cytotoxicity and can stop the growth of human cell lines. The evidence makes it abundantly evident that BHA causes human cells to undergo both apoptosis and toxicity. The distribution of DNA content in the cell cycle phases of Caco-2 and ABC-1 cell lines was considerably impacted by exposure to BHA at a concentration of 50 µg/ml, according to flow cytometry analysis. This was especially evident in the G0/G1 phase population, which was reduced when compared to untreated controls.

The expression of important apoptosis-related genes was assessed using quantitative real-time PCR (qPCR) in order to better investigate BHA-induced apoptosis. After BHA treatment, the anti-apoptotic gene *Bcl-2* was significantly downregulated, but *Bax* and *p53* were upregulated in both Caco-2 and ABC-1 cell lines. The notion that BHA predominantly causes cell death by activating pro-apoptotic pathways and suppressing anti-apoptotic signals is supported by these molecular studies.

All of these findings point to the possibility that BHA, a widely used food ingredient, may damage human cell lines by causing apoptosis and interfering with normal cell cycle development. Because BHA has been shown to have cytotoxic and genotoxic properties, its use in consumer goods should be strictly controlled. To reduce any health concerns and maintain public safety, stringent allowable limits must be established.

In conclusion, our work offers strong evidence that BHA and other food additives can have detrimental effects on human health by lowering cell viability and changing apoptosis-related gene expression. These results underline how crucial it is to assess the biosafety of food additives and back the necessity of strict regulations to reduce human exposure from dietary and manufacturing sources.

Acknowledgments

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Conflicts of Interest

Author declares no conflict of interests.

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Machine Learning Approach to Identify and Evaluate the High protein-yielding Varieties of *Glycine max*

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Abstract

Soybeans are valued worldwide as legumes with exceptional health benefits, high nutritional value, and protein content. Though well-studied in many regions, there is limited information on the nutritional status of indigenous soybeans cultivated in the Uttarakhand region of the western Himalayan range in India, despite their prominence in the traditional food system. The present study aimed to assess protein content in indigenous soybean germplasms and use a machine learning algorithm to classify the nutritionally superior soybean samples. 50 samples were collected from different regions of the hilly areas of the state and evaluated for protein content using the Kjeldahl method, followed by an HPLC analysis to ascertain the amino acid content in germplasms with high protein content. Machine learning method-the Decision Tree algorithm (J48) in WEKA (Waikato Environment for Knowledge Analysis) software was used to analyse the attributes like location, seed traits, temperature, and nutrient content. The ten-fold cross-validation achieved 94 % accuracy in classifying germplasms according to the protein content, as shown in the confusion matrix. The study showed that maximum protein content in the analyzed samples ranges from 36.96% to 43.56%, mostly in samples collected at an elevation of 345 m – 1021 m, and 1699 m to 2044 m, latitudinal range of 28° to 30.9°, and longitudes 77.5° to 79.52°. HPLC analysis confirmed histidine as the most prominent amino acid, with the highest amount being 0.688 g/100g.

Keywords: Bhat, *Glycine max*, Food security, amino acids profiling, Machine learning.

1. Introduction

Soybean (*Glycine max*) is a leguminous crop (40-50% content) with significantly high protein, fat content, and is considered a potential solution to global food security (Keatinge et al., 2011; Tanwar and Goyal, 2021; Florentino et al., 2022). The protein content is attributed to its nitrogen-fixing capacity (Carter et al., 2016; Cafaro et al., 2020) that peaks during flowering and pod filling (Ciampitti et al., 2021; Kubar et al., 2021). Considering the high protein content, soy offers scope for diverse soy-based dietary and other consumable products to meet the nutritional needs and highlighting soybeans as an excellent source to address the issues of food security (Thrane et al., 2017; Qin et al., 2022). Soybeans also provide all nine essential amino acids, including histidine, isoleucine, lysine, methionine, phenylalanine, threonine, and tryptophan, supporting human health and development (Singer & Zhang, 2020; Hu et al., 2023). This further enhances the need for inclusion of soybean in the diet since the essential amino acids need to be taken from the diet; while non-essential amino acids can be produced internally (Thanapornpoonpong et al., 2008; Holeček 2020; Lopez & Mohinuddin, 2024).

Uttarakhand is a hilly state in Northern India where the native soybean is called 'bhat' and is a very popular cuisine. However, there is a scarcity of information on the nutritional profiling of this crop. This has led to lack of general awareness on the need for planned cultivation and utilization of soybean in the region. Plant-based proteins are considered a cheaper and more sustainable source of protein with high nutritional and nutraceutical potential. Asians typically prefer plant-based protein in several traditional cuisines due to its long shelf life and high nutritional value (Qin et al., 2022). In recent times, there has been a growing trend towards vegan and vegetarian lifestyles, and increased consumption of soy has been observed due to its high protein concentration (Tamang, 2012). A variety of products, such as sausages, bacon, burger patties, etc., containing soy protein are available in the market and readily consumed by consumers due to their low cost, and health benefits like less risk of cardiovascular ailments (Akintola et al., 2022; Chetachukwu et al., 2022). In this scenario, a focused study on indigenous soybean germplasms and analysis of protein content become important to identify superior germplasms and utilise them as a cost-effective protein source for a healthy diet, addressing issues of food security.

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The present study was conducted to establish the protein profile of soybean germplasm from the Garhwal region of Uttarakhand state in India and further assess the amino acid content in the samples with high protein content.

2. Materials and Methods

2.1. Protein estimation using Kjeldahl method

In this study, a total of 50 soybean cultivars (approximately 100 gm of each sample) were collected from different Uttarakhand regions and sun-dried for 3-4 days. The sample was further processed to prepare analytical sample (fine dry powder) of particle size 0.8-1mm using electric grinders. Estimation of Crude protein and nitrogen content was performed using the Kjeldahl method. The basic principle involves the digestion of the material in H_2SO_4 and conversion of protein 'N' to ammonium sulfate $(NH_4)_2SO_4$ at an elevated boiling point in the presence of potassium sulphate K_2SO_4 and copper (Cu) catalyst to enhance the reaction rate. Ammonia is released and subsequently steam distilled into boric acid under alkaline conditions, and then quantified by titration with standardized Hydrochloric acid (HCl). The methodology involves two steps: digestion and distillation. Block digester was switched on and heated up to $420^\circ C$. 2 g 'bhat' sample (10 gm) with sulphuric acid H_2SO_4 and potassium sulphate and copper sulphate as catalysts (7.0 g K_2SO_4 + 0.8 g $CuSO_4$) were added into the digestion tubes. This process of digestion was carried out for 50-60 minutes. The samples were cooled down for another 15-20 min, and they were proceeded further for the distillation process. Digested Kjeldahl tubes were connected to the distillation unit along with a 40% NaOH solution in the alkali tank. The unit contains an Erlenmeyer titration flask (capacity: 500 ml) filled with 30ml boric acid (H_3BO_3) solution along with an indicator. As soon as the distillation process begins the procedure of the automatic titration system immediately starts the titration inside the Kjeldahl tube. It is steam distilled until ≥ 150 ml of distillate is collected in the titration flask. H_3BO_3 acts as an adsorbent receives the distillate from the distillation unit and facilitates titration to estimate the quantity of nitrogen and protein content in the sample using 0.1000M HCl standard solution. The whole procedure was carried out automatically by using a steam distiller with automatic titration. The total Nitrogen content present in the crude sample is thus calculated using equation 1 and further crude protein % is estimated using equation 2 mentioned below.

Equation 1

$$Kjeldahl\ nitrogen\ \% = \frac{(V_s - V_B) \times M \times 14.01}{W \times 6.38}$$

Equation 2

$$Crude\ protein\ \% = \% Kjeldahl\ N \times F$$

where V_s = Volume (ml) of standardized acid used to titrate a test
 V_B = Volume (ml) of standardized acid used to titrate reagent blank; M = HCl molarity; 14.01 = Molecular weight of N
W = Weight (g) of test sample; 6.38 = Factor to convert mg/g to percent; and F = 6.38 (Factor to convert N to protein)
Goulding et al., 2020; Licon, 2022)

2.2. Amino acid analysis

The top five performing samples based on their protein content were evaluated for their amino acid profiles using the HPLC technique with a UV-Vis fluorescence detector. All the amino acids were prepared with 0.25mM/L concentration (Conc.) standards, followed by crushing of 'bhat' samples into fine powder. The sample was then digested using HCl acid overnight. 100 mg sample was weighed and added with 300 μ l of 6N HCl in digestion bottles and kept in the oven at $110-120^\circ$ for 24 hours. After 24 hours, the tubes were taken out of the oven and added with 2ml of 0.1N HCl solution and filtered with a 0.22 μ m syringe filter. A final volume of 1.0 μ l was injected into the HPLC, and the program for the amino acid analysis was set and run under the conditions mentioned in Table 1 (Lamp et al., 2018).

Table 1 Different chromatographic parameters involved in separation of amino acids using HPLC

Column	Phenomenex Gemini-NX C18 3 μ m C183 μ m, 150mmL x3. 0mmID/Shim pack scepter C183 μ m, 150MMLX3.0mmI		
Mobile Phase A	25mM DI potassium dihydrogen phosphate pH 7		
Mobile Phase B	Water-acetonitrile methanol (15:45:40v/v/v)		
Flow rate	0.7ML/min		
Auto sampler temp	15 $^\circ C$		
Column temp	40 $^\circ C$		
Injection volume	1.0 μ l		
Rinse RO	Water: methanol (20:80v/v)		
Rinse R3	Water: acetonitrile (80:20) v/v)		
Detection conditions	Detector: Shimadzu fluorescence detector RF-20AXS		
Run time	50MIN		
Time program	TIME	A Conc%	B Conc%
	0.00	90.0	10.0
	30.0	55.0	45.0
	35.0	0	100.0
	42.0	0	100.0
	43.0	90.0	10.0
	50.0	90.0	10.0

2.3. Statistical analysis

The different values of the amino acids obtained for the respective samples were further analyzed to ascertain the significant difference among the samples. One-way ANOVA statistical analysis tool was used for the purpose. LSD was performed at a significance level of 0.05. P-values less than 0.05 were considered significant, while

those with p-values greater than 0.05 were considered non-significant. The post-hoc analysis was further conducted using python to determine statistically significant differences in amino acid concentrations among the surveyed villages. Tukey's HSD test was used to compare group means while controlling for family-wise error, making it appropriate for biochemical and nutraceutical datasets involving multiple group comparisons.

2.4. Machine learning analysis of protein content in seed through a decision tree algorithm implemented in WEKA

The data generated by Kjeldahl method was used to train the machine learning models. The dataset was loaded into WEKA's Explorer interface, and it was ensured that the data was formatted properly (Frank et al., 2016). The data was pre-processed by normalizing the data and handling any missing values. Feature selection was done to configure the J48 classifier by selecting it from the "Classify" tab and setting the appropriate parameters, such as the confidence factor and the minimum number of instances per sample. The data was divided into training and test sets after cross-validation. The J48 decision tree algorithm was trained by analysing metrics such as the confusion matrix, accuracy, precision, recall, and F-measure. The J48 decision tree algorithm implemented in WEKA was employed for the classification of seed samples obtained from different sites in Uttarakhand classified into high, medium, and low. The samples with the best protein and nitrogen content as well as attributes like the number of seeds in 10 grams were found to be decisive for the classifying samples into high, medium and low yielding. Other parameters that were included in the classifier were geographical, environmental, and physical factors like latitude, longitude, altitude, seed coat colour, average temperature from, average temperature to, protein percentage, shape, size and class. The Decision Tree algorithm in WEKA represents a very strong method of classification and regression in particular, useful for data analysis like that in our research for protein content estimation in seeds. This is a class of non-parametric supervised learning methods that construct models to predict the value of a target variable using simple decision rules inferred from the features of the data. Algorithms in WEKA include J48, which is a realization of C4.5, RandomTree, and REPTree. The process starts by importing data in ARFF or CSV format into WEKA, followed by preprocessing stages including missing values handling, normalization, and feature selection. The actual decision tree classifier is then selected from the Classify menu, configured for a confidence factor and pruning options, and trained on the dataset. It is evaluated using cross-validation and analyzed with the performance metrics and confusion matrices given by WEKA. Such a generated decision tree can be visualized, thus allowing interpretation of decision rules and feature importance. For seed classification, this model can classify the seed samples under classes such as "good" or "medium" according to various attributes such as percentage of nitrogen, protein percentage, and geographical features. Patterns and insights were taken from the findings indicating considerable attributes that distinguish seed quality.

3. Results and Discussion

3.1. Protein content

The present study aims to evaluate the protein content and amino acid profile in soybean by using the machine learning approach after obtaining data from the Kjeldahl method of protein estimation and amino acid quantification using the HPLC technique. Overall Protein content among all the villages were observed to ranges from 36.13% (New Tehri) to 43.56 % (Gwad Khirsu) with nitrogen content ranging from 5.78% to 6.97% for the same villages. (Fig.1). Studies suggests that high nitrogen content increases the protein content in cereals (Kaufman et al.,2013) and similar results were observed in the current study. The highest concentration of protein was found in S15 Gwad Khirsu with 43.56% followed by Maral S27 with 43.18% indicating that soybean from Pauri Garhwal could be the highest potential source of protein. After S5 & S27 the other highest protein content carrying villages with protein percentage more than the checks were S43 Joshimath (42.64%), S5 Pratapnagar (42.63%) and S28 Sripur (42.31%) (Table 2). The top five samples with the highest protein content, along with the nitrogen content, were observed to surpass the checks with high margins and emerge as the promising sources of protein. The villages with the highest percentage of protein were found to have protein content ranging from 42.31% to 43.56 % w/w, which is useful for meeting the food security issues prevailing in hilly areas.

Table 2 The top five villages with the highest protein and nitrogen content along with their 11 validated attributes

S.no	1	2	3	4	5
Village	Gwad Khirsu	Maral	Joshimath	Pratapnagar	Sripur
Latitude	30°1	29°48	30°53	30°3	30°75
Longitude	78°52	78°36	79°51	78°29	78°6
Altitude(m)	1699.56	1650	1874.52	2620	1765.9
Seed coat colour	Yellowish white	Yellowish white	Greenish yellow	Yellowish white	Yellowish white
Average temp from (° c)	15° C	17° C	14° C	15° C	15° C
Average temp to (° c)	25° C	28° C	21° C	18° C	23° C
No of seeds in 10gm	97	60	97	86	80
Nitrogen %	6.97	6.91	6.82	6.82	6.77
Protein %	43.5625	43.1875	42.64	42.625	42.312
Seed Shape size	Round medium	Round large	Round large	Oval large	Oval medium
Class	Good	Good	Good	Good	Good

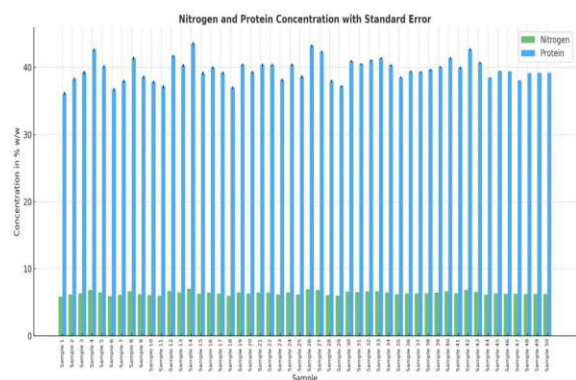


Figure 1. Nitrogen and Protein content in Soybean samples from different villages of Uttarakhand

According to studies, protein percentage in meat is approximately 18-25% (Ahmad et al., 2018; Widodo et al., 2022). On the other hand, pulses and legumes contain 18% to 40% protein, respectively (Erbersdobler et al., 2017). Studies performed by Conte et al. (2014) Gálová et al. (2019) Parveen et al. (2022) Varalaxmi et al. (2024) have reported the protein content ranging from 20-26% in pulses and 18-26 % in other cereals such as maize oats, barley, millets, rice, etc., which is considerably lower than the values observed in the present study. The present study shows a protein content of up to 43.56% in 'bhat', which is substantially higher than the reported content in previous studies. This study backs up Hoffman and Falvo (2004) research, where they found soybeans have a higher protein percentage of ~40-50% and less carbohydrate than other legumes. Similarly, Dhull et al. (2023) reported a lower percentage of protein (24%) and a higher content of carbohydrates (63%), indicating a higher protein percentage in the present study. The reported amount of protein in the current study falls under the recommended dietary allowance of 0.8-1.2gm/kg weight for elderly people for proper body functioning and avoiding muscle mass loss, as suggested by Traylor et al. (2018)

3.2. Amino acid content

All the top five performing samples with the highest content of protein were further evaluated for the presence of amino acids. The samples were found to have all the essential and non-essential amino acids such as threonine(T) tryptophan (W) isoleucine(I), leucine(L), lysine(K) and histidine (H), arginine (R), aspartic acid(D), glutamine(Q), glycine(G) ornithine, gamma aminobutyric acid (GABA) and cysteine (C) except methionine (Table 3). Reported studies show that non-essential amino acids are synthesized inside the body but their synthesis is limited at the time of illness or stress, therefore, consumed through diet (Shine & Rostom, 2021). The study reported the absence of methionine but detected cysteine, another sulfur-containing amino acid. Cysteine plays a significant role in various biological reactions. It helps in the formation of disulfide bonds, playing a key role in protein binding, systematic protein folding, and formation of tertiary and quaternary protein structures (Brosnan & Brosnan, 2006). Cysteine is an essential substrate for the formation of glutathione and taurine, which are biological antioxidants that protect the cells from cell damage and cell toxicity (Serenio et al., 2014). The current study revealed that Pratapnagar was seen to have nine amino

acids with histidine as the most present amino acid, with a concentration of 0.516g/100g at retention time (Rt) of 6.216 (Fig 2). Gawad Khirsu was found to have a total of twelve amino acids (Fig 3). Maral had shown the presence of a total of eleven amino acids with histidine being the one with highest concentration at Rt 6.16 (Fig 4). Stripur similar to Gawad khirsu had twelve amino acids (Fig 5), while Joshimath showed the presence of ten amino acids (Fig 6). On comparing all five samples, S15 Gwad Khirsu and S28 Stripur were found to have the maximum count (12) of amino acids. In terms of total amino acid content, S15 Gawad Khirsu (at an elevation of 1699m) scored the highest concentration of 3.183 ± 0.6 g/100g followed by Pratapnagar (at an elevation of 2620m) with 1.057 ± 0.7 g/100g content (Table 3). The study showed that samples at the highest altitude/low temperature and lowest altitude/high temperature were observed to have the highest total amino acid content. Almost all the samples were found to be enriched with the amino acid Histidine (H) with the highest content of 0.688g/100g from S15 Gawad Khirsu with a retention time peak of 6.104. Histidine is an essential amino acid that serves many benefits, including antioxidant and anti-inflammatory properties. It is known to reduce the effect of stress and anxiety and improve sleep quality (Thalacker-Mercer, 2020). Studies have suggested its potential role in treating rheumatoid arthritis and chronic renal failure (Holeček, 2020; Thalacker-Mercer, 2020).

There have been some studies (García et al., 1998; Lopez et al., 2024) where the content of different amino acids was evaluated, and it was found that the content of leucine, lysine, valine isoleucine phenylamine was found to be 8 g, 6.5 g, 5g, 5g, 4g/100 g of protein which were relatively higher than the present study. Similarly, another recent study performed by Custodio-Mendoza et al., (2024) showed the amino acid content to be higher than in the present study. A study done by Kudelka et al., (2021) reported amino acids with values lower than those of the present study. Nizkii et al., (2020) performed a similar study and obtained results similar to those obtained in the current study.

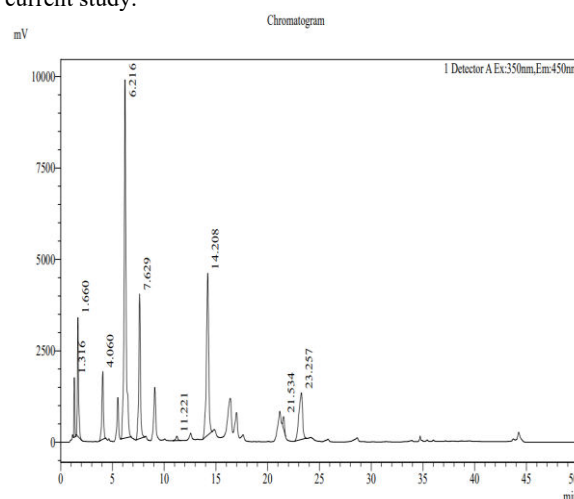


Figure 2 HPLC Chromatogram of S1pratapnagar showing the separation peaks of Aspartic acid (D), Glutamic acid (E), Histidine (H), Threonine (T), Tyrosine (Y), Tryptophan (W), Isoleucine (I), Arginine (R), Aminobutyric acid (GABA)

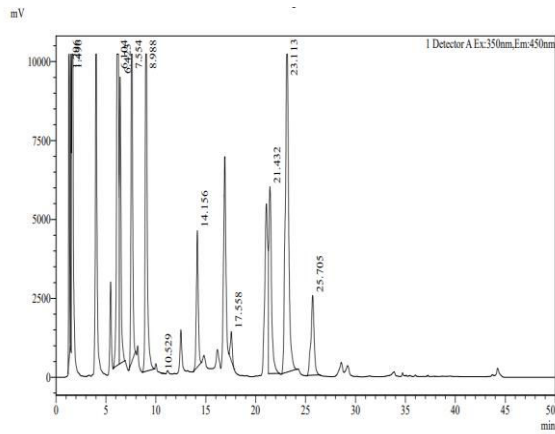


Figure 3 HPLC Chromatogram of S2 Gawad Khirsu showing the separation peaks of Aspartic acid (D), Glutamic acid (E), Histidine (H), Glycine (G), Threonine (T), Arginine (R), Alanine (A), Tyrosine (Y), Cysteine (C), Tryptophan (W), Isoleucine (I), Ornithine (ORN)

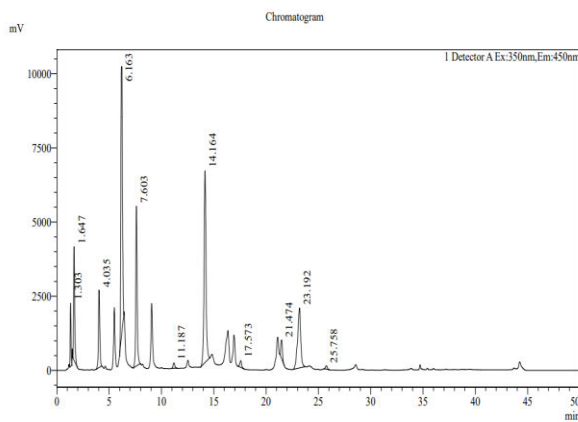


Figure 4 HPLC Chromatogram of S3 Maral showing the separation peaks of Aspartic acid (D), Glutamic acid (E), Histidine (H), Glycine (G), Tyrosine (Y), Cysteine (C), Tryptophan (W), Isoleucine (I), Ornithine (ORN), Arginine (R), Aminobutyric acid (GABA)

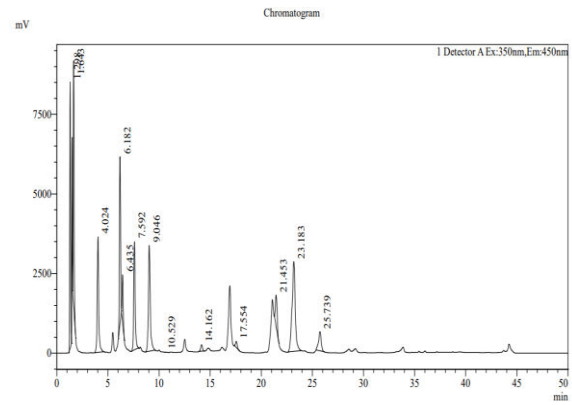


Figure 5 HPLC Chromatogram of S4 Sripur showing the separation peaks of Aspartic acid (D), Glutamic acid (E), Histidine (H), Glycine (G), Threonine (T), Arginine (R), Alanine (A), Tyrosine (Y), Cysteine (C), Tryptophan (W), Isoleucine (I), ornithine (ORN)

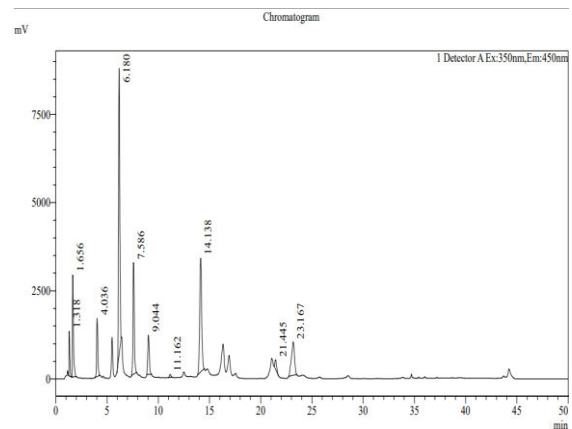


Figure 6 HPLC Chromatogram of S5 Joshimath showing the separation peaks of Aspartic acid (D), Glutamic acid (E), Histidine (H), Threonine (T), Alanine (A), Tyrosine (Y), Tryptophan (W), Isoleucine (I), Arginine (R), Aminobutyric acid (GABA)

Table 3 Amino acid profile of all the top 5 villages with highest concentration of protein

		VILLAGES				
S.no	Amino acid	Pratapnagar g/100g	Gawad Khirsu	Maral g/100g	Sripur g/100g	Joshimath g/100g
		Mean $\pm$ SD	g/100g Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
1	Aspartic acid	0.030 $\pm$ 0.01	0.241 $\pm$ 0.2	0.039 $\pm$ 0.1	0.149 $\pm$ 0.5	0.024 $\pm$ 0.4
2	Glutamic acid	0.119 $\pm$ 0.2	0.225 $\pm$ 0.3	0.129 $\pm$ 0.1	0.217 $\pm$ 0.3	0.092 $\pm$ 0.2
3	Histidine	0.516 $\pm$ 0.1	0.688 $\pm$ 0.3	0.398 $\pm$ 0.2	0.167 $\pm$ 0.1	0.284 $\pm$ 0.1
4	Glycine	0.0000	0.071 $\pm$ 0.3	0.038 $\pm$ 0.2	0.0080.4	0.000
5	Threonine	0.182 $\pm$ 0.12	0.516 $\pm$ 0.2	0.000	0.138 $\pm$ 0.1	0.117 $\pm$ 0.02
6	Arginine	0.000	0.248 $\pm$ 0.1	0.000	0.068 $\pm$ 0.1	0.018 $\pm$ 0.03
7	Alanine	0.000	0.000	0.000	0.0000.2	0.000
8	Tyrosine	0.164 $\pm$ 0.2	0.139 $\pm$ 0.1	0.201 $\pm$ 0.2	0.006 $\pm$ 0.4	0.103 $\pm$ 0.1
9	Cysteine	0.000	0.016 $\pm$ 0.3	0.004 $\pm$ 0.2	0.003 $\pm$ 0.1	0.0000.4
10	Tryptophan	0.011 $\pm$ 0.1	0.353 $\pm$ 0.2	0.021 $\pm$ 0.3	0.037 $\pm$ 0.2	0.010 $\pm$ 0.2
11	Isoleucine	0.000	0.372 $\pm$ 0.19	0.064 $\pm$ 0.1	0.094 $\pm$ 0.2	0.030 $\pm$ 0.2
12	Ornithine	0.000	0.314 $\pm$ 0.2	0.008 $\pm$ 0.4	0.061 $\pm$ 0.7	0.000
13	Asparagine	0.018 $\pm$ 0.02	0.000	0.030 $\pm$ 0.2	0.000	0.040 $\pm$ 0.2
14	GABA	0.018 $\pm$ 0.019	0.000	0.020 $\pm$ 0.09	0.000	0.029 $\pm$ 0.3
	Total	1.057 $\pm$ 0.7	3.183 $\pm$ 0.6	0.952 $\pm$ 0.29	0.949 $\pm$ 0.33	0.747 $\pm$ 0.26
	Average	0.0755 $\pm$ 0.05	0.2274 $\pm$ 0.04	0.0680 $\pm$ 0.02	0.0678 $\pm$ 0.02	0.0533 $\pm$ 0.01
	S.D.	YS	YS	YS	YS	YS
	LSD	0.05	0.05	0.05	0.05	0.05

Results of statistical analysis using ANOVA revealed a significant difference in amino acid concentration with $P < 0.05$ value in 'bhat' samples collected from different locations. Post hoc test revealed arginine as the most significant amino acid. Multiple pairwise comparisons showed highly significant differences, particularly between Gawad Khirsu and Joshimath, Maral, Pratapnagar, and Sripur, with Gawad Khirsu consistently having lower Arginine levels ($p < 0.001$). The factors that contribute to the variation in amino acid content include genotype, environmental factors and soil conditions (Goldflus et al., 2006). With the change in temperature and elevation, the change in total amino acid concentration was observed, indicating the direct relation of abiotic factors like temperature and altitude in determining the concentration of amino acids in soybean 'bhat'. Mourtzinis et al. (2017) also reported such variations in protein and amino acid content with temperature. The present study confirms that temperature and altitude could be one of the reasons for variation in amino acid profile in soybean 'bhat', indicating them as essential parameters to determine the amino acid content. Another factor that could contribute to the variations in amino acid profile is its relationship with nitrogen. Reports suggest that high nitrogen levels increase the amino acid content. Applying nitrogen in the form of fertilizer can impact the amino acid composition and protein content in plants (Thanapornpoonpong, 2008); therefore, high content of nitrogen in Gawad Khirsu could be the most acceptable reasoning for high concentration of amino acids (Atanasova, 2008). Apart from these factors, there is a slight possibility of amino acid hydrolysis and modification due to acid (HCL) digestion during the extract preparation, which would have led to loss of amino acids.

3.3. WEKA analysis: Seed quality assessment through machine learning

The decision tree algorithm implemented in Weka software has been used in this study to analyze and understand the influence of various parameters or combinations of various parameters that can be decisive in determining the seed quality or classifying seeds based on different attributes into medium or good quality seed. With the J48 decision tree algorithm in Weka, an open-source data mining and machine learning tool, a classification accuracy of 94% was obtained for the seed data set on performing ten-fold cross-validation (Table 4). The classifier was trained with seed data obtained from 50 different geographical locations in Uttarakhand. The seed samples with protein content ≤ 39.95 were placed under the class medium (total instances or samples 29.0); the seed samples with protein content > 39.95 were considered as belonging to class good (total instances or number of samples 21.0).

Table 4. Stratified Cross-Validation Metrics for Model Performance Evaluation

Correctly Classified Instances	47 (94%)
Incorrectly Classified Instances	3 (6%)
Kappa statistic	0.8792
Mean absolute error	0.06
Root mean squared error	0.2449
Relative absolute error	12.2822%
Root relative squared error	49.546%
Total Number of Instances	50

The confusion matrix revealed that in 50 samples, all 21 samples designated as good variety seeds were correctly classified or cross-validated as good variety seeds, but 26 samples out of 29 medium variety seeds could only be classified correctly, and 3 were classified incorrectly, resulting into 94% validation accuracy estimated by the matrix (Fig 7). However, 94% validation is considered highly significant and highlights its reliability in class prediction of different quality groups of seeds. This high degree of accuracy has demonstrated the major influence of geographical, environmental and physiological characteristics in determining the quality of seeds. Factors including longitude, altitude, seed colour, seed shape, number of seeds in 10 gm, average temperature have emerged as pivotal factors in determining the seed quality. The results suggest that the use of these attributes can be further considered in similar studies for various other seed-related assessments (Fig 8).

The analysis reveals that 11 attributes (latitude, longitude, altitude, seed coat colour, average temperature, seed shape and size, number of seeds in 10 gm, nitrogen percentage, protein percentage, class of seed) selected for the analysis were effective in differentiating between the good and medium types of 'bhat' seeds, making them suitable for further similar analysis. The analysis also demonstrated the high degree of overlapping among the

different samples, indicating the similar values of attributes shared between the different soybean cultivars.

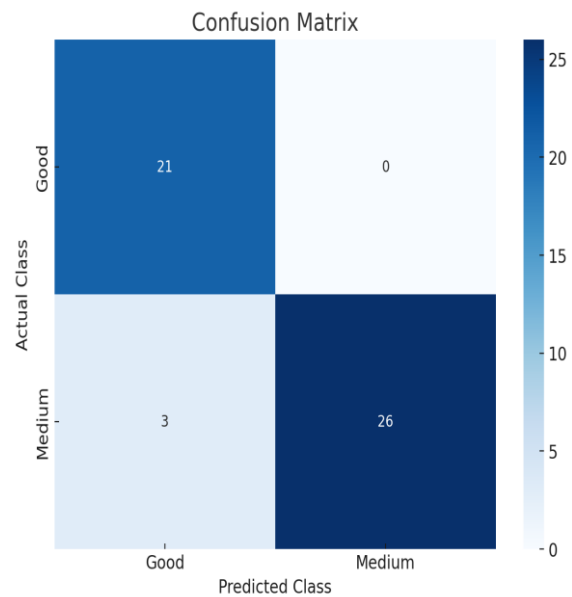


Figure 7 Confusion Matrix Showing Actual versus Predicted Class Distribution of the Model

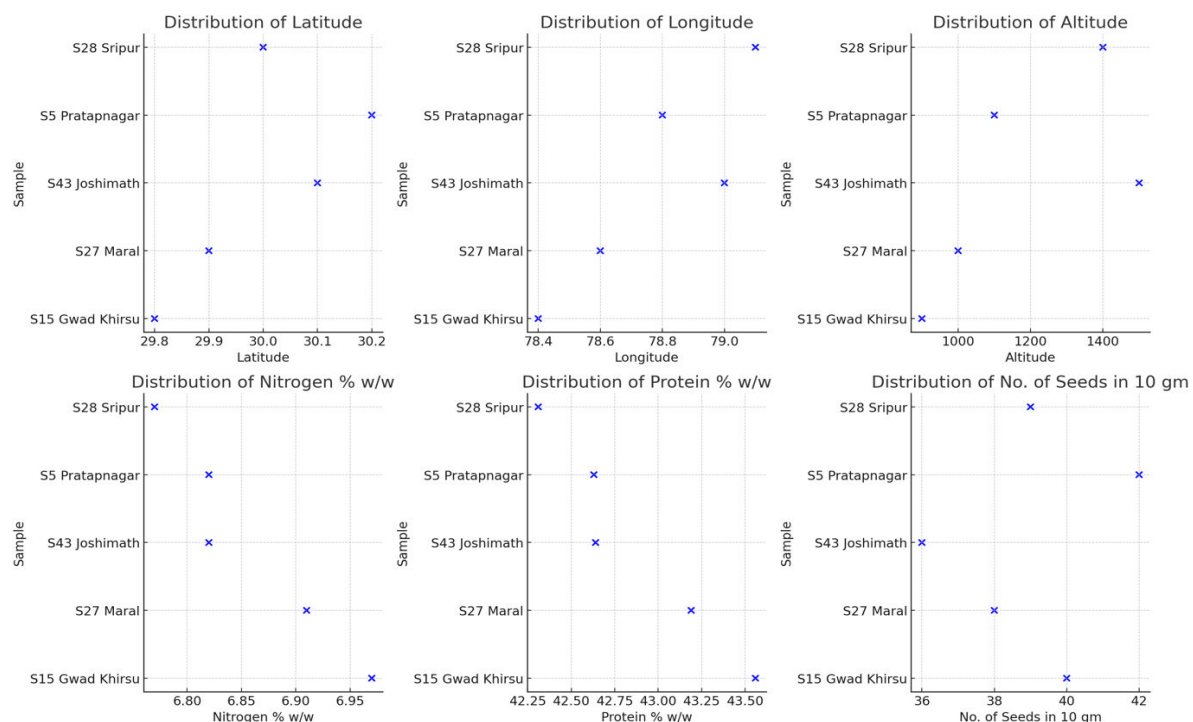


Figure 8. Distribution of bhat samples according to various attributes: latitude, longitude, altitude, nitrogen % w/w, protein % w/w and no of seeds in 10 gm

Results revealed that the maximum number of samples fell within the latitude range of 29.45-30.9° and the longitude range of 78.2°-79.52° in an altitudinal gradient ranging from 345-1021 m and 1699-2044 m. The results show that the protein content ranges from 39% to 40%. The top five seed samples with the highest protein and nitrogen content were identified as S15 Gwad Khirsu, S27 Maral, S43 Joshimath, S5 Pratapnagar, and S28 Sripur, which ranged between 42.31% - 43.56% w/w (Table 2).

The machine learning approach used here enabled quick and efficient data analysis, pattern identification, and

classification of seed samples based on various attributes, such as nitrogen and protein percentages. The decision tree model helps visualize rules and how features can be ranked in importance, which leads to seeds being classified as 'good' or 'medium'. Besides saving time, the auto-analysis provides a robust framework for decision-making by using data to identify the best seeds to be cultivated. Therefore, machine learning is an invaluable tool in agricultural research, enabling the development of sustainable, high-yield farming practices.

4. Conclusion

Machine learning algorithms were used to identify protein content in soybean seeds collected from Garhwal region of Uttarakhand, India. The study confirmed Sample 15 from Gwad Khirsu to have the highest protein content of 43.56% followed by the sample from Maral village, having 43.18% protein. All the essential amino acids were present in the analysed top five protein-containing samples, except for methionine. Histidine was further identified as the most prominent amino acid. The statistical analysis confirmed that the variations in nutrient content in the samples relate to environmental factors, such as temperature and altitude. The WEKA analysis found the best protein content (39-40%) in the samples collected from villages at altitudinal ranges of 345-1021m and 1699-2286 m. This approach can be used to analyse the effects of environmental factors on crop varieties, revealing patterns that are difficult to analyse beyond manual methods. Using advanced machine learning for seed quality and nutrient content offers new insights, promoting reproducibility, productivity, and quality in agriculture, hence ensuring global food security.

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Conflicts of Interest

There are no competing interests that need to be disclosed by the authors.

Author's Contribution

TN: Methodology, data curation, validation, writing-original draft, MP: conceptualisation, experimentation, resource management, writing-review and editing, KP: investigation and formal analysis, ASN: resource management, project administration, investigation, PN: project administration, data curation, AK: Writing-review and editing, visualization. All authors have read and approved the final manuscript.

Ethical issues

There are no ethical issues that need to be disclosed by the authors.

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Virtual Prediction of King Cobra (*Ophiophagus hannah*) Neurotoxin Epitopes for Vaccine-Based Antitoxin Development

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Abstract

The King Cobra (*Ophiophagus hannah*) possesses a highly neurotoxic venom where long-chain neurotoxins (LNTX) are the primary lethal components. Current antivenoms, such as OHMAV and SABU, have constrained efficacy and regional availability, highlighting the need for innovative solutions. This study aimed to computationally predict conserved and immunogenic epitopes from LNTX proteins for vaccine development. Using IEDB tools and ProtParam, potential epitopes were screened for immunogenicity, conservancy, physicochemical properties, and their MHC-II (HLA-DRB1*07:01) binding affinity was analyzed. Three lead epitopes were identified, namely 26-WCDGFCSSRGKRIDL-40, 27-CDGFCSSRGKRIDL-41, and 25-TWCDGFCSSRGKRIDL-39. These candidates demonstrated low allergenicity, favorable physicochemical properties, and broad population coverage. Structural analysis confirmed that the epitopes, connected by a linker, docked stably with the MHC-II molecule. This interaction remained stable throughout a 20 ns molecular dynamics simulation. These results provide a robust foundation for developing a novel, epitope-targeted vaccine against king cobra venom, offering a promising alternative to traditional antivenoms.

Keywords: LNTX, Neurotoxin, *Ophiophagus hannah*, Southeast Asia, Vaccine-based antivenom

1. Introduction

One of the biggest and most deadly venomous snake species in the world is the King Cobra (*Ophiophagus hannah*) (Le *et al.*, 2021; Simangunsong *et al.*, 2024). This species belongs to the Elapidae family, with a total body length of up to 5 metres (Gowri *et al.*, 2021). This species is distributed widely throughout South and Southeast Asia, including Indonesia, China, Bangladesh, India, Nepal, Bhutan, Pakistan, and the Indochina and Indo-Malaya areas. In Indonesia, this species can be found in Sumatra, Java, Borneo, Bali, and Sulawesi (Amat and Escoriza, 2022; Charlton, 2018; Das *et al.*, 2024; C. H. Tan *et al.*, 2021). King Cobra predominantly inhabited primary tropical forest and shrubland habitats and could also be found in cultivated fields and suburban environments (Marshall *et al.*, 2018). Its presence in rural areas poses a significant threat, especially to those who work as farmers, who are most at risk of envenomation (Adiwinata and Nelwan, 2015; Dafa and Suyanto, 2021; Simangunsong *et al.*, 2024). Snakebites from King Cobra and other venomous species cause many cases of snakebite envenomation in tropical regions due to treatment delays and a lack of medical resources (Adiwinata and Nelwan, 2015; Tan *et al.*, 2021). Despite this, envenomation snakebite cases are still not a concern for the government and public health community. In 2017, snakebite

envenomation was reinstated according to the WHO as a neglected tropical disease, and it continues to be an open problem (Tan *et al.*, 2021).

The venom of *O. hannah* is composed of several protein families, including three-finger toxins (3FTxs) specifically long neurotoxin (LNTX) and short neurotoxin (SNTX) dominating its proteome at 33,5% and 11,9%, respectively. Other protein families that can be found on *O. hannah* venom are LAAO, Snake Venom Metalloproteinase-Disintegrin (SVMP), and cysteine-rich secretory proteins (CRISPs) (Rajendiran *et al.*, 2024; C. H. Tan *et al.*, 2021). These neurotoxins are the primary contribution to the venom's high lethality. The long-chain neurotoxins (LNTXs) act by binding to and blocking the nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction, leading to paralysis and death. Therefore, LNTXs are an important contributor to neurotoxic effects as they produce a stronger and reversible blockage of nAChRs compared to the short-chain neurotoxins (SNTXs) (Huyh *et al.*, 2022; Wong *et al.*, 2021). Based on this, the effectiveness of antivenom therapy is determined by the ability of antibodies to neutralize the envenoming toxin at any rate (Danpaiboon *et al.*, 2014).

The most recent monospecific antivenom specifically made against King Cobra envenomation is OHMAV, which is produced using King Cobra Venom (KCV) of Thai origin and is used in Thailand and Malaysia. On the

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other hand, OHMAV is not provided in China and Indonesia. Seeing that geographically widespread species like King Cobras may have probably speciated, the efficacy of OHMAV might not be as high against populations elsewhere, as in China or Indonesia, or even Malaysia, because of the change in venom composition and, consequently, the immune response (Tan *et al.*, 2020). In Indonesia, there is a commercially available antivenom, namely SABU; also, little scientific study has been conducted regarding the efficacy of this antivenom. SABU has been depicted as unproductive or even almost ineffective in neutralising the king cobra venom (*O. hannah*) since it lacks antibodies to the main neurotoxins of long-chain (Tan *et al.*, 2016).

The specificity and scalability of traditional antivenoms for King Cobra (*O. hannah*) envenomation are limited. Recent discoveries were able to foresee the presence of stable T-cell epitopes by related elapid venoms using immunoinformatics, which also demonstrates that immunoinformatics is promising as an alternative (Chan *et al.*, 2023; Grahadi *et al.*, 2022). Thus, this research paper aims to determine conserved and immunogenic T-cell epitopes that are based on the predominant long-chain neurotoxins (LNTXs) of *O. hannah* venom, the goal of which is to create a vaccine-based antivenom using computational methods. The methodology that is used in this study consists of epitope prediction of high MHC-II binding affinity, evaluation of immunogenicity, population coverage, conservancy, allergenicity potential, and determination of stability and molecular interactions using dynamics simulations and structural modelling. The final goal of this report is to come up with a platform that will provide an enhanced defense against the venom of King Cobras in a more efficient, secure, and widely applicable manner.

2. Materials and methods

2.1. Prediction of LNTX protein epitopes

The amino acid sequences of Long Neurotoxin (LNTX) were obtained using accession codes A01657 from the NCBI and UniProt protein databases (He *et al.*, 2004). Only sequences originating from Southeast Asia were used in this study. Epitopes for LNTX were predicted using the IEDB MHC II binding prediction tool (<http://tools.iedb.org/mhcii>) (Grahadi *et al.*, 2022; He *et al.*, 2004; Wang *et al.*, 2008; Wang *et al.*, 2010). Epitope prediction was carried out using the IEDB-provided HLA reference allele collection, which represents the polymorphism of human MHC class II molecules, such as HLA-DRB1*07:01 (Fathollahi *et al.*, 2021; Grahadi *et al.*, 2022). Epitopes exhibiting a percentile rank below 5 meet the required criteria and will be utilised for further analysis (Grahadi *et al.*, 2022). BepiPred 2.0 Linear Epitope Prediction was utilised to predict B-cell epitopes, utilising the IEDB B-cell analysis tool, which may be found at <http://tools.iedb.org/bcell/> (Dhanda *et al.*, 2018; Grahadi *et al.*, 2022).

2.2. Prediction of epitope of epitopes conservancy and immunogenicity

The IEDB (Immune Epitope Database) tools were used for the prediction of epitopes' immunogenicity (<http://tools.iedb.org/CD4episcore/>) and calculation of

epitopes' conservancy (<http://tools.iedb.org/conservancy/>). Epitopes were considered to be immunogenic if their immunogenicity score exceeded 70% (Dhanda *et al.*, 2018). The epitopes with IEDB scores exceeding 70% were considered to be conserved (Grahadi *et al.*, 2022). The epitopes that meet both conservation and high immunogenicity parameters are then subjected to further analysis (Dhanda *et al.*, 2018; Kurniawan *et al.*, 2020).

2.3. Allergenic properties of epitope and population coverage

Algpred

(<https://webs.iitd.edu.in/raghava/algpred/submission.html>) was used to predict the allergenic characteristics of the epitope by mapping the IgE epitopes and PID, MEME/MAST motif, and Blast search on the allergen representative peptides (ARPs) menu. These predictions are carried out to ensure safety when administered to humans. IEDB was used to calculate population coverage (<http://tools.iedb.org/population/>) (Grahadi *et al.*, 2022). Predictions of population coverage were limited to areas in Southeast Asia that are the natural habitat of *O. hannah*.

2.4. Estimation of physicochemical characteristics

The ProtParam program on the ExPASy server (<https://web.expasy.org/protparam/>) was used to examine the physicochemical characteristics of the chosen epitopes (Duvaud *et al.*, 2021). Molecular weight, isoelectric point (pI), instability index, aliphatic index, predicted half-life, and grand average of hydropathicity (GRAVY) were among the physicochemical parameters that were calculated.

2.5. Epitope structure modeling

The three-dimensional structure of the selected epitope was modeled using PEP-FOLD3 (<https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3>). PEP-FOLD3 is a *de novo* peptide structure prediction tool. The simulation provided a structural model crucial for the subsequent analysis (Lamiabile *et al.*, 2016).

2.6. Molecular docking

The selected epitope's three-dimensional structure, obtained from the PEP-FOLD3 model, was converted into PDB format and then optimized through energy minimization using PyRx v0.8 (Lamiabile *et al.*, 2016; Mao *et al.*, 2023). MHC class II molecules and selected epitopes were docked using HDock (Ruaro-Moreno *et al.*, 2023). Discovery Studio was used to depict the interactions between certain epitopes and MHC class II molecules (PDB ID 3C5J) (Naveed *et al.*, 2022).

2.7. Molecular dynamics simulation

YASARA software was used to run the simulation for 20 nanoseconds. The simulation environment conditions were adjusted to physiological conditions, namely at pH 7.4 and NaCl concentration of 0.9%. The temperature was kept at 310°K (equivalent to human body temperature), while the water density was set at 0.997 g/L to evaluate the stability of the epitope backbone, the root-mean-square deviation (RMSD) is examined. The epitope is considered stable if the resulting RMSD value does not exceed 3 Å, showing that during the simulation, the epitope structure relatively stays the same (Naveed *et al.*, 2022; Ruaro-Moreno *et al.*, 2023).

3. Results

3.1. Prediction of LNTX protein epitopes

Based on percentile rank values less than 5, 13 epitopes from the LNTX protein for human MHC class II (HLA-DRB1*07:01) were projected to satisfy the selection criteria (Sup. Table 1). Percentile rank values are used to predict MHC class II epitopes and evaluate how strongly a peptide binds to MHC molecules. Lower percentile values indicate higher binding affinity (Rezaldi *et al.*, 2021). One of the HLA Class II alleles that was extensively dispersed in Southeast Asia is HLA-DRB1*07:01, which was used in this investigation (Gustiananda *et al.*, 2021). In addition, HLA-DRB1*07:01 was used in this study because the prediction results of HLA-DRB1*15:02 and HLA-DRB1*12:02 that were previously used did not show good results. All epitopes had amino acid sequence lengths varying from 15 to 18 residues, with the core peptide sequence consisting mostly of the sequence "FCSSRGKR". Then these 13 epitopes can be further analyzed (Table 1). Four possible epitope candidates have been identified from the B-cell epitope prediction. Only the second epitope out of the four exhibits sequence similarity to MHC class II epitopes (Table 2).

Table 1. Selected MHC class II epitopes from LNTX protein.

Allele	Core Sequence	Peptide Sequence	Percentile Rank
HLA-DRB1*07:01	FCSSRGKR	27-CDGFCSSRGKRIDL-41	3.7
		25-TWCDGFCSSRGKRID-39	3.7
		26-WCDGFCSSRGKRIDL-40	3.7

Table 2. Selected B cell epitopes from LNTX protein.

Start	End	Peptide	Length
6	19	TPDATSQTCPDGQD	14
27	39	CDGFCSSRGKRID	13
48	54	CPKVKPGVD	9
61	69	DNCNPFPTW	9

bold letters indicate epitopes that have sequence similarity to MHC class II epitopes

3.2. Prediction of epitope conservation and immunogenicity

The analysis of epitope conservation and immunogenicity was performed using the IEDB. The results indicated that 12 out of 13 predicted epitopes were conserved (Sup. Table 2), exhibiting a conservancy value of 83.33%. Most of the predicted LNTX epitopes were used in further analysis, except for the thirteenth epitope KTWCDGFCSSRGKRID, which was not categorized as conserved with a value of 66.67%. The identification of conserved epitopes with a minimal number of mutations is of importance when designing an appropriate vaccine to target a wide range of variants of *O. hannah* toxin (Bagherzadeh *et al.*, 2022). The predicted LNTX epitopes were found to be immunogenic, as indicated by an immunogenicity value that was higher than 98% (Table 3).

Activating CD4⁺ T cells requires recognizing the immunogenic LNTX epitope on MHC class II. Once activated, these helper T cells interact with B cells. This interaction initiates an immune response that results in the production of antibodies (Xu *et al.*, 2022).

Table 3. Conservancy and immunogenicity of LNTX *O. hannah* MHC II predicted epitopes.

Epitope Sequences	Percent of protein sequence matches at identity <= 100% (%)	Immunogenicity Score
27-CDGFCSSRGKRIDL-41	83.33 (5/6)	98.57
25-TWCDGFCSSRGKRID-39	83.33 (5/6)	98.74
26-WCDGFCSSRGKRIDL-40	83.33 (5/6)	98.76

3.3. Allergenic properties of epitope and population coverage

AlgPred was used for this study to predict the allergenic characteristics in order to make sure that the anticipated LNTX epitopes would not cause a reactogenic reaction in humans (Ahmad *et al.*, 2021). Three primary computational methods were used to predict the allergenic potential of LNTX epitopes and IgE epitope mapping in AlgPred, namely homologue search using BLAST against the representative allergen peptide database, Support Vector Machine (SVM) based classification analyzing single amino acid and dipeptide composition, and allergenic motif search using the MEME/MAST tool (Hayes *et al.*, 2015). Considering no IgE epitopes were detected, the data demonstrated that the predicted epitopes do not have the potential to be allergenic. MAST and BLAST results also showed that the predicted epitopes were categorized as non-allergens (Table 4). This non-allergenic criterion was required to produce immunoreactive peptides (Khamjan *et al.*, 2023).

Table 4. The Allergenic Properties of LNTX *O. hannah* epitopes.

Amino Acid Sequences	Mapping IgE epitope	MAST Results	BLAST Result
27-CDGFCSSRGKRIDL-41			
25-TWCDGFCSSRGKRID-39	Does not contain experimentally proven IgE epitope	Non-Allergen	Non-Allergen
26-WCDGFCSSRGKRIDL-40			

To assess the compatibility of predicted LNTX epitopes with HLA alleles that are common in Southeast Asia, which is *O. hannah*'s natural environment (Indonesia, Malaysia, the Philippines, Singapore, Thailand, and Vietnam), population coverage was estimated using IEDB methods. The prediction of population coverage was important in epitope-based vaccine design to ensure that immune recognition is maximized in target populations, as

the heterogeneity of HLA allele diversity across diverse geographical regions (Khamjan *et al.*, 2023). The results showed that the predicted epitopes had limited coverage, reaching only 11.26% of the population in eight Southeast Asian countries. Among these countries, Indonesia had the highest population coverage of 20.49%, while other countries ranged from 1.22% to 15.91% (Table 5). This suggested that the highly variable HLA alleles of most Southeast Asian populations were unable to detect the predicted LNTX epitopes due to their narrow range. One of the HLA Class II alleles that was extensively dispersed throughout Southeast Asia and was prevalent in Indonesia with a frequency of 13.7% is HLA 07:01, which was used in this study (Gustiananda *et al.*, 2021), but other countries may have different more dominant HLA alleles so to overcome the low population coverage, future designs could integrate several Southeast Asia-specific HLA frequency data.

Table 5. The population coverage of LNTX *O. hannah* epitopes.

Countries	Population Coverage (%)
Indonesia	20.49
Malaysia	6.42
Singapore	15.91
Thailand	14.21
Philippines	1.22
Vietnam	9.28

Table 6. Physicochemical characteristics of epitopes.

Physicochemical Characteristic	26-WCDGFCSSRGKRIDL-40	27-CDGFCSSRGKRIDL-41	25-TWCDGFCSSRGKRIDL-39
Molecular Weight	1742.99	1613.83	1730.93
Theoretical pI	8.06	8.06	7.74
Estimated half-life in mammalian cells (Hours)	2.8	1.2	7.2
Estimated half-life in yeast	3 Min	> 20 Hours	> 20 Hours
Estimated half-life in <i>E. coli</i>	2 Min	> 10 Hours	> 10 Hours
Instability index	58.27	58.27	48.25
Stability	Unstable	Unstable	Unstable
Aliphatic index	52	52	26
Grand average of hydropathicity (GRAVY)	-0.473	-0.44	-0.773

3.5. Epitope Structure Modelling

PEP-FOLD3 was used to model the selected epitope after the assessment of its physicochemical characteristics, immunogenicity, allergenicity, population coverage, and conservancy. Based on the amino acid sequences of peptides, PEP-FOLD3 is a proven method for predicting their three-dimensional structures. Due to its favorable energy profile and higher structural stability, Model 1 (Sup. Figure A1) was selected for further analysis among the models that were generated. Molecular docking studies were then conducted using this model, which was provided in Protein Data Bank (PDB) format. PEP-FOLD3's prediction process consists of three primary phases. A Monte Carlo approach is used to develop and refine various models after the support vector machine first predicts the structural alphabet of peptide fragments from the input sequence. Afterwards, the top five conformations are chosen (Sánchez-Hernández *et al.*, 2021). Typically, the best model for epitope modeling is the top-ranked

3.4. Estimation of physicochemical characteristics

The ExPASy ProtParam tool revealed significant variability in the properties of peptide epitopes due to differences in sequence composition, length, and terminal residues. Molecular weight (161.38–202.93 Da) and isoelectric point (pI 7.74–8.96) depended on amino acid composition, with most epitopes carrying a slight negative charge at physiological pH, enhancing solubility and immunological interactions (Devi *et al.*, 2021; Khamjan *et al.*, 2023). The instability index (>40) indicated that all epitopes were susceptible to degradation (Raza *et al.*, 2021).

Half-life varies according to the N-terminal residue: Thr/Gly epitopes persist longer (~7.2 h in mammalian cells), while Lys/Asp epitopes degrade rapidly (~1.1–1.3 h) (Khamjan *et al.*, 2023; Raza *et al.*, 2021). High aliphatic index (26–52) and negative GRAVY scores (–0.473 to –0.773) (Table 6) confirm these epitopes are hydrophilic (Kaynarov *et al.*, 2024). Stable hydrophilic epitopes (e.g., 25-TWCDGFCSSRGKRIDL-39) are promising vaccine candidates, while unstable epitopes may require structural optimization (Devi *et al.*, 2021).

conformation among these clusters, chosen based on criteria such as the lowest coarse-grained energy (sOPEP score) or the highest predicted TM-score. This ensures the selection of the most stable and native-like peptide structure, which is then utilized for downstream analyses like molecular docking (Lamiable *et al.*, 2016).

3.6. Validation of epitope interaction with MHC class II through molecular docking

Molecular docking performed between HLA DRB1*07:01 and epitopes that had been previously modeled was done by a specific docking method. Specific docking was performed on active sites of HLA DRB1*07:01 which are GLU88, THR90, LEU92, ILE106, PHE108, ASP110, LYS111, PHE148, THR145, LEU147, ILE148, HIS149, ASN150. Molecular docking results showed differences in binding scores across all epitopes, ranging from -134 Kcal/mol to -186 Kcal/mol (Sup. Table 3). The three epitopes that have the most negative docking score are 26-26-WCDGFCSSRGKRIDL-40-40 (-186.77

Kcal/mol), 27-CDGFCSSRGKRIDL-41 (-160.15 Kcal/mol), and 25-TWCDGFCSSRGKRID-39 (-156.10 Kcal/mol) (Figure 1). A more negative binding score indicates that the model has a higher probability of binding (Dey *et al.*, 2023; Wibowo *et al.*, 2021). The epitope 26-WCDGFCSSRGKRIDL-40-40 interacts with various types of bonds, with the most dominant interactions being hydrogen bonds and electrostatic bonds that bind to the binding site residues of HLA. The epitope 27-

CDGFCSSRGKRIDL-41 uses hydrophobic interactions as the primary mechanism for interacting with HLA. Meanwhile, the epitope 25-TWCDGFCSSRGKRID-39 interacts through hydrogen bonds, hydrophobic interactions, and electrostatic bonds (Table 7). An increased negative docking score indicates a more likely binding model, suggesting that the three epitopes will be examined further.

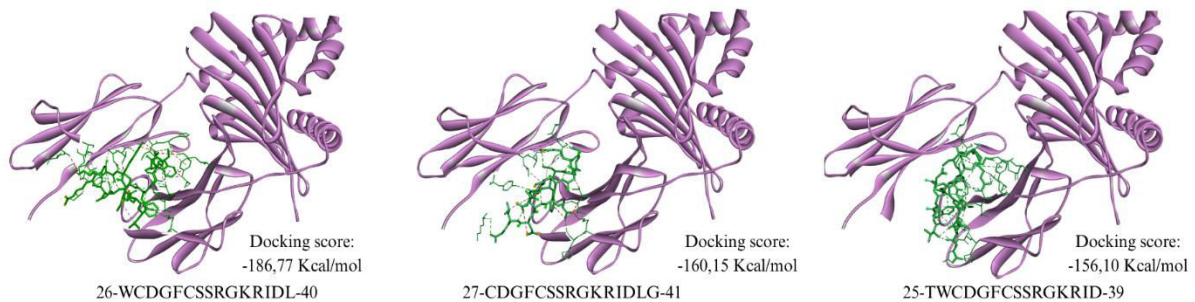


Figure 1. Top three interactions between epitopes and HLA class II.

Table 7. Top three interactions between epitopes and HLA class II

MHC II	Epitope	Docking Score (Kcal/mol)	Interaction	Amino Acids
HLA DRB1*07:01	26-WCDGFCSSRGKRIDL-40	-186,77	Electrostatic	A:LYS111, B:GLU162
			Hydrogen Bond	A:LYS111, A: THR90, A: LYS176, B: TYR102, B:HIS149, B:ALA104, B:THR145, B:LEU158
			Hydrophobic Bond	A:LEU92, B: ALA104, B:VAL143, B:LEU158, B:VAL116, A:VAL91, A:LYS176
			Other	A:PHE108
	27-CDGFCSSRGKRIDL-41	-160,15	Hydrogen Bond	B:TYR102, B:THR145, B:LEU147, B:THR145, A:HIS177, B:LYS105, B:ILE148, B:THR145, A:THR90
			Hydrophobic Bond	B:LEU158, B:ILE148
	25-TWCDGFCSSRGKRID-39	-156,1	Electrostatic	A:GLU88, A:ASP110, A:PHE108, A:TRP178
			Hydrogen bond	A:THR90, A:LYS111, A:GLU179, A:THR93, A:VAL91, A:TRP178, A:GLU88, A:PHE108, A:HIS177
			Hydrophobic Bond	B:ILE148, A:PHE108

Bold letters indicate the same amino acid residue as the active site

3.7. Evaluation of the stability of epitope-MHC class II complexes with molecular dynamics simulations

The three epitopes with the highest docking scores, which are 26-WCDGFCSSRGKRIDL-40, 27-CDGFCSSRGKRIDL-41, and 25-TWCDGFCSSRGKRID-39, were used for molecular dynamics simulations with the HLA DRB1*07:01 complex to determine their stability. The analysis was conducted by monitoring RMSD (Root Mean Square Deviation) fluctuations over 20 ns. The analysis results showed that all three epitopes had an average RMSD value below 3 Å. Although they had average values below 3 Å, the three epitopes exhibited different fluctuation patterns. The 26-WCDGFCSSRGKRIDL-40 epitope demonstrated the best stability with an average RMSD of 2.344 Å, with one peak reaching 3.122 Å at 8.125 ns. 27-

CDGFCSSRGKRIDL-41 exhibits similar stability with an average of 2.395 Å, but there is fluctuation at 5.6 ns, where the RMSD value reaches 3.177 Å. Meanwhile, the 25-TWCDGFCSSRGKRID-39 epitope is the least stable among the two epitopes. The average RMSD of the epitope is 2.479 Å and exhibits instability at 6.35 ns and 4–7 ns, where the maximum RMSD value reaches 3.311 Å (Figure 2). The epitope is considered stable because the resulting RMSD value does not exceed 3 Å. This indicates the epitope structure is relatively unchanged through the simulation (Dey *et al.*, 2023; Naveed *et al.*, 2022).

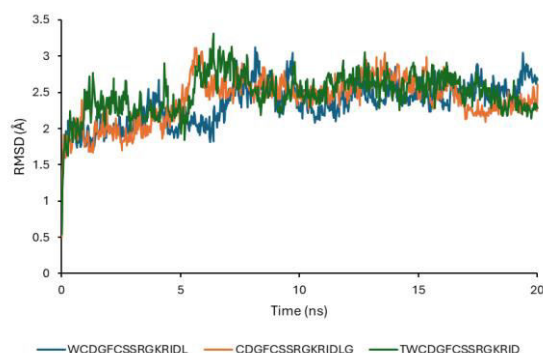


Figure 2. Simulation of the molecular dynamics of the interaction between HLA DRB1* 07:01 and the epitopes.

4. Discussion

This study predicted the potential epitopes of LNTX as the dominant component in *O. hannah* toxin. This prediction represents the initial step in the development of vaccine-based antivenoms, which are expected to prevent the increase in mortality from *O. hannah* bites with greater specificity and safety. The selection of epitopes that exhibited overlap between MHC II epitopes and B cell epitopes was a key consideration in the design of peptide-based vaccines, as it promoted effective cooperation between B cells and CD4⁺ T helper cells. This paper determines essential requirements of induction of high-affinity antibody responses and development of humoral immune memory (Li *et al.*, 2014). The first model of the predicted epitopes included 13 MHC class II epitopes and 4 linear B cell epitopes. Among them, 12 MHC II epitopes were present at locations that overlapped with the predicted B cell epitopes, implying their ability to induce T-cell-mediated, along with antibody-mediated immunity, and therefore their suitability to be used as a candidate in the development of peptide-based vaccines. However, the epitopes that were further developed to undergo further analysis were restricted to three, since the epitopes were the only ones that satisfied all of the necessary conditions, which are conservancy, immunogenicity, allergenicity, and population coverage. The rest of the epitopes were omitted owing to non-adherence to one or more of these parameters.

The chosen epitopes were also preserved in various sequences of LNTX toxin in the proteomes of different species of *O. hannah*. Recovery of epitopes where there is a minimum change in sequence is critical in achieving universal coverage of toxin variants (Bagherzadeh *et al.*, 2022). The high immunogenicity shown by the selected epitopes indicates their ability to activate CD4⁺ T cells and thereby initiate downstream immune responses, including B cell activation and antibody production (Xu *et al.*, 2022). The safety of the selected epitopes to human use was also assessed by means of allergenicity. This assessment used several approaches, such as allergenic motif scanning, SVM-based classification, and homology searches (Ahmad *et al.*, 2021). The findings showed no signs of an allergenic nature or a possibility of IgE-binding activity of the chosen epitopes, which implies they should be regarded as non-allergenic and can be safely used in the creation of a vaccine (Hayes *et al.*, 2015).

The three chosen epitopes were identified as the most promising candidates because they possess very good immunogenicity, but the key weakness of the present work is that the coverage of the population is limited, and this is a critical limitation in vaccine development. Combined, these epitopes represent just 11.26 of the combined populations of eight Southeast Asian countries, a fact owing to much HLA heterogeneity in the area. The highest coverage rate was in Indonesia (20.49 %), but it was still fairly low, and other countries had even lower levels (between 1.22 % and 15.91 %). The limitation is due to the fact that the study only focuses on one, region-specific HLA allele, HLA-DRB107-01, with a prevalence of 13.7 per cent in Indonesia (Gustiananda *et al.*, 2021). Other alleles, including HLA-DRB112:02 (36.8 %) and HLA-DRB115:02 (24.1 %), which are more frequent (Hayes *et al.*, 2015; Rezaldi *et al.*, 2021), were excluded because of their low predicted immunogenicity. In turn, the dependence on a single allele with immunogenicity highlights one of the major tradeoffs and indicates that a single-allele approach cannot be used to protect a wide region. The future vaccine designs should thus be multi-allele. Incorporating a panel of the most frequent and immunogenic HLA Class II alleles from across Southeast Asia is the essential next step to engineer a vaccine with robust, pan-regional efficacy.

The physicochemical characteristics of the epitope were predicted to determine the stability of the epitope. Based on the results, it is known that the three selected epitopes fall into the unstable category, as shown by the stability index, where the three epitopes have a value above 40. The stability index value that exceeds 40 indicates that the epitope is unstable and easily degraded (Raza *et al.*, 2021). Epitopes with longer half-lives remain in circulation for extended periods, thereby providing a greater opportunity to be recognized by immune cells. This prolonged exposure enhances the likelihood of effective antigen presentation and subsequent immune activation (Mathur *et al.*, 2016). This study found that epitope 25-TWCDGFCSSRGKRIDL-39 showed the highest estimated half-time value among the three selected epitopes, either in mammalian cells (7.2 h), yeast (>20 h), or *E. coli* (>10 h). As indicated by the relatively high range of aliphatic index (26-52), the selected epitopes were shown to possess thermostability over a broad temperature range. The selected epitopes were hydrophilic or globular (negative GRAVY), therefore, suggesting better solubility in aqueous environments (Panda and Chandra, 2012). These acceptable stability criteria showed the potential suitability of the selected epitopes as a stable candidate for a peptide-based vaccine, while unstable epitopes may require structural optimization (Devi *et al.*, 2021).

To overcome this problem, linkers can be added to the epitope to increase their stability. The increase in protein stability after the addition of linkers is due to the spatial structure of the linkers, which provides sufficient space for the protein to fold properly, thus it can function independently (Guo *et al.*, 2021). EAAAK linker is a rigid alpha helix peptide responsible for maintaining the functional properties of the epitope (Fadilah *et al.*, 2023). In this study, the EAAAK linker was added to the N-terminal and C-terminal of the epitope (Figure 3). The addition of the linker to the epitope successfully increased the stability of the epitope and maintained its physicochemical characteristics (Table 8).

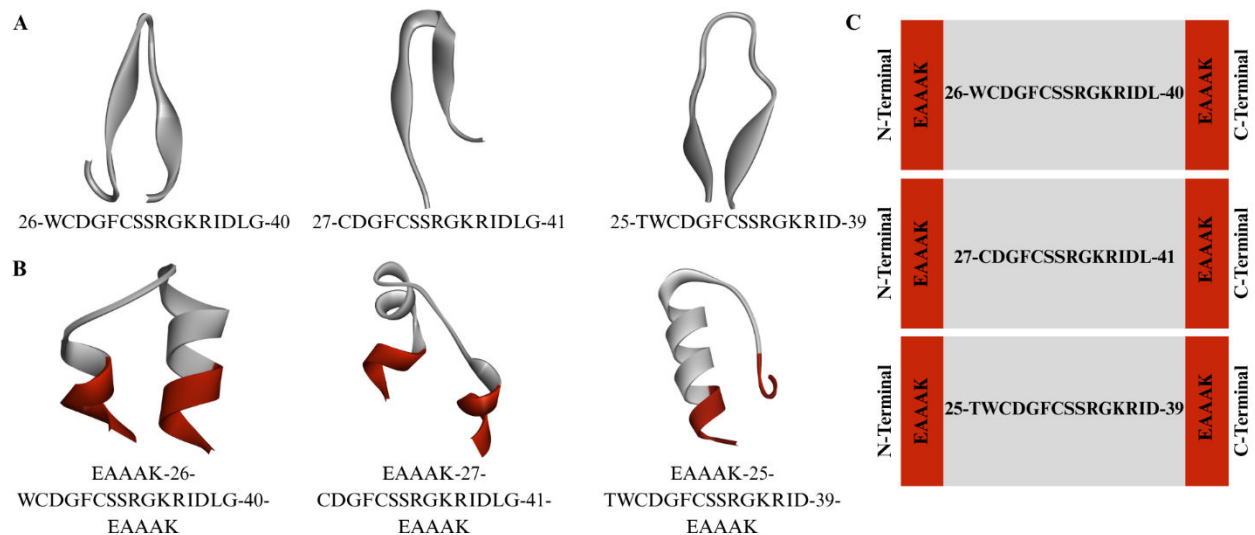


Figure 3. Design of linker conjugated epitopes. A) Initial epitope structure modeled by PEP FOLD3, B) Epitope with linker, C) Epitope sequence with linker. Red color indicated the EAAAK linker and grey color indicated the epitope sequence.

Table 8. Physicochemical characteristics of linker conjugated epitopes.

Physicochemical Characteristic	EAAAKWCDGFCSSRGKRIDL EAAAK	EAAAKCDGFCSSRGKRIDL EAAAK	EAAAKTWCDGFCSSRGKRIDE AAAK
Molecular Weight	2684.04	2554.88	2671.99
Theoretical pI	8.12	8.12	8.12
Estimated half-life in mammalian cells (Hours)	1	1	1
Estimated half-life in yeast (Min)	30	30	30
Estimated half-life in <i>E. coli</i>	10 min	> 10 Hours	> 10 Hours
Instability index	38.96	35.94	32.95
Stability	Stable	Stable	Stable
Aliphatic index	55.20	55.20	39.60
Grand average of hydropathicity (GRAVY)	-0.444	-0.44	-0.624

Molecular docking was performed to predict how ligands and targets will align to form a stable compound (Batra *et al.*, 2022). In this study, molecular docking was used to determine the ability of three epitope candidates, namely 26-WCDGFCSSRGKRIDL-40, 27-CDGFCSSRGKRIDL-41, and 25-TWCDGFCSSRGKRID-39, to bind to the binding groove of HLA DRB1*07:01, which is human MHC-II. All three epitopes are capable of binding to HLA DRB1*07:01 in the binding groove through several kinds of bonding, including hydrophobic interactions, electrostatic bonds, and hydrogen bonds (Table 7). Among the three epitopes, the epitope 26-WCDGFCSSRGKRIDL-40 has the highest docking score.

The ability of epitopes to bind to MHC class II is linked to the role of antigens in generating antibodies (Hanyu *et al.*, 2019). With varying affinities, the three identified epitopes can bind to HLA DRB1*07:01. The potential of epitopes to bind to MHC class II increases for epitopes with higher negative docking scores (H. Li *et al.*, 2022). On the surface of antigen-presenting cells (APCs), CD4⁺ T cells will recognize epitopes that bind to the MHC II complex. This recognition aids in the differentiation of B cells into plasma cells, which in turn aids in the production of antigen-specific memory B cells and antibodies against the specific antigen by initiating an

immune cascade. Because of their capacity to bind to MHC II binding sites, these three epitopes may therefore be able to initiate the production of antibodies (Moten *et al.*, 2023).

The interplay between the epitopes and HLA molecules can only be elucidated by an evaluation of both structural stability and conformational flexibility, which can be measured quantitatively using the simulation of molecular dynamics (Salo-Ahen *et al.*, 2020). Simulation findings show that the average root-mean-square movement (RMSD) of the three epitopes is below 3 Å in the mean, which shows a general structural integrity, although there are occasional exceedances of the limit. The RMSD profile in an epitope thus shows stability, which is a measure of its energy to form strong and sufficient interactions and have high functional efficacy. According to Hassan *et al.* (2024), an epitope's bonding ability and potential efficacy increase with its RMSD value stability. The most stable epitope is 26-WCDGFCSSRGKRIDL-40. Consequently, it possesses the most promise as a possible epitope for vaccine development.

5. Conclusions

This study provides a novel *in silico* framework for a vaccine-based antivenom by identifying three

conserved, immunogenic, and stable epitopes from the LNTX of *O. hannah* venom, specifically designed to interact with the prevalent HLA-DRB1*07:01 allele. These results offer a targeted alternative to conventional antisera. Future directions must include *in vitro* and *in vivo* immunological assays to confirm neutralization efficacy, alongside the incorporation of a broader panel of HLA alleles to overcome population coverage limitations and achieve a truly pan-regional vaccine for Southeast Asia.

Data Availability

All data supporting the findings of this study are available in Figshare with the identifier DOI: 10.6084/m9.figshare.30749081

(<https://doi.org/10.6084/m9.figshare.30749081>). The dataset can be accessed publicly and includes all processed data and supplementary materials used in the analysis.

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Effect of Antioxidants at varying Concentrations on Sperm Quality of Naleh Fish (*Barbonymus* sp.) Post-cryopreservation

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Abstract

Cryopreservation can generate oxidative stress, which in turn may harm cellular structures and diminish sperm quality upon thawing. Several studies highlight that antioxidants are pivotal in reducing the harmful effects of Reactive Oxygen Species (ROS). However, the ideal type and concentration depend on the fish species. Thus, the goal of this research is to determine the most effective type and concentration of antioxidants in maintaining the quality of post-cryopreservation sperm of naleh fish (*Barbonymus* sp.). The procedures were conducted in 2 stages using a Completely Randomized Design (CRD) that included 5 treatments with 4 replications for each. In the first stage, 5 types of antioxidants, namely ascorbic acid, β -carotene, glutathione, butylated hydroxytoluene (BHT), and myo-inositol, were tested at a concentration of 10%. The most effective antioxidant from this stage was then tested again at 5 different concentrations (0%, 5%, 10%, 15%, and 20%) in the second stage. Ringer's solution, 10% dimethyl sulfoxide (DMSO), and 5% egg yolk served as extenders and cryoprotectants. The sperm were stored at -196°C in liquid nitrogen for a duration of 14 days. The results indicated that both the antioxidant type and concentration had a significant impact on sperm motility, motility duration, viability, abnormalities, and fertilization rate ($P < 0.05$). Glutathione showed the highest effectiveness in maintaining sperm quality. Further test results showed that glutathione at a concentration of 10% could better maintain sperm quality. The analysis of naleh fish sperm DNA integrity following antioxidant supplementation demonstrated the absence of DNA fragmentation across all samples, these findings indicate that the sperm DNA remained undamaged in both fresh and post-cryopreservation conditions. Therefore, 10% glutathione was recommended for use in the cryopreservation process of naleh fish sperm.

Keywords: Naleh Fish, Antioxidant, Glutathione, DMSO, Ringer

1. Introduction

As a native freshwater species of Indonesia, the naleh fish (*Barbonymus* sp.) represents a valuable resource with high economic value and potential for development as a target for cultivation, both for food and ornamental purposes (Muchlisin *et al.*, 2015). The natural population of the species is found in the inland waters of the Southwest Aceh region, specifically in Nagan Raya Regency (Batubara *et al.*, 2018). However, high levels of exploitation without regard for catch time and size have led to population decline or overfishing (Batubara *et al.*, 2019). This shows that commercial cultivation is necessary to meet increasing market demand and maintain the sustainability of its natural stock.

One challenge in cultivating naleh fish is the limited availability of superior seeds due to the lack of development of seeding technology. This is partly due to the scarcity of quality broodstock, which is dependent on the natural spawning season that lasts from March to June (Efizon *et al.*, 2021). During this period, the broodstock of naleh fish becomes mature, making artificial spawning impossible throughout the year. To overcome the

challenge, sperm cryopreservation represents a strategic solution, particularly for species with short and seasonal reproductive periods such as naleh fish (Maulida *et al.*, 2021). This technology ensures the year-round availability of viable male gametes for artificial fertilization (Muthmainnah *et al.*, 2019). In addition, sperm available through cryopreservation can also be used to support breeding and genetic conservation programs, as well as gamete exchange between regions (Muchlisin, 2005; Zairin *et al.*, 2005; Maulida *et al.*, 2020).

The effectiveness of sperm cryopreservation is highly influenced by the correct blend of storage media, which involves the choice of extenders, dilution ratios, cryoprotectants, and the inclusion of antioxidants (Lestari *et al.*, 2013; Maulida *et al.*, 2020; Maulida *et al.*, 2023). Extenders function to prolong viability and prevent premature activation, while cryoprotectants such as DMSO, glycerol, and extracellular substances, including egg yolk or skim milk, protect cells from damage due to freezing (Sieme *et al.*, 2016; Muthmainnah *et al.*, 2019). During fish sperm cryopreservation, increased ROS production commonly occurs from the cooling phase through thawing, inducing oxidative stress that may compromise sperm quality (Sandoval-Vargas *et al.*, 2021).

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Because many fish spermatozoa, particularly those of cyprinid species, are rich in polyunsaturated fatty acids, they are highly prone to oxidative lipid damage, making antioxidant supplementation a critical component for preserving post-thaw sperm quality (Castro *et al.*, 2025). Antioxidants, including glutathione, vitamins C, and E, play an essential role in neutralizing ROS and minimizing oxidative damage to sperm membranes, mitochondria, and DNA during cryopreservation (Feradis, 2009; Kaltsas, 2023).

Research has explored using antioxidants in the cryopreservation media for fish sperm, such as ascorbic acid (Vitamin C) introduced in Atlantic salmon (*Salmo salar*) (Figueroa *et al.*, 2018), butylated hydroxytoluene (BHT) in depik fish (*Rasbora tawarensis*) (Muchlisin *et al.*, 2023), as well as glutathione and myo-inositol in betok fish (*Anabas testudineus*) (Maulida *et al.*, 2024). Muthmainnah *et al.* (2019) reported that supplementation with glutathione maintained the percentage of sperm motility in seurukan fish (*Osteochilus vittatus*), while melatonin supplementation improved post-thaw motility and membrane integrity in the freshwater fish *Prochilodus lineatus* (Motta *et al.*, 2022). In addition, inositol supplementation has been shown to significantly enhance post-thaw motility, membrane integrity, and overall sperm quality in Mesopotamian catfish (*Silurus triostegus*) (Doğu *et al.*, 2021). The combination of β -carotene and α -tocopherol in cryopreservation media in trout pelangi fish (*Oncorhynchus mykiss*) helped protect sperm membrane from oxidative damage, thereby improving quality after freezing (Kutluyer *et al.*, 2014). Maulida *et al.* (2021) examined the suitability of cryoprotectant types and concentrations, and reported that DMSO was the best cryoprotectant for fish. Diani (2024) also concluded that a 10% DMSO concentration was the most effective for cryoprotecting naleh fish sperm. Despite the existing literature, research concerning the suitability of antioxidants and their concentrations for cryopreservation of naleh fish sperm have never been conducted. Consequently, the present study assessed various antioxidants and their concentrations to identify the most effective combination for supporting post-thaw sperm performance in naleh fish (*Barbonymus* sp.).

2. Materials and Methods

2.1. Time and Site

The present study was undertaken in April 2025 at Gampong Meunasah Krueng in the Beutong District of Nagan Raya Regency, Aceh Province, and at the Fish Hatchery and Breeding Laboratory of the Faculty of Marine and Fisheries, Syiah Kuala University.

2.2. Experimental Design

In this research, a Completely Randomized Design (CRD) was used as the basis for the experimental setup, comprising five treatment levels with four replications. The first stage tested the effectiveness of five types of antioxidants: ascorbic acid, β -carotene, glutathione, BHT, and myo-inositol. Each was used at a concentration of 10% to determine the most effective type of antioxidant. The second stage continued with testing 5 levels of concentration, namely 0%, 5%, 10%, 15%, and 20% of the

best antioxidant obtained in the first stage, specifically glutathione.

2.3. First Experiment Procedure

2.3.1. Parent provision

Fish samples for the experiment were sourced from the Nagan River within the Nagan Raya Regency area. Approximately 20 male and 10 female broodstock of final gonad maturity were used, with males averaging 16 cm in length and 36 grams in weight. The broodstock individuals were kept in a pond for 14 days, receiving natural food, including silkworms, provided twice daily, in the morning (07:00 Western Indonesian Time) and afternoon (18:00 Western Indonesian Time).

2.3.2. Sperm retrieval

Ten mature male naleh fish broodstock were weighed and injected intramuscularly with Ovaprim (0.5 ml/kg) in the back at a 45° angle, then placed in a broodstock tank. Sperm were collected by gently massaging the abdomen 7 to 8 hours after injection and then aspirated using a separate syringe for each broodstock. This was collected in an ice box at 4°C, exhibiting $\geq 70\%$ motility, and was used. Good-quality sperm were collected in a petri dish and temporarily stored at 4°C.

2.3.3. Preparation of extenders, cryoprotectants, and antioxidants

Ringer's solution served as the extender, with several compositions including 3.25 g NaCl, 0.125 g CaCl₂, 0.1 g NaHCO₃, which was then added with distilled water to 100 ml. The sperm was then combined with the extender in a 1:60 ratio (1.67 ml sperm: 100 ml Ringer). Furthermore, the cryoprotectant used consisted of 1% DMSO and 5% egg yolk, achieved by adding 10 mL of DMSO and 5 mL of egg yolk, respectively. Sperm mixture was divided into 20 cryotubes, each containing 1.5 mL. Subsequently, 0.15 mL of different antioxidants (ascorbic acid, β -carotene, glutathione, BHT, and myo-inositol) was added to achieve a concentration of 10% in each treatment, with 4 cryotubes serving as replicates.

2.3.4. Sperm freezing and thawing

A total of 20 cryotubes filled with sperm samples were first stored in a chilled box (4°C) for 5 minutes, followed by exposure to the vapor phase of liquid nitrogen (-79°C) for 5 minutes, and frozen in liquid nitrogen (-196°C) for 2 weeks. After this procedure, the samples were thawed gradually according to concentration using a temperature-controlled bath maintained at 32°C for 3 minutes, and then macroscopic as well as microscopic examination of sperm quality was carried out.

2.4. Second Experiment Procedure

2.4.1. Preparation of extenders, cryoprotectants, and antioxidants

The procedures for providing broodstock and sperm collection, preparing extenders, and adding cryoprotectants were similar to those used in the first phase of the study. Approximately 1.67 ml of sperm was combined with 100 ml of Ringer's solution (1:60 ratio), followed by 10% DMSO and 5% egg yolk as cryoprotectants. This mixture was divided into 20 cryotubes (1.5 ml each), and the best antioxidant from the first phase of the study, glutathione,

was added. One set of cryotubes was left without antioxidant addition, while the other four sets were added with 0.075 ml, 0.15 ml, 0.225 ml, and 0.3 ml of glutathione, respectively, to produce concentrations of 5%, 10%, 15%, and 20%, respectively.

2.4.2. Sperm freezing and thawing

In this study, 20 cryotubes with sperm samples inside were stored in a chilled box (4°C) for 5 minutes, then evaporated in the neck of a nitrogen tank (-79°C) for 5 minutes and frozen in liquid nitrogen (-196°C) for two weeks. Subsequently, the samples were thawed in stages according to concentration in a water bath (32°C) for 3 minutes, and then macroscopic and microscopic sperm quality examinations were carried out after freezing (Maulida *et al.*, 2024).

2.5. Assessment of Sperm Quality Parameters

2.5.1. Macroscopic and Microscopic Observations of Sperm

Macroscopic observation: Macroscopic observations were conducted on fresh sperm, including assessment of color, pH, and consistency. Furthermore, its consistency was assessed based on the rate of sperm flow in the test tube. This was categorized as thin (rapid flow to the bottom of the tube), moderate (slow flow and some of sperm adhered to the walls of the tube), and thick (very slow flow and much of sperm remained at the edges of the tube) (Ariefantini, 2012). Sperm pH was evaluated by dropping a sample onto pH paper and then comparing the color change with a standard pH scale. The normal pH value for fish sperm was 6.5 (Muchlisin *et al.*, 2020). **Microscopic observation** an analysis was conducted on fresh and post-cryopreserved sperm, including observations of motility, viability, and abnormalities. Sperm motility was assessed by positioning a drop of the sample onto a glass slide, adding one to three drops of water to initiate sperm activation, and then observing it using a microscope and Spermvision software (Minitube, Germany). Sperm were categorized as motile when moving in a straight, progressive, agile, and non-rotating manner. Motility was calculated using the formula of Muchlisin *et al.* (2004):

$$\text{Motility (\%)} = \frac{\text{Number of sperm that move straight forward}}{\text{Number of observed sperm}} \times 100$$

Motility duration was assessed by recording the period between the beginning and the end of movement, using a microscope magnification of 100× or 400×. Viability was analyzed by mixing a drop of sperm with a drop of 2% eosin, then making a smear, drying it, and observing it under a microscope. Live sperm were transparent white, while dead sperm were red. To quantify viability, the formula outlined by Ax *et al.* (2000) was applied:

$$\text{Viability (\%)} = (\text{Number of live sperm} / \text{Number of observed sperm}) \times 100$$

Abnormality monitoring was performed on the same specimens as viability. Sperm were considered abnormal when abnormalities were observed in the head and tail. Abnormality was assessed based on the formula of Akbar *et al.* (2015).

$$\text{Sperm abnormalities (\%)} = (\text{Number of abnormal sperm} / \text{Number of observed sperm}) \times 100$$

2.5.2. Fertilization

Fertilization was performed using 10 mature female broodstock, which were administered Ovaprim at a dosage of 0.5 ml/kg body weight and allowed to undergo ovulation for 7 hours. Eggs were removed by stripping and collected in a petri dish. One ml of eggs (250 to 400 eggs) was mixed with 1 ml of sperm (1:1 ratio), then 2 to 5 drops of water were applied to trigger sperm motility, and the preparation was gently stirred using sterilized chicken feather. The eggs were left for 4 minutes to fertilize sperm, then incubated at 28°C for 48 hours. Successful fertilization was observed after 6 hours, as fertilized eggs appeared clear, while unfertilized eggs appeared cloudy white. The following equation, adapted from Muchlisin *et al.* (2015), was used to determine the fertilization rate:

$$\text{Fertilization (\%)} = (\text{Number of fertilized eggs} / \text{Number of eggs observed}) \times 100$$

2.5.3. DNA Fragmentation

DNA Extraction and Electrophoresis

Sterile microcentrifuge tubes were used to transfer the sperm samples, which were then spun at 14,000 rpm for 3 minutes to create a pellet. Around 100 µl of this pellet was placed in a new sterile tube, after which 300 µl of cell lysis buffer was added. The suspension was carefully inverted 5–6 times to ensure even consistency and was left to incubate at ambient temperature for 10 minutes. The solution was subjected to another spin (at 14,000 rpm for 20 seconds), and the supernatant fluid was removed. A 15-second vortexing step was performed to fully resuspend the pellet, followed by the addition of 100 µl of nuclei lysis solution and subsequent vortexing (Promega, 2017).

Next, 35 µl of a protein precipitation mixture was introduced and mixed on a vortex machine for 20 seconds, then spun in a centrifuge at 14,000 rpm for 3 minutes. The resulting clear supernatant was carefully transferred to a fresh tube preloaded with 100 µl of isopropanol and mixed by vortexing for 15 seconds. This was followed by another centrifuge step at 14,000 rpm for 1 minute at ambient temperature. The supernatant was thrown away, and a 70% ethanol solution (in a 1:1 ratio) was used to rinse the DNA pellet, then centrifuged for another minute. Following ethanol removal, the pellet was air-dried for 15 minutes, rehydrated with 35 µl of DNA rehydration solution, and subsequently incubated at 65 °C for 1 hour. The final measurement involved determining DNA concentration and purity using a Nanodrop C2000 spectrophotometer (Chen *et al.*, 2018).

For the electrophoresis procedure, 1.5 grams of agarose was completely dissolved in 100 milliliters of TBE buffer by heating clear and boiling. After cooling slightly, 2 microliters of DNA dye was pipetted to the mix, and transferred into a gel mold to solidify for about 30 minutes. The prepared agarose gel had a concentration of 1.5% and was placed in an electrophoresis chamber filled with 1× TBE buffer. Next, 10 microliters of DNA sample were blended with 22 microliters of loading dye and then loaded into the wells, alongside 5 microliters of DNA marker. The electrophoresis was conducted at 135 volts for 30 minutes, after which the DNA bands were visualized and captured using a UV-Doc transilluminator.

2.6. Data Analysis

Descriptive analysis was performed on sperm data, including pH, color, consistency, and DNA fragmentation, while data on percentage of motility, duration of motility, viability, abnormality, fertilization, and egg hatching were statistically analyzed using SPSS version 25. The data were initially tested for normality, and when normally distributed and homogeneous, a one-way ANOVA was performed. When the treatment had a significant effect, Duncan's test was applied to evaluate the most effective treatment.

3. Results

The fresh sperm of naleh fish appeared milky white, had a pH of 7, and exhibited a medium level of consistency. Furthermore, the average sperm density was 263×10^6 cells/mL, with percentages of motility, motility duration, viability, sperm abnormalities, and fertility of 98.2%, 464.58 seconds, 93%, 0.2% and 89.2%, respectively (Table 1). The results of observations on naleh fish sperm showed sperm morphology with indications of abnormal sperm marked with red arrows. Abnormalities detected included asymmetrical head shape and bent tail. For sperm viability, dead sperm were black, while live sperm were white or appeared transparent (Figure 1).

Table 1. Characteristics of fresh sperm quality of naleh fish (*Barbonymus sp.*)

Parameter	Result
Color	Milky White
Average pH	7
Average consistency	Medium
Average sperm concentration (cells/mL)	263×10^6
Average fresh sperm motility (%)	98.2 ± 1.40
Average motility duration (seconds)	464.58 ± 45.55
Average fresh sperm viability (%)	93 ± 1.15
Average fresh sperm abnormality (%)	0.2 ± 0.4
Average egg fertility (%)	89.2 ± 4.41

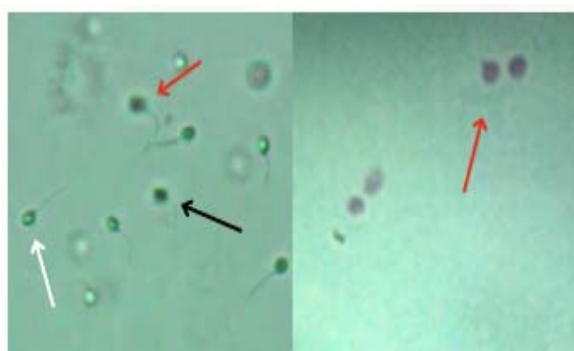


Figure 1. Sperm morphology of naleh fish observed under 400× microscopic magnification, with red arrows indicating abnormal sperm, black arrows indicating dead sperm, and white arrows indicating live sperm.

Analysis of variance indicated that the type of antioxidants had a significant effect on motility, duration of motility, viability, sperm abnormalities, and fertility of naleh fish eggs ($P < 0.05$). According to Duncan's further test results, the highest motility and viability were found with the use of glutathione (98.5% and 92.8%, respectively). These rates were significantly different compared to β -carotene and BHT treatments, but not different from the ascorbic acid and myo-inositol treatments. The highest motility duration was also observed with the use of glutathione (428.75 seconds), a value different from those of the other treatments. Glutathione resulted in the minimal level of sperm abnormalities (0.2%) and achieved the highest fertilization success (88.6%). Nevertheless, these results were statistically comparable to those recorded for ascorbic acid, BHT, and myo-inositol (Table 2). Therefore, the best sperm quality was obtained with the use of glutathione.

In the Glutathione test with several concentration levels, based on the ANOVA test results, differences in glutathione concentration significantly influenced sperm motility, motility duration, viability, sperm abnormalities, and fertility of naleh fish eggs ($P < 0.05$). The Duncan test indicated that the 10% concentration resulted in the highest motility (98.7%) and viability (91.7%). This value was significantly distinct from the 20% and 0% treatments, but not significantly different from the 5% and 15% treatments. Furthermore, the highest motility duration was also found at the 10% concentration, specifically 452.5 seconds, which differed significantly from the other treatments. The lowest sperm abnormality value (0.2%) was observed at the 10% concentration treatment, although it did not differ significantly from the 5% and 15% treatments. Meanwhile, the highest fertilization rate was also found at the 10% Glutathione concentration (84.9%), a value that was significantly different from the other concentrations, except for 5% (Table 3). In general, the best quality was obtained when using 10% glutathione.

Gel electrophoresis results demonstrated that sperm DNA from all treatment groups, including the antioxidant treatments (Figure 2) and the various glutathione concentrations (Figure 3), remained intact, with no observable DNA fragmentation. These results indicate an absence of DNA damage or degradation in both fresh and thawed sperm, suggesting that neither the treatments nor the storage process compromised the DNA quality of naleh fish sperm.

Table 2. Percentage±SD of spermatozoa motility, motility duration, viability, sperm abnormality, and fertility of naleh fish (*Barbonymus sp.*) post cryopreservation with combination of ringer extender, 5% egg yolk, and 10% DMSO using different types of antioxidants. Values within the same column for each respective antioxidants followed by different superscripts indicate a significant difference ($P<0.05$).

Antioxidant	Motility (%)	Motility Duration (sec)	Viability (%)	Sperm Abnormality (%)	Fertility (%)
Ascorbic Acid	92.2±2.87 ^{bc}	228.75±54.5 ^a	89.3±2.49 ^{bc}	1.2±0.95 ^{ab}	82.6±4.33 ^{bc}
Beta Carotene	71.0±9.5 ^a	237.50±18.4 ^a	81.1±7.23 ^a	1.5±0.57 ^b	72.2±5.93 ^a
Glutathione	98.5±0.57 ^c	428.75±90.4 ^b	92.8±1.31 ^c	0.2±0.50 ^a	88.6±4.19 ^c
BHT	79.5±3.10 ^a	231.25±25.6 ^a	84.3±5.12 ^{ab}	2.0±0.81 ^b	76.7±3.16 ^{ab}
Myo-inositol	89.2±7.80 ^b	226.25±52.1 ^a	89.2±3.40 ^{bc}	1.2±0.5 ^{ab}	81.0±1.91 ^b

Table 3. Percentage results of motility, motility duration, viability, sperm abnormality, and fertility of naleh fish (*Barbonymus sp.*) post cryopreservation with combination of ringer extender, 5% egg yolk, and 10% DMSO using different concentrations of antioxidants. The values in the same column at respective concentration followed by a different superscript denote significant difference ($P<0.05$).

Concentration (%)	Motility (%)	Motility Duration (sec)	Viability (%)	Sperm Abnormality (%)	Fertility (%)
0	73.5±12.2 ^a	265.0±17.7 ^a	78.8±7.43 ^a	2.5±1.29 ^{bc}	37.1±0.55 ^a
5	94.0±6.78 ^b	336.2±49.2 ^b	90.3±4.32 ^b	1.2±0.95 ^{ab}	79.4±1.38 ^d
10	98.7±0.50 ^b	452.5±56.7 ^c	91.7±1.75 ^b	0.2±0.50 ^a	84.9±4.40 ^d
15	91.7±6.39 ^b	335.0±30.0 ^b	86.7±2.62 ^b	1.5±1.29 ^{ab}	72.5±5.49 ^c
20	65.2±6.13 ^a	260.0±25.8 ^a	72.6±5.72 ^a	4.0±0.81 ^c	45.2±5.14 ^b

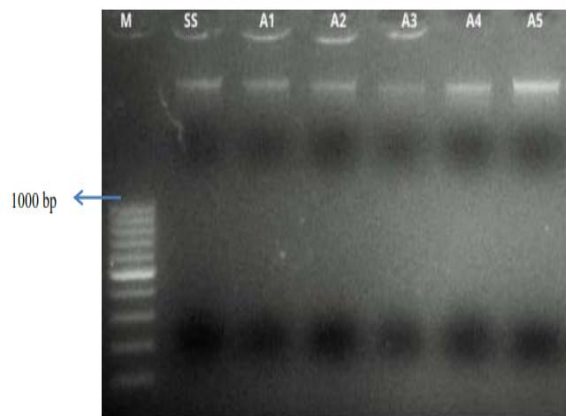


Figure 2. Gel electrophoresis results of naleh fish sperm samples from the first stage (comparison of antioxidant types). Note: M = Marker; SS = Fresh sperm; A1 = Ascorbic acid; A2 = Beta-carotene; A3 = Glutathione; A4 = BHT; A5 = Myo-inositol.

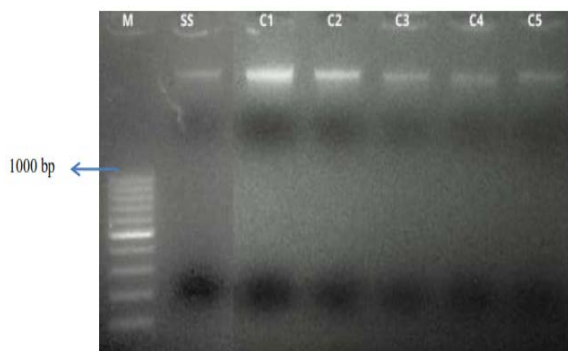


Figure 3. Gel electrophoresis results of naleh fish sperm samples from the second stage (comparison of glutathione concentrations). Note: M = Marker; SS = Fresh sperm; C1 = Glutathione (0%); C2 = Glutathione (5%); C3 = Glutathione (10%); C4 = Glutathione (15%); C5 = Glutathione (20%).

4. Discussion

According to the study findings, the fresh sperm presented a milky-white appearance, neutral pH (7), and moderate consistency. Concentration of fresh sperm from naleh fish was 263×10^6 cells/mL, with the percentage of motility, motility duration, viability, sperm abnormalities, and fertility, respectively, of 98.2%, 464.58 seconds, 93%, 0.2% and 89.2%. Based on these results, the quality of fresh sperm was suitable for use in the cryopreservation procedure (Di Santo *et al.*, 2012; Bier *et al.*, 2025). Motility was one important parameter in the study of sperm quality, both fresh and post-freezing (Sarosiek *et al.*, 2012). Whaley *et al.* (2021) reported that fresh sperm motility must exceed 70%.

The data suggested that the presence of antioxidants ascorbic acid and glutathione maintained post-freezing sperm motility equivalent to that of fresh sperm without freezing. However, the use of β -carotene, BHT, and myo-inositol resulted in slightly lower sperm quality compared to these 2. The results showed that all sperm treatments supplemented with antioxidants resulted in motility above 70%. This suggests that antioxidant supplementation could effectively maintain sperm quality, with the best sperm quality obtained with the addition of Glutathione. Differences in the suitability of the type of antioxidants and concentration used provided varying effectiveness, depending on the type and concentration suitable for use in naleh fish sperm. According to Li *et al.* (2017), each different type and concentration of antioxidant had different effects on the 3 sturgeon species tested. Further analysis showed that 10% glutathione was the optimal concentration, representing the most suitable antioxidant treatment for naleh fish sperm cryopreservation.

This study demonstrated glutathione's superior effectiveness in maintaining naleh fish sperm quality compared to other antioxidants and doses. Beyond naleh fish, glutathione has proven successful in cryopreserving sperm from other species, including betok fish (*Anabas testudineus*) (Maulida *et al.*, 2024), depik fish (*Rasbora tawarensis*) (Muchlisin *et al.*, 2023), and seurukan fish

(*Osteochilus jeruk*) (Muthmainnah *et al.*, 2018). As a non-enzymatic antioxidant, glutathione's efficiency in cryopreservation stems from its multifaceted mechanism against oxidative stress. Its main role is to alleviate oxidative stress by neutralizing reactive oxygen species (ROS), particularly superoxide anions (O_2^-), and converting them into less harmful molecules such as water (H_2O_2) (Panday *et al.*, 2020). This action is vital because lipid peroxidation can damage sperm membrane structure, mitochondria, and acrosome integrity, all critical for successful fertilization (Smits *et al.*, 2019; Félix *et al.*, 2020).

Glutathione protected DNA and sperm chromatin structure from oxidative damage, while maintaining mitochondrial function, which was crucial for energy production (ATP) and sperm motility (Ribeiro *et al.*, 2022). Another advantage of glutathione was its ability to be regenerated from its oxidized form (GSSG) to the active form (GSH) by the enzyme glutathione reductase, making it resistant to long-term oxidative damage (Halliwell dan Gutteridge, 2015). In the context of fish sperm cryopreservation, Yang *et al.* (2025) on mandarin fish (*Siniperca chuatsi*) and Muthmainnah *et al.* (2018) on seurukan fish (*Osteochilus jeruk*) noted that the addition of glutathione in the freezing medium significantly maintained sperm motility, viability, and fertility compared to other antioxidants, specifically at concentration of 10%.

In addition to glutathione, the use of 10% ascorbic acid produced results that were not significantly different from those of glutathione. Therefore, ascorbic acid was also able to maintain the quality of naleh fish sperm after thawing. Ascorbic acid or vitamin C, with its antioxidants properties, was known to be effective in neutralizing free radicals, specifically ROS, through the mechanism of electron donation to pro-oxidant compounds, thereby preventing the chain reaction of lipid peroxidation in sperm cell membranes and maintaining their quality and viability (Li *et al.*, 2010). Muthmainnah *et al.* (2018) also stated that ascorbic acid could increase sperm motility and viability post-cryopreservation in seurukan fish (*Osteochilus vittatus*), although the effect was less significant than glutathione. The effectiveness of ascorbic acid has been previously reported, for instance by Maulida *et al.* (2024) in betok fish (*Anabas testudineus*) and in the sperm of atlantic salmon (*Salmo salar*) (Figueroa *et al.*, 2018).

Although its value was lower than that of glutathione and ascorbic acid, the use of myo-inositol, β -carotene, and BHT also yielded satisfactory results, as sperm motility and viability were above 70%. Myo-inositol, with its antioxidants properties, increased osmotic pressure in sperm, enhanced sperm mitochondrial function by modulating intracellular calcium ions, and improved redox homeostasis during the cryopreservation process (Doğu *et al.*, 2021). The effectiveness of myo-inositol had also been reported by several authors in betok fish (*Anabas testudineus*) (Maulida *et al.*, 2024) and catfish (*Silurus triostegus*) (Doğu *et al.*, 2021).

BHT was reported to be effective in curbing oxidative stress by donating hydrogen atoms to free radicals, thereby breaking the chain of lipid peroxidation in cell membranes during the cryopreservation process, and its effectiveness was dose-dependent (Mostafa *et al.*, 2019). β -carotene

played an important role in safeguarding cell membrane integrity and reproductive function, enabling this compound to integrate into phospholipid membranes and provided direct protection against lipid peroxidation (Martín-San Juan *et al.*, 2025; Sereti *et al.*, 2025). Therefore, all types of antioxidants used in this study yielded positive results for the quality of naleh fish sperm after freezing, but glutathione provided the greatest efficacy.

Sperm motility and viability improved with glutathione up to a 10% concentration, but decreased slightly at 15% and more sharply at 20%. Moreover, although all antioxidant treatments maintained post-thaw fertility above 70%, the consistently superior motility, viability, and fertility observed at 10% glutathione clearly demonstrate that this concentration provides the highest level of cryoprotection. These results indicate that 10% glutathione is not only adequate but optimal, as it yields post-thaw fertility values closest to those of fresh sperm and outperforms all other antioxidants tested. The marked decline in sperm quality at concentrations above 10% further highlights the importance of this threshold. While other antioxidants produced acceptable fertility levels, none achieved the stability or post-thaw functional integrity afforded by 10% glutathione. Collectively, these results justify the recommendation of 10% glutathione as the most effective and biologically appropriate concentration for naleh fish sperm cryopreservation.

Kolyada *et al.* (2023) explained that while antioxidants are beneficial, excessive concentrations can induce reductive stress, inhibiting crucial cellular signaling and negatively impacting sperm. Therefore, 10% Glutathione is ideal for naleh fish sperm. Other antioxidants show similar dose-dependent toxicity. Martín-San Juan *et al.* (2025) found that β -carotene at roughly 10% could reduce sperm motility and damage mitochondrial function, even acting as a pro-oxidant. Kutluyer *et al.* (2014) also reported no positive effect from β -carotene in rainbow trout (*Oncorhynchus mykiss*) sperm cryopreservation. According to Lahnsteiner dan Mansour (2010), correct antioxidant dosing improves frozen sperm quality, but incorrect doses cause harmful toxic effects.

A similar phenomenon was also reported by Banihani and Alawneh (2019), who stated that high antioxidants concentrations could cause reductive stress, negatively impacting mitochondrial function and sperm motility after thawing. The results of study by Yang *et al.* (2025) on mandarin fish (*Siniperca chuatsi*) at the 2 highest doses (4 and 8 mM) showed a decrease in sperm quality compared to the lower dose (2 mM). These results showed that excessively high doses caused redox stress, a disturbance in the balance within cells that disrupted normal body function due to excessive antioxidants levels or cellular toxicity. In addition to fish, the toxic effects of glutathione at high concentrations have also been noted in terrestrial animals, such as goat sperm (Zou *et al.*, 2021) and Murrah bull semen (Gangwar *et al.*, 2018).

DNA is a highly sensitive cellular component that is prone to damage during cryopreservation, making the preservation of its integrity crucial for maintaining sperm quality (Santi *et al.*, 2018). Sperm cells experience extreme environmental stress during freezing and thawing, leading to disruption of cellular structures and metabolic pathways. Such damage is typically characterized by

reduced motility and impaired plasma membrane integrity, ultimately decreasing fertilization potential (Khan *et al.*, 2021; Min *et al.*, 2024). During cryopreservation, spermatozoa experience osmotic, thermal, and oxidative stresses that predominantly affect the plasma membrane, leading to potential alterations in lipid composition and impairments in functional physiology (Sieme *et al.*, 2015; Castro *et al.*, 2025). Therefore, the inclusion of cryoprotectants and antioxidants is important for alleviating cryogenic damage and shielding sperm cells from reactive oxygen species (ROS).

Analysis of DNA integrity in naleh fish sperm following cryopreservation with antioxidant supplementation (Figures 2 and 3) revealed intact DNA bands across all treatments (A1–A5, C1–C5) as well as fresh sperm (SS), with no evidence of smearing or fragmentation. This suggests that DNA remained structurally intact, and that antioxidant supplementation contributed to maintaining DNA stability during the freezing process. These findings are consistent with Huang *et al.* (2022), who noted that cryoprotective agents (CPAs) incorporated into sperm diluents can lessen cellular injury by limiting ice crystal formation and preserving membrane integrity and cellular structure, critical aspects, particularly in slow-freezing methods that are prone to mechanical and osmotic stress. Similarly, Berean *et al.* (2024) highlighted the protective role of antioxidants in preserving motility, membrane integrity, and DNA structure, particularly in the context of small animal sperm cryopreservation. Hence, antioxidants represent an key factor in maintaining sperm DNA integrity throughout cryopreservation. It is noteworthy that gel electrophoresis, while widely applied in forensic and biological research to assess DNA degradation, primarily provides qualitative results (band patterns or smearing). Its sensitivity is limited, especially in detecting subtle or early-stage DNA damage (Bhoyar *et al.*, 2024).

5. Conclusion

In conclusion, variations in antioxidant type and concentration significantly affect the post-cryopreservation quality of naleh fish sperm ($P < 0.05$). Antioxidant supplementation helps preserve sperm quality, and glutathione is the most effective type of antioxidants. When glutathione was used, post-cryopreservation sperm quality increased up to the concentration of 10%, then decreases as concentration increases. Sperm DNA integrity analysis showed that none of the sperm DNA samples exhibited fragmentation, suggesting that no DNA damage was present in any of the samples evaluated. Consequently, 10% glutathione is recommended as an antioxidant for the cryopreservation of naleh fish sperm.

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Conflict of interests

The authors state that no conflicts of interest are associated with this publication.

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Morphological and Molecular Characterization of Moss Species Widespread in Syria

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Abstract

This study addresses a significant knowledge gap in Syria's bryophyte diversity by conducting an integrated analysis of moss species from two climatically distinct regions: the drier inland area of Al-Zabadani and the more humid coastal region of Al-Haffah. By combining morphological examination with molecular techniques, we characterized 16 Bryopsida species using 12 morphological parameters and the RAPD method with 10 primers. Our results clearly show that the humid conditions of Al-Haffah support a greater diversity of mosses compared to the drier Al-Zabadani region. While morphological analysis successfully grouped the species into four clusters at the order level, the molecular data provided a higher resolution, distinguishing five distinct groups at the family level and revealing a high degree of genetic polymorphism (701 bands, PIC values 0.28-0.37). Methodologically, we successfully extracted high-purity DNA using the CTAB method and found the RAPD technique to be highly effective, with primer OP-R11 performing particularly well (25 markers and 120 bands). By establishing the first detailed moss database for these Syrian regions and validating a combined morphological-molecular framework, this study provides essential foundations for advancing bryophyte conservation and ecological research in similar Mediterranean environments.

Keywords: Bryopsida, acrocarpous, pleurocarpous, RAPD, cluster analysis, Syria.

1. Introduction

Bryophytes are important worldwide plant groups, they constitute the second most diverse group of land plants after flowering plants. Taxonomically, Bryophytes are situated between the algae and the pteridophytes. Their life cycle includes two generations: a gametophyte (haploid) and a sporophyte (diploid) stages. They are morphologically divided into three phyla: Marchantiophyta (liverworts), Anthocerotophyta (hornworts) and Bryophyta (mosses). They are small plants that grow on various types of substrates such as moist soil, tree trunks and branches, and rocks. They can be found in all ecosystems except marine environments; however, they prefer moist and shady habitats (Dziwak *et al.*, 2022; Dhyani *et al.*, 2025). In Syria, it is widespread throughout the western part, from the Turkish border in the north to Al-Golan in the south, including Syrian coastal strip forests and mountainous regions (Ismaeel *et al.*, 2024a).

Bryophytes hold significant value in both traditional and modern medicine. Historically used across various cultures for treating ailments like skin wounds and liver disorders, their medicinal potential was first documented in 1957. Contemporary research confirms their role as sources of antibiotics, antioxidants, and potential anti-cancer agents (Mandić *et al.*, 2021; Motti *et al.*, 2023; Ismaeel *et al.*, 2024b). Furthermore, they have emerged as

promising biopharmaceutical platforms, with *Physcomitrella patens* being the first plant to produce human proteins " such as vascular endothelial growth factor (VEGF) and alpha-galactosidase (α -Gal)", positioning bryophytes as potential candidates for vaccine production (Reski *et al.*, 2015).

Beyond their medicinal value, bryophytes serve important economic and ecological roles. Economically, they are utilized as heating fuel, food, and animal feed (Klavina, 2015; Ismaeel *et al.*, 2022). Ecologically, they significantly contribute to humid environment biodiversity through their exceptional water retention capacity and play crucial roles in nutrient cycling, particularly for carbon and nitrogen (Condes *et al.*, 2023). Furthermore, their effectiveness as bioindicators for pollution monitoring has been well documented (Ismaeel, 2017; Nurkassimova *et al.*, 2024).

While morphological criteria have traditionally served as the primary basis for plant classification, the growing need for precise species identification to support conservation efforts has driven the adoption of molecular techniques in recent decades. Molecular markers such as RAPD, ISSR, and SSR have become pivotal tools in phylogenetic and taxonomic studies (Crespo Pardo *et al.*, 2014; Dhyani *et al.*, 2025). These molecular markers "defined as heritable DNA sequences at specific genomic loci" provide high resolution data that elucidating taxonomic relationships between genera and species, often obscured by morphological analysis alone (Vanderpoorten

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and Jacquemart, 2004; Lang *et al.*, 2015; Schlesak *et al.*, 2018).

Among these methods, Random Amplified Polymorphic DNA (RAPD) has proven particularly valuable in bryological research since its introduction in the 1990s. Despite its limitations, including band irreproducibility under low stringency conditions, dominant inheritance patterns, uncertain amplification products, and co-migration of non-homologous fragments of similar size, the RAPD technique remains highly suitable for moss studies. This suitability stems from the small, often unsequenced genomes and haploid nature of mosses, which avoids complications of dominant marker expression common in diploid organisms (Arif *et al.*, 2010; Crespo Pardo *et al.*, 2014). By amplifying random DNA fragments using short primers, RAPD enables genetic diversity assessment without prior sequence knowledge and requires minimal sample material (Dale *et al.*, 1999).

The field of bryophyte molecular research has expanded significantly since the pioneering DNA sequencing study in 1983, accumulating approximately 382 studies by 2009 (Stech and Quandt, 2010). The RAPD technique has been widely applied to elucidate intraspecific genetic variation across diverse ecological contexts. Initial investigations on *Hennediella heimii* demonstrated population level genetic differentiation (Dale *et al.*, 1999). Findings later extended to related mosses species, including *Bryum argenteum*, *B. pseudotriquetrum*,

and *Ceratodon purpureus* from Antarctic environments (Selkirk *et al.*, 1998; Skotnicki *et al.*, 1998; Clarke *et al.*, 2009). Collectively, these studies establish RAPD as an effective methodology for resolving genetic relationships among bryophytes.

In response to the scarcity of data on Syrian bryophytes, which has historically been confined to morphological descriptions, this study provides a taxonomic and genetic survey of moss species from the ecologically contrasting regions of Al-Zabadani and Al-Haffah. The study involves identification using bryological taxonomic keys, complemented by a molecular analysis using RAPD markers to determine interspecific genetic relationships. The morphological documentation will subsequently be compared with cluster analysis results from the genetic data to establish a unified classification.

2. Materials and methods

2.1. Sample Collection

Mosses samples were collected from Al-Zabadani "Damascus countryside" (33°06'62"N 36°08'66"E) and Al-Haffah "Latakia" (35°05'58"N 36°05'72"E) regions in Syria during multiple field trips between January and April 2023 (Figure 1), coinciding with the annual growth and spread gametophyte generations of mosses associated with suitable precipitation and temperature conditions. Samples were then transferred to the laboratory and stored.



Figure 1. General location of the studied areas (Al-Zabadani and Al-Haffah regions).

2.2. Morphological and taxonomic study

Morphological analyses were conducted at the Plant Biology Department, University of Damascus. Species identification was performed using light macroscopy and microscopy, with taxonomic classification based on morphological and anatomical characteristics commonly used in bryophytes classification, including leaf and costa length, leaf margins, capsule shape, stem branching, and more. The observed features were systematically compared with standard bryological taxonomic keys and literature (Kurschner, 2006, 2007, 2008; Goffinet and

Shaw, 2008). Twelve morphological parameters were evaluated using SPSS V22 (fifty replicates each). The quantified characteristics encompassed sporophyte dimensions (sporophyte plant, seta, and capsule length), leaf length, costa length, costa width, and cellular characteristics (basal, lamina, and apical cell measurements, including length, and length-to-width ratios), as these morphological criteria are commonly used in classification.

2.3. Molecular study (Molecular Characterization)

The molecular study was conducted to investigate the genetic relationships and species clustering. Molecular analysis was carried out in the Graduate Studies Laboratory (Department of Plant Biology, Faculty of Science, Damascus University), using RAPD technique, as follows:

2.3.1. DNA extraction

Samples were thoroughly cleaned with distilled water, and old parts (brown parts) were removed. DNA extraction was performed using CTAB method, as described by Skotnicki *et al.* (2005), with minor modifications. A freshly prepared 1% CTAB extraction solution was used, prepared as follows: 2.5 mL of 1M Tris-HCl, 2.5 mL of 0.5M EDTA, 3.5 mL of 5M NaCl, 250 mg of CTAB, 250 mg of PVP, and 25 μ L of β -mercaptoethanol, with the final volume adjusted to 25 mL using distilled water (Table 1).

Table 1. Composition and final concentrations of the 1% CTAB extraction buffer.

Material	Volume/Weight for 25 mL	Final concentrations
Tris-HCl (1M) - pH:7.5	2.5 mL	100 mM
EDTA (0.5 M) - pH: 8	2.5 mL	50 mM
NaCl (5M)	3.5 mL	1.4 M
CTAB	250 mg	1%
PVP	250 mg	1%
β -mercaptoethanol	25 μ L	0.1%
Distilled water	Add to 25 mL	

For acrocarpous species, DNA was extracted using single shoots to avoid contamination from species overlap within moss mats, with each species' individual placed in a separate tube (Figure 2). For pleurocarpous species, where there is no risk of interspecies interference, DNA extraction was performed using 100 mg of material per species.



Figure 2. Samples of single shoots of some acrocarpous species in Eppendorf tubes.

2.3.2. Quantitative and Purity Analysis of DNA

DNA concentration and purity were assessed using a UV-Visible spectrophotometer (Mecasys - Korea). Absorbance was measured at 260 nm (OD_{260}) and 280 nm (OD_{280}), with concentration calculated according to the standard formula (Bessey, 2014): DNA Concentration (μ g/mL) = $OD_{260} \times 50 \times$ Dilution Factor, while DNA purity was evaluated using the OD_{260}/OD_{280} ratio, with values

between 1.8 and 2.0 indicating high purity DNA. To verify these measurements, DNA concentration was additionally quantified using a NanoDrop 2000 spectrophotometer "Thermo Fisher Scientific" (Shen *et al.*, 2022).

2.3.3. Amplification Using RAPD Technique

The Random Amplified Polymorphic DNA (RAPD) technique was employed for molecular characterization, genetic distance estimation among collected moss species, as well as for cluster analysis. DNA amplification was performed using ten randomly selected primers from Alpha DNA (Operon), as detailed in Table 2.

Table 2. RAPD Primers used for Polymerase Chain Reaction (PCR) amplification:

Primer Number	Primer Code	Sequences (5'-3')	Annealing Temperature ($^{\circ}$ C)
1	OP-Z14	TCGGAGGTTC	32
2	OP-R15	GGACAACGAG	32
3	OP-R11	GTAGCCGTCT	32
4	OP-R07	ACTGGCCTGA	32
5	OP-E12	TTATCGCCCC	32
6	OP-E01	CCCAAGGTCC	34
7	OP-E18	GGACTGCAGA	32
8	OP-Z13	GACTAAGCCC	32
9	OP-R04	CCCGTAGCAC	34
10	OP-R08	CCCGTTGCCT	34

RAPD reactions were conducted using GoTaq® Green Master Mix (2X) (Promega, USA) in a 25 μ L final reaction volume, with modifications introduced to the manufacturer's protocol to optimize reaction conditions. Each reaction contained: 12.5 μ L master mix, 2 μ L DNA template (approximately 100 ng μ L⁻¹), 1.5 μ L primer, 9 μ L distilled water. Reaction mixtures were overlaid with 40 μ L of sterile mineral oil to prevent evaporation during thermal cycling.

Amplification was conducted in a GenePro Thermocycler (BIOER, USA) programmed as follows:

- Initial denaturation: 94 $^{\circ}$ C for 3 minutes.
- Amplification: 45 cycles of:
 1. Denaturation: 94 $^{\circ}$ C for 1 minute.
 2. Primer annealing: 32-34 $^{\circ}$ C for 1 minute (primer specific).
 3. Elongation: 72 $^{\circ}$ C for 2 minutes.
- Final elongation: 72 $^{\circ}$ C for 10 minutes.

The annealing according temperature was optimized to the specific primers employed in this study (32 - 34 $^{\circ}$ C). It should be noted that annealing temperature varies depending on the nucleotide composition of individual primers.

2.3.4. Electrophoresis of amplification products

Amplification products were separated on a 1.8% (w/v) agarose gel in 1X TBE buffer containing 5 μ L of ethidium bromide (10 mg/mL). Samples were electrophoresed at 100 volts for 2 hours (Electrophoresis Power Supply-Consort EV243) in TBE buffer. DNA bands were visualized by staining with ethidium bromide and photographed using a gel documentation system (Cleaver Scientific - UK) equipped with a UV filter. Additionally, 5 μ L of a 100 bp DNA ladder (GeneDirex - USA) was loaded onto the gel as a molecular weight standard. Following amplification,

the resulting DNA profiles were examined under UV light and photographed for analysis.

2.4. Statistical study

Statistical analysis of the morphological parameters for the all species was performed using SPSS V22 software. Means were calculated, and differences between them were assessed for statistical significance using a One-Way ANOVA test, with a significance level of $P < 0.05$ considered statistically significant at a 95% confidence level. Additionally, a dendrogram was constructed based on morphological criteria using SPSS V22 (fifty replicates each).

The Polymorphism Information Content (PIC) represents a fundamental metric for assessing a genetic marker's ability to detect polymorphisms, thereby serving as a critical tool in marker selection for genetic research (Kulibaba *et al.*, 2025). PIC is calculated using the established formula: $PIC_i = 2f_i(1-f_i)$, where PIC_i represents the degree of variation between species for molecular index i , f_i represents the frequency of bands presence, while $1-f_i$ corresponds to the frequency of band absence. A dendrogram was constructed using the Jaccard similarity coefficient with the paired groups algorithm (UPGMA) in Past Statistical Software Version 1.92. Our team manually scored only the clear and reproducible amplification bands, with multiple observers independently visually inspecting and cross-checking the results to minimize bias. Each band was manually recorded as present (1) or absent (0) without using automated scoring software. We then built a data matrix from these binary scores and performed a cluster analysis to construct a genetic similarity dendrogram (Mokrani and Al-Ashkar, 2022).

3. Results

3.1. Climate study

Climate data from 2010 to 2022 indicated that the average relative humidity in Al-Haffah ranged from 62% to 76%, with a total precipitation of 998 mm, and a temperature average varying between 10°C and 27°C. The actual dry period during summer season lasted for three months. However, in Al-Zabadani, precipitation totaled approximately 662 mm, with an average temperature ranging from 4°C to 22°C. Relative humidity varied between 46% and 76%. The actual dry period during summer season extended to five months. These values are consistent with several local studies for both regions (Ismaeel, 2017; Saeed and Hasso, 2021).

3.2. Classification study

This study aims to complement previous research on mosses in Syria, with a specific focus on the Al-Zabadani (Damascus countryside Governorate) and Al-Haffah (Latakia Governorate) regions. A comprehensive morphological analysis was conducted for all collected specimens, with classification based on standardized morphological and anatomical criteria as detailed in the Materials and Methods. Taxonomic identification was verified through comparison with established taxonomic keys and relevant literature.

The investigation revealed that all identified species belong to the class Bryopsida, comprising sixteen distinct species: ten acrocarpous species and six pleurocarpous mosses. Distribution analysis showed twelve species in Al-Zabadani and eleven in Al-Haffah, with several species common to both regions (Tables 3-4).

Table 3. Categorization of species based on their distribution in the study areas.

Study areas	Species	No.
Al-Zabadani region-specific species	<i>Barbula unguiculata</i> Hedw.	1
	<i>Didymodon fallax</i> (Hedw.) R.H.Zander	2
	* <i>Amblystegium riparium</i> (Hedw.) Schimp.	3
	* <i>Hygroamblystegium tenax</i> (Hedw.) Jenn.	4
	* <i>Brachythecium erythrorrhizon</i> Schimp.	5
Species common to both regions	<i>Grimmia pulvinata</i> (Hedw.) Sm.	6
	<i>Schistidium apocarpum</i> (Hedw.) Bruch & Schimp.	7
	<i>Syntrichia ruralis</i> (Hedw.) F.Weber & D.Mohr	8
	<i>Bryum argenteum</i> Hedw.	9
	<i>Bryum caespitium</i> Hedw.	10
	<i>Bryum capillare</i> Hedw.	11
	* <i>Eurhynchium praelongum</i> (Hedw.) Schimp.	12
Al-Haffah region-specific species	<i>Timmiella anomala</i> (Bruch & Schimp.) Limpr.	13
	<i>Tortella fragilis</i> (Drumm.) Limpr.	14
	* <i>Leptodictyum riparium</i> (Hedw.) Warnst.	15
	* <i>Rhynchostegium murale</i> (Hedw.) Schimp.	16

The asterisk (*) denotes pleurocarpous species.

Table 4. The species distributed in the two study regions.



Didymodon fallax



Barbula unguiculata



Schistidium apocarpum



Grimmia pulvinata



Bryum argenteum



Tortella fragilis



Timmiella anomala



Syntrichia ruralis



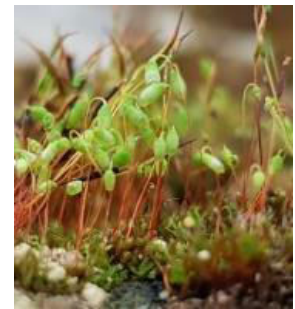
Hygroamblystegium tenax



Amblystegium riparium



Bryum capillare



Bryum caespiticium



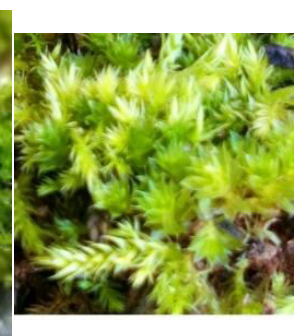
Rhynchosstegium murale



Eurhynchium praelongum



Brachythecium erythrorrhizon



Leptodictyum riparium

The species distribution between sites is presented below, where the equals symbol (=) indicates taxonomic synonyms:

- Species found in Al-Zabadani region
 1. *Grimmia pulvinata* (Hedw.) Sm.
 2. *Schistidium apocarpum* (Hedw.) Bruch & Schimp.
 3. *Barbula unguiculata* Hedw.
 4. *Didymodon fallax* (Hedw.) R.H.Zander
= *Geheebia fallax* (Hedw.) R.H.Zander
 5. *Syntrichia ruralis* (Hedw.) F.Weber & D.Mohr
 6. *Bryum argenteum* Hedw.
 7. *Bryum caespitium* Hedw.
= *Ptychostomum imbricatum* (Müll.Hal.) Holyoak & N.Pedersen
 8. *Bryum capillare* Hedw.
= *Rosulabryum capillare* (Hedw.) J.R.Spence
 9. *Amblystegium riparium* (Hedw.) Schimp.
= *Leptodictyum riparium* (Hedw.) Warnst.
 10. *Hygroamblystegium tenax* (Hedw.) Jenn.
 11. *Brachythecium erythrorrhizon* Schimp.
 12. *Eurhynchium praelongum* (Hedw.) Schimp.
= *Kindbergia praelonga* (Hedw.) Ochyra

Species found in Al-Haffah region

1. *Grimmia pulvinata* (Hedw.) Sm.
2. *Schistidium apocarpum* (Hedw.) Bruch & Schimp.
3. *Syntrichia ruralis* (Hedw.) F.Weber & D.Mohr
4. *Timmia anomala* (Bruch & Schimp.) Limpr.
5. *Tortella fragilis* (Drumm.) Limpr.
6. *Bryum argenteum* Hedw.
7. *Bryum caespitium* Hedw.
= *Ptychostomum imbricatum* (Müll.Hal.) Holyoak & N.Pedersen
8. *Bryum capillare* Hedw.
= *Rosulabryum capillare* (Hedw.) J.R.Spence
9. *Leptodictyum riparium* (Hedw.) Warnst.
10. *Eurhynchium praelongum* (Hedw.) Schimp.
= *Kindbergia praelonga* (Hedw.) Ochyra
11. *Rhynchostegium murale* (Hedw.) Schimp.

The statistical study was conducted using morphological criteria with 12 parameters and 50 replicates (as previously mentioned), employing One-way ANOVA at a significance level of $P < 0.05$, indicating statistical significance at a 95% confidence level. Our statistical analysis revealed clear morphological divergence between pleurocarpous and acrocarpous species (see table 5 for pleurocarpous; tables 6-7 for acrocarpous), underscoring the distinct phenotypic profiles that characterize these two growth forms.

Table 5. Statistical study of morphological criteria for pleurocarpous species.

Species	S.P.L/ mm	S.L/ mm	C.L/ mm	L.L/ mm	CO.L/ mm	CO.W/ μ m
<i>Hygro. tenax</i> -Z	16.5 $\pm$ 0.71 ^a	14.5 $\pm$ 0.72 ^a	2 $\pm$ 0.14 ^a	1.85 $\pm$ 0.1 ^a	1.5 $\pm$ 0.11 ^a	78 $\pm$ 4.89 ^a
<i>A. riparium</i> -Z	20.7 $\pm$ 1.61 ^b	18.2 $\pm$ 1.59 ^b	2.4 $\pm$ 0.11 ^b	2.16 $\pm$ 0.11 ^b	1.3 $\pm$ 0.1 ^b	46 $\pm$ 4.88 ^b
<i>L. riparium</i> -L	17.3 $\pm$ 1.26 ^c	15.8 $\pm$ 1.24 ^c	1.5 $\pm$ 0.12 ^c	1.42 $\pm$ 0.05 ^c	1.1 $\pm$ 0.08 ^c	47 $\pm$ 7.23 ^b
<i>B. erythrorrhizon</i> -Z	13.6 $\pm$ 0.8 ^d	11.5 $\pm$ 0.83 ^d	2.1 $\pm$ 0.18 ^d	2.43 $\pm$ 0.18 ^d	1.2 $\pm$ 0.11 ^d	48 $\pm$ 4.77 ^b
<i>R. murale</i> -L	20.1 $\pm$ 1.7 ^e	17.8 $\pm$ 1.72 ^e	2.4 $\pm$ 0.15 ^e	1.60 $\pm$ 0.08 ^e	1.2 $\pm$ 0.1 ^b	56 $\pm$ 4.99 ^c
<i>E. praelongum</i> -Z	14.6 $\pm$ 1.52 ^f	12.7 $\pm$ 1.51 ^f	1.9 $\pm$ 0.12 ^f	1.15 $\pm$ 0.09 ^f	1.1 $\pm$ 0.12 ^c	47 $\pm$ 5.71 ^b
<i>E. praelongum</i> -L	12.1 $\pm$ 0.73 ^g	10.4 $\pm$ 0.74 ^g	1.8 $\pm$ 0.1 ^g	1.34 $\pm$ 0.06 ^g	1 $\pm$ 0.06 ^c	52 $\pm$ 4.06 ^d
Species	B.C.L/ μ m	B.C.L/B.C.W	L.C.L/ μ m	L.C.L/L.C.W	A.C.L/ μ m	A.C.L/A.C.W
<i>Hygro. tenax</i> -Z	39.6 $\pm$ 3.8 ^a	7.14 $\pm$ 1.4 ^a	72.3 $\pm$ 3.72 ^a	13.4 $\pm$ 1.94 ^a	56.4 $\pm$ 5 ^a	9.3 $\pm$ 2.01 ^a
<i>A. riparium</i> -Z	69 $\pm$ 5.08 ^b	6 $\pm$ 0.94 ^b	91.7 $\pm$ 4.33 ^b	11.7 $\pm$ 1.42 ^b	71.6 $\pm$ 5.58 ^b	9.5 $\pm$ 2.86 ^{a,b}
<i>L. riparium</i> -L	61 $\pm$ 5.82 ^c	9 $\pm$ 1.72 ^c	105.6 $\pm$ 7.1 ^c	19.3 $\pm$ 3.33 ^c	71.8 $\pm$ 4.4 ^b	14.2 $\pm$ 3.33 ^c
<i>B. erythrorrhizon</i> -Z	61.3 $\pm$ 4.55 ^c	8.1 $\pm$ 1.84 ^d	98 $\pm$ 4.74 ^d	14.1 $\pm$ 2.49 ^a	72.3 $\pm$ 4.22 ^b	10.2 $\pm$ 2.24 ^b
<i>R. murale</i> -L	53.5 $\pm$ 9.15 ^d	10.6 $\pm$ 2.29 ^c	75 $\pm$ 4.49 ^c	16.2 $\pm$ 2.79 ^d	32.9 $\pm$ 6.8 ^c	5.4 $\pm$ 1.69 ^d
<i>E. praelongum</i> -Z	26.7 $\pm$ 4.3 ^c	3.9 $\pm$ 1 ^f	44.1 $\pm$ 4.74 ^f	8.1 $\pm$ 1.57 ^c	33.4 $\pm$ 2.85 ^c	5.1 $\pm$ 0.95 ^d
<i>E. praelongum</i> -L	19.9 $\pm$ 2.74 ^f	1.8 $\pm$ 0.36 ^g	34 $\pm$ 4.49 ^g	8.8 $\pm$ 3.45 ^c	25.6 $\pm$ 4.78 ^d	4.3 $\pm$ 1.15 ^c

(S.P.L: sporophyte Plant length, S.L: seta length, C.L: capsule length, L.L: leaf length, CO.L: costa length, CO.W: costa width, B.C.L: length of basal cells in the leaf, B.C.L/B.C.W: ratio of basal cells length to width, L.C.L: length of lamina cells in the leaf, L.C.L/L.C.W: ratio of lamina cells length to width, A.C.L: length of apical cells in the leaf, A.C.L/A.C.W: ratio of apical cells length to width)

Table 6. Statistical study of some morphological criteria for acrocarpous species (1).

Species	S.P.L/mm	S.L/mm	C.L/mm	L.L/mm	CO.L/mm	CO.W/ μ m
<i>S. ruralis</i> -L	19.7 $\pm$ 0.85 ^a	16.4 $\pm$ 0.77 ^a	3.31 $\pm$ 0.28 ^a	3.31 $\pm$ 0.17 ^a	3.31 $\pm$ 0.17 ^a	56 $\pm$ 4.82 ^a
<i>S. ruralis</i> -Z	22 $\pm$ 1.73 ^b	17.1 $\pm$ 1.52 ^b	4.94 $\pm$ 0.48 ^b	4.14 $\pm$ 0.22 ^b	4.14 $\pm$ 0.22 ^b	52 $\pm$ 4.94 ^a
<i>To. fragilis</i> -L	12.5 $\pm$ 0.65 ^c	10.5 $\pm$ 0.62 ^c	2.08 $\pm$ 0.16 ^c	2.27 $\pm$ 0.12 ^c	2.27 $\pm$ 0.11 ^c	75 $\pm$ 4.95 ^b
<i>Bar. unguiculata</i> -Z	21.4 $\pm$ 1.09 ^b	16.5 $\pm$ 0.99 ^a	4.9 $\pm$ 0.79 ^b	2.86 $\pm$ 0.19 ^d	2.86 $\pm$ 0.19 ^d	90 $\pm$ 7.56 ^c
<i>Ti. anomala</i> -L	23.5 $\pm$ 2.11 ^d	18.2 $\pm$ 2.04 ^d	5.3 $\pm$ 0.35 ^d	4.48 $\pm$ 0.21 ^c	4.48 $\pm$ 0.21 ^c	212 $\pm$ 9.15 ^d
<i>D. fallax</i> -Z	20.2 $\pm$ 0.97 ^a	17.6 $\pm$ 0.93 ^b	2.59 $\pm$ 0.26 ^c	2.42 $\pm$ 0.12 ^f	2.42 $\pm$ 0.12 ^f	102 $\pm$ 15.9 ^c
<i>Sch. apocarpium</i> -L	3.7 $\pm$ 0.15 ^e	1.4 $\pm$ 0.1 ^e	2.31 $\pm$ 0.09 ^f	2.59 $\pm$ 0.2 ^g	2.59 $\pm$ 0.2 ^g	77 $\pm$ 6.56 ^b
<i>Sch. apocarpium</i> -Z	2.6 $\pm$ 0.1 ^f	1.1 $\pm$ 0.09 ^e	1.52 $\pm$ 0.06 ^g	2.47 $\pm$ 0.16 ^f	2.47 $\pm$ 0.16 ^f	80 $\pm$ 7.62 ^b
<i>G. pulvinata</i> -L	5.3 $\pm$ 0.38 ^g	3.8 $\pm$ 0.33 ^f	1.54 $\pm$ 0.12 ^g	2.44 $\pm$ 0.21 ^f	2.44 $\pm$ 0.21 ^f	60 $\pm$ 5.56 ^a
<i>G. pulvinata</i> -Z	5 $\pm$ 0.32 ^g	3.1 $\pm$ 0.24 ^g	1.91 $\pm$ 0.14 ^h	2.26 $\pm$ 0.17 ^c	2.26 $\pm$ 0.17 ^c	59 $\pm$ 5.52 ^a
<i>Bry. caespitium</i> -L	16.1 $\pm$ 0.7 ^h	14 $\pm$ 0.676 ^h	2.06 $\pm$ 0.12 ^c	1.32 $\pm$ 0.05 ^h	1.32 $\pm$ 0.05 ^h	55 $\pm$ 4.99 ^a
<i>Bry. caespitium</i> -Z	19.5 $\pm$ 1.1 ^a	17.2 $\pm$ 1.1 ^b	2.3 $\pm$ 0.18 ^f	1.52 $\pm$ 0.13 ⁱ	1.52 $\pm$ 0.13 ⁱ	85 $\pm$ 7.3 ^f
<i>Bry. capillare</i> -L	24.9 $\pm$ 1.62 ⁱ	20.7 $\pm$ 1.54 ⁱ	4.15 $\pm$ 0.23 ⁱ	3.31 $\pm$ 0.19 ^a	3.31 $\pm$ 0.19 ^a	111 $\pm$ 13.52 ^g
<i>Bry. capillare</i> -Z	25 $\pm$ 3.32 ⁱ	20.7 $\pm$ 3.27 ⁱ	4.31 $\pm$ 0.26 ^j	2.89 $\pm$ 0.19 ^d	2.6 $\pm$ 0.17 ^g	127 $\pm$ 9 ^h
<i>Bry. argenteum</i> -L	16.1 $\pm$ 1.65 ^h	14.7 $\pm$ 1.64 ^j	1.44 $\pm$ 0.16 ^g	0.99 $\pm$ 0.03 ^j	0.6 $\pm$ 0.02 ^j	52 $\pm$ 3.91 ^a
<i>Bry. argenteum</i> -Z	16 $\pm$ 1.83 ^h	14 $\pm$ 1.82 ^h	1.98 $\pm$ 0.26 ^{c,h}	0.94 $\pm$ 0.07 ^j	0.56 $\pm$ 0.04 ^j	48 $\pm$ 5.25 ⁱ

(S.P.L: sporophyte Plant length, S.L: seta length, C.L: capsule length, L.L: leaf length, CO.L: costa length, CO.W: costa width)

Table 7. Statistical study of some morphological criteria for acrocarpous species (2).

Species	B.C.L/ μ m	B.C.L/B.C.W	L.C.L/ μ m	L.C.L/L.C.W	A.C.L/ μ m	A.C.L/A.C.W
<i>S. ruralis</i> -L	52.7 $\pm$ 7.99 ^a	3.5 $\pm$ 0.46 ^a	11.9 $\pm$ 1.19 ^a	1.15 $\pm$ 0.17 ^a	9.7 $\pm$ 1.3 ^a	1.11 $\pm$ 0.21 ^a
<i>S. ruralis</i> -Z	48.5 $\pm$ 5 ^b	2.9 $\pm$ 0.54 ^b	11.7 $\pm$ 1.4 ^a	1.1 $\pm$ 0.16 ^a	12.4 $\pm$ 1.71 ^b	1.2 $\pm$ 0.17 ^{a,b}
<i>To. fragilis</i> -L	52.8 $\pm$ 6.38 ^a	6.2 $\pm$ 1.22 ^c	8.1 $\pm$ 1.08 ^b	1.09 $\pm$ 0.26 ^a	7.7 $\pm$ 0.79 ^c	1.1 $\pm$ 0.27 ^a
<i>Bar. unguiculata</i> -Z	24.3 $\pm$ 2.58 ^c	1.8 $\pm$ 0.22 ^{d,f}	14.4 $\pm$ 1.11 ^{c,d}	1.2 $\pm$ 0.19 ^a	12.3 $\pm$ 1.1 ^b	1.2 $\pm$ 0.14 ^{a,b}
<i>Ti. anomala</i> -L	39.2 $\pm$ 2.24 ^d	3.4 $\pm$ 0.41 ^a	6.5 $\pm$ 1.15 ^b	1.15 $\pm$ 0.28 ^a	7.4 $\pm$ 0.85 ^c	1.2 $\pm$ 0.27 ^{a,b}
<i>D. fallax</i> -Z	14.8 $\pm$ 3.62 ^c	1.6 $\pm$ 0.51 ^d	11.3 $\pm$ 1.62 ^a	1.45 $\pm$ 0.33 ^b	8.4 $\pm$ 1.73 ^c	1.3 $\pm$ 0.4 ^b
<i>Sch. apocarpium</i> -L	30.7 $\pm$ 3.71 ^f	2.7 $\pm$ 0.39 ^c	11.7 $\pm$ 1.69 ^a	1.3 $\pm$ 0.23 ^c	12.2 $\pm$ 1.62 ^b	1.3 $\pm$ 0.27 ^b
<i>Sch. apocarpium</i> -Z	21.6 $\pm$ 2.52 ^g	1.9 $\pm$ 0.27 ^f	7.8 $\pm$ 0.8 ^b	1.14 $\pm$ 0.22 ^a	11 $\pm$ 1.17 ^{a,b}	1.3 $\pm$ 0.2 ^b
<i>G. pulvinata</i> -L	33.7 $\pm$ 3.68 ^{h,i}	2.5 $\pm$ 0.33 ^e	12.1 $\pm$ 1.18 ^a	1.1 $\pm$ 0.17 ^a	10.1 $\pm$ 1 ^a	1.1 $\pm$ 0.19 ^a
<i>G. pulvinata</i> -Z	23.1 $\pm$ 2.89 ^{c,g}	2.1 $\pm$ 0.36 ^g	13.1 $\pm$ 1.29 ^d	1.2 $\pm$ 0.18 ^a	10 $\pm$ 0.99 ^a	1.1 $\pm$ 0.17 ^a
<i>Bry. caespitium</i> -L	40.4 $\pm$ 4.96 ^d	2.6 $\pm$ 0.51 ^e	61.8 $\pm$ 7.88 ^c	5.3 $\pm$ 0.85 ^d	46.3 $\pm$ 3.92 ^d	5.7 $\pm$ 0.71 ^c
<i>Bry. caespitium</i> -Z	34.5 $\pm$ 2.83 ⁱ	2.3 $\pm$ 0.29 ^g	58.8 $\pm$ 4.77 ^f	6 $\pm$ 1.1 ^e	42.7 $\pm$ 2.39 ^e	5 $\pm$ 0.73 ^d
<i>Bry. capillare</i> -L	75.4 $\pm$ 5.94 ^j	3.8 $\pm$ 0.43 ^h	57.1 $\pm$ 9.06 ^g	2.6 $\pm$ 0.47 ^f	49.2 $\pm$ 5.24 ^f	2.7 $\pm$ 0.36 ^e
<i>Bry. capillare</i> -Z	74.8 $\pm$ 5.42 ^j	4.1 $\pm$ 0.66 ⁱ	63.8 $\pm$ 4.11 ^h	3.3 $\pm$ 0.43 ^g	63.3 $\pm$ 5.4 ^g	3.2 $\pm$ 0.47 ^f
<i>Bry. argenteum</i> -L	31.5 $\pm$ 2.91 ^{f,h}	1.8 $\pm$ 0.25 ^{d,f}	55.1 $\pm$ 5.93 ⁱ	5.5 $\pm$ 0.97 ^h	66.3 $\pm$ 3.24 ^h	5.3 $\pm$ 0.71 ^g
<i>Bry. argenteum</i> -Z	43.8 $\pm$ 5.06 ^k	3.6 $\pm$ 0.64 ^a	52.1 $\pm$ 4.1 ^j	5 $\pm$ 0.83 ⁱ	64.9 $\pm$ 6.57 ⁱ	5.8 $\pm$ 1.05 ^c

(B.C.L: length of basal cells in the leaf, B.C.L/B.C.W: ratio of basal cells length to width, L.C.L: length of lamina cells in the leaf, L.C.L/L.C.W: ratio of lamina cells length to width, A.C.L: length of apical cells in the leaf, A.C.L/A.C.W: ratio of apical cells length to width)

Hierarchical cluster analysis of morphological characters grouped the studied species into four distinct clusters, which corresponded directly to their taxonomic orders (Figure 3). The classification was as follows: the first group contained pleurocarpous mosses of the order Hypnales (*Amblystegium riparium*, *Leptodictyum riparium*, *Hygroamblystegium tenax*, *Brachythecium erythrorrhizon*, *Eurhynchium praelongum*, and

Rhynchostegium murale). The second group comprised two species of Grimmiales (*Grimmia pulvinata* and *Schistidium apocarpum*). The third group included five species of Pottiales (*Barbula unguiculata*, *Timmiella anomala*, *Tortella fragilis*, *Didymodon fallax*, and *Syntrichia ruralis*). Finally, the fourth group comprised three Bryales species: *Bryum argenteum*, *B. capillare*, and *B. caespitium*.

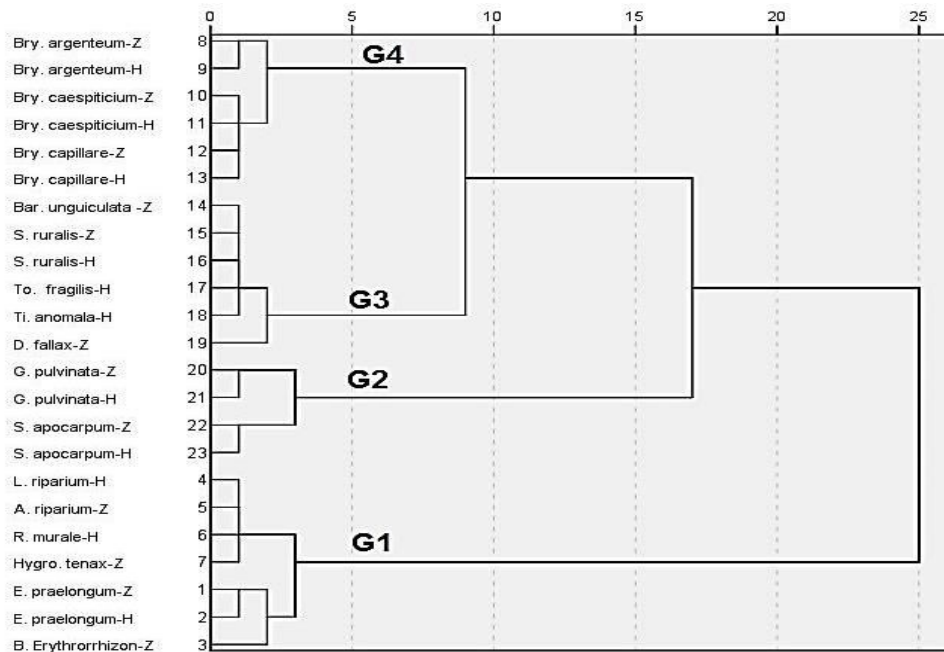


Figure 3. Dendrogram of the species based on morphological criteria using SPSS V22 (Hierarchical Clustering). (The letter at the end of each species name denotes the collection area: 'H' denotes Al-Haffah region and 'Z' denotes Al-Zabadani region).

3.3. Molecular analysis

Molecular characterization was conducted to study the genetic distance between moss species found in Al-Zabadani and Al-Haffah regions. DNA concentration in pleurocarpous moss species was higher when compared to acrocarpous species, with values exceeding $100 \text{ ng.}\mu\text{l}^{-1}$ for all pleurocarpous species, while acrocarpous species had concentrations below $100 \text{ ng.}\mu\text{l}^{-1}$ (Table 8). This difference is attributed to the smaller amount of plant material from which DNA was isolated in acrocarpous species in comparison to pleurocarpous species. DNA samples had A260/280 ratios higher than 1.8, indicating high purity. Furthermore, the results confirmed that CTAB method was highly efficient for extracting DNA from moss samples, as supported by several previous studies (Schlink and Reski, 2002; Baraki *et al.*, 2018).

Table 8. DNA concentration and purity of the studied Moss Species.

Species		Purity	Concentration $\text{ng.}\mu\text{l}^{-1}$
Pleurocarpous moss species	<i>Rhynchostegium murale</i>	1.96	481.8
	<i>Eurhynchium praelongum</i>	1.82	341.8
	<i>Leptodictyum riparium</i>	2	130.1
	<i>Amblystegium riparium</i>	1.94	125.7
	<i>Brachythecium erythrorrhizon</i>	1.99	118.5
	<i>Hygroamblystegium tenax</i>	1.94	112
Acrocarpous moss species	<i>Grimmia pulvinata</i>	1.84	76.5
	<i>Bryum caespitium</i>	1.92	52.4
	<i>Bryum capillare</i>	1.94	48
	<i>Didymodon fallax</i>	1.78	44.6
	<i>Schistidium apocarpium</i>	1.98	36.1
	<i>Tortella fragilis</i>	1.81	35.7
	<i>Syntrichia ruralis</i>	1.78	26.3
	<i>Barbula unguiculata</i>	2.1	25.4
	<i>Bryum argenteum</i>	1.97	22.8
	<i>Timmia anomala</i>	1.84	19.5

We employed the Random Amplified Polymorphic DNA (RAPD) technique for molecular characterization. It was accomplished using 10 primers produced by Alpha DNA, all of which yielded reliable results in elucidating the genetic variation among the studied species. Figure 4 shows the electrophoresis results on a 1.8% agarose gel using primers OP-Z13 and OP-R11.

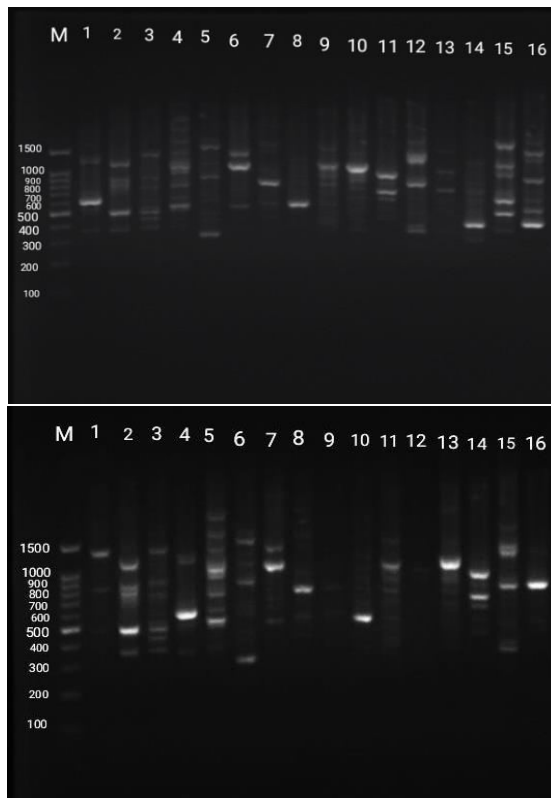


Figure 4. Gel Electrophoresis results using primers OP-Z13 and OP-R11.

(Molecular weights on the left, at the top the studied species as follows: 1: *A. riparium*, 2: *E. praelongum*, 3: *Sch. Apocarpium*, 4: *G. pulvinata*, 5: *Bar. unguiculata*, 6: *D. fallax*, 7: *Ti. anomala*, 8: *To. fragilis*, 9: *Bry. argenteum*, 10: *Bry. capillare*, 11: *R. murale*, 12: *Bry. caespitium*, 13: *Hygro. tenax*, 14: *S. ruralis*, 15: *L. riparium*, 16: *B. erythrorrhizon*)

Table 9 presents amplification results using RAPD, showing a total of 163 molecular markers, with an average of 16.3 markers per primer. The total number of bands was 701, averaging 70.1 bands per primer. The table also indicates that primer OP-R11 yielded the highest number of molecular markers, totaling 25 markers and 120 bands. However, primers OP-E18 and OP-E12 yielded the lowest number of molecular markers, with 12 markers each. PIC values were consistent across all primers, ranging from 0.28 to 0.37, with an average of 0.34. These results indicate the efficiency of RAPD technique as well as the primers employed in this study to reveal the genetic diversity among species.

Table 9. Number of molecular markers and bands yielded by each primer, with PIC values.

Num	Primer Code	Number of molecular markers	Number of Resulting Bands	PIC
1	OP-Z14	17	67	0.35
2	OP-R15	15	74	0.35
3	OP-R11	25	120	0.36
4	OP-R07	18	56	0.28
5	OP-E12	12	53	0.37
6	OP-E01	14	47	0.31
7	OP-E18	12	56	0.37
8	OP-Z13	19	118	0.36
9	OP-R04	14	57	0.33
10	OP-R08	17	53	0.29
Total		163	701	3.37
Average		16.3	70.1	0.34

Hierarchical cluster analysis (Dendrogram) was conducted using Past Version 1.92 software to determine genetic similarity or divergence among the studied species, based on the presence or absence of bands resulting from RAPD amplification. The dendrogram analysis revealed the distribution of species into five groups (clusters) according to their respective families (Figure 5). The first group included five Pottiaceae species: *Barbula unguiculata*, *Tortella fragilis*, *Timmiella anomala*, *Didymodon fallax*, and *Syntrichia ruralis*. The second group comprised three species of Bryaceae: *Bryum caespitium*, *B. capillare*, and *B. argenteum*. The third group included three species of pleurocarpous mosses belonging to Brachytheciaceae: *Eurhynchium praelongum*, *Rhynchostegium murale*, and *Brachythecium erythrorrhizon*. The fourth group also contained three species of pleurocarpous mosses, but belonging to Amblystegiaceae: *Leptodictyum riparium*, *Hygroamblystegium tenax*, and *Amblystegium riparium*. Finally, the fifth group consisted of two Grimmiaceae species: *Schistidium apocarpum* and *Grimmia pulvinata*.

Cluster analysis results of molecular and morphological data yielded congruent results, both grouping the studied species according to their established orders and families. While morphological clustering effectively delineated taxa at the ordinal level, molecular data provided finer resolution, clearly distinguishing species at the family level.

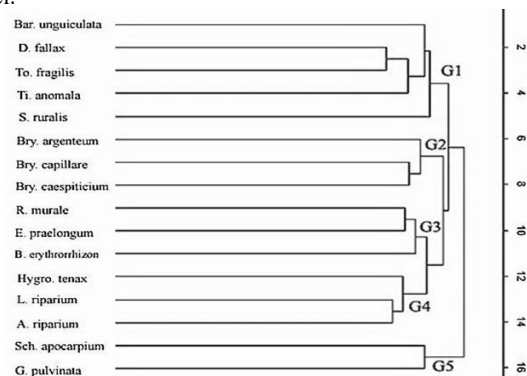


Figure 5. Dendrogram representing the studied species based on the Jaccard coefficient (paired groups algorithm).

4. Discussion

Syria's diverse landscapes provide rich habitats for a wide array of plant species, including bryophytes. However, the taxonomic study of Syrian mosses remains notably limited. This gap highlights the significant potential of regional surveys, especially in humid areas with conditions favorable for moss growth, to yield new species records and substantially enrich our understanding of the national flora.

4.1. Climatic Impact on Moss Distribution

Our analysis of climate data reveals a clear contrast between the two study regions. Al-Haffah experiences high humidity and extended rainfall for most of the year, with only a short three-month dry interval (Saeed and Hasso, 2021). In contrast, Al-Zabadani endures a more extended dry period of five months (Ismaeel, 2017). The significant differences in climate are directly mirrored by variations in moss diversity and abundance. The consistently humid conditions in Al-Haffah foster a significantly richer and more abundant moss community than the drier environment of Al-Zabadani; these findings align with Tizini *et al.* (2012) and Zhang *et al.* (2023), who identified moisture availability as a primary factor governing moss establishment and proliferation. Furthermore, as Al-Araj (2007) suggested, the favorable conditions "including optimal temperature, high humidity, and ample precipitation" enhance critical developmental stages like protonemal rhizoid formation, thereby supporting new gametophyte growth. The denser vascular plant canopy in Al-Haffah, also noted for its role in moisture retention and protection from solar radiation, creates a microclimate that further amplifies this effect (Al-Araj, 2007; Zhang *et al.*, 2023).

4.2. Morphological Diversity and Phenotypic Clustering

Statistical evaluation of the morphological data revealed significant phenotypic variation, not only among different species but also within populations of the same species across the two regions. This points to a strong environmental influence on morphological criteria, driven by factors like topography, rainfall, and humidity. Hierarchical clustering based on these morphological criteria grouped the species into four distinct clusters, which aligned well with established taxonomic orders. Hierarchical clustering based on these morphological traits grouped the species into four distinct clusters, which aligned well with established taxonomic orders. This congruence with standard morphological classifications validates the utility of our selected morphological criteria for reliable species grouping and differentiation (Goffinet and Shaw, 2008).

4.3. Genetic Variation and Molecular Marker Efficacy

Molecular characterization of moss species from Al-Zabadani and Al-Haffah regions revealed significant insights into their genetic variation. Our data consistently show that species adapted to dry environments contain minimal DNA, unlike their moisture-dependent relatives. This pattern indicates that a reduced genome has been selectively advantageous in arid habitats. Furthermore, the higher DNA yield we obtained from pleurocarpous species is likely a methodological artifact. Their distinctive growth

forms simply provide more biomass for extraction and should not be misinterpreted as evidence of a larger genome size (Li *et al.*, 2023). The high purity of DNA samples supports the effectiveness of CTAB method, which is consistent with previous studies that highlight its superiority over commercial kits in terms of both concentration and purity (Schlink and Reski, 2002). Our results confirm that RAPD is an effective method for detecting genetic variation in mosses. The primers produced a rich set of molecular markers, consistent with previous applications of this technique. For instance, Soni *et al.* (2014) successfully used RAPD to reveal genetic patterns in *Plagiochasma appendiculatum* across Himalayan elevation gradients. Similarly, Dale *et al.* (1999) demonstrated its effectiveness in detecting variation among *Hennediella heimii* populations from ecologically distinct regions. The molecular data grouped the species into five distinct genetic clusters, which largely matched their existing family level classifications. This agreement between DNA data and morphological characteristics also seen in studies on *Didymodon manhanensis* and *Syntrichia* spp. highlights how combining molecular methods with traditional taxonomy gives us a more complete picture (Gallego *et al.*, 2022; Feng *et al.*, 2022). Together, these approaches create a stronger foundation for defining species boundaries and understanding evolutionary relationships, leading to a more accurate classification system.

4.4. Broader Implications and Future Perspectives

Our results provide valuable insights into the geographic distribution patterns of mosses in Syria. The distinct genetic clusters found between the two regions support the idea that geographic isolation is a key driver in shaping moss biodiversity, which helps us better understand the evolutionary history of plants in the Mediterranean basin. While our findings are promising, they should be considered in light of some limitations. The relatively small number of sampling sites and limited sample sizes from certain populations may affect how broadly our results can be generalized. Furthermore, while the RAPD technique was effective for revealing genetic variation, newer high-resolution sequencing technologies (e.g., RAD-seq, SSR, ISSR) could provide a more detailed understanding of the genomic architecture underlying adaptation. Building on these results, we propose several promising future directions. First, a comprehensive national survey is urgently needed to fully document and map Syria's bryophyte diversity, especially in unexplored regions. Second, integrating our data with phylogeographic models would help reconstruct predict their responses to future environmental change.

5. Conclusion

This study addresses a critical gap in the floristic knowledge of Syria by providing the first comprehensive assessment of moss diversity across two distinct biogeographical regions, Al-Zabadani (Damascus countryside) and Al-Haffah (Latakia). Sixteen moss species were identified, and analysis revealed a clear correlation between their distribution and local environmental gradients, offering a baseline model for predicting bryophyte responses to ecological changes. The

high concordance between morphological and molecular cluster analyses validates the use of classical taxonomic methods while revealing the superior precision of molecular markers in distinguishing species at the family level. This dual-methodological approach provides taxonomists with a validated framework for accurate species identification, especially for morphologically variable taxa. For ecologists, the clear relationship between species distribution and environmental conditions provides a powerful tool for biomonitoring and assessing habitat quality. The successful application of the RAPD technique and the specific primers confirmed their high efficiency in detecting intraspecific genetic variation. The successful application of the RAPD technique and the specific primers confirmed their high efficiency in detecting intraspecific genetic variation. To build on these findings, future studies should broaden sampling to cover additional Syrian ecoregions and integrate additional molecular markers, such as ITS or rbcL. This will enable the construction of a comprehensive DNA barcode library for Syrian bryophytes, ultimately leading to a more robust phylogenetic understanding and effective conservation planning for this critical component of plant diversity.

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DNA damage in *Halophila stipulacea* as pollution bioindicator in the gulf of Aqaba/Jordan

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Abstract

The aim of this study is to evaluate genotoxicity in *Halophila stipulacea* (Forsk.) Aschers. found in the Gulf of Aqaba as a pollution bioindicator. We used Comet assay to assess the degree of DNA damage in *Halophila stipulacea* (Forsk.) Aschers. Samples were collected from various regions in the Gulf of Aqaba. The results showed significant differences in DNA damage score, index and frequency among the studied sites (Tala Bay, Old Phosphate Port, Marine Science Station (MSS), Big Bay and the Industrial area). Significantly, the highest DNA damage score and damage index were found in Old Phosphate Port (1.78 and 49.7 respectively). The highest DNA damage frequency was reported in Old Phosphate Port and Industrial area (82.3% and 82.9%, respectively). Samples collected from the industrial area showed Moderate DNA damage score (1.62). Samples collected from Big Bay, MSS, Tala Bay and Industrial area showed similar DNA damage index (39.7, 41.5, 42.3 and 43.7 respectively). The lowest DNA damage score was observed in MSS, Big Bay, and Tala Bay (0.62, 0.72 and 0.73 respectively), and the lowest DNA damage frequency was in MSS (47.9%). The highest MDA content was found in Industrial area (0.277 nmole g⁻¹ FM), and the lowest MDA content was in MSS (0.158 nmole g⁻¹ FM). In addition, analysis of heavy metals showed that plants collected from Industrial area and Old Phosphate Port had the highest Al, Cr, Fe, Cu, Zn, Cd and Pb contents. Significant positive correlation was found between DNA damage index, DNA damage frequency, lipid peroxidation rate and heavy metal analysis, which indicates that pollution with heavy metals is the main cause of genotoxicity for *Halophila stipulacea* (Forsk.) Aschers in gulf of Aqaba. Our results indicate that Tala Bay, Big Bay and Marine Science Station (MSS) are the least polluted areas in the gulf of Aqaba based on plant comet assay.

Keywords: *Halophila stipulacea*; seagrass; heavy metals; pollution; Aqaba; genotoxicity.

1. Introduction

Aqaba is the sole maritime port in Jordan, located on the Red Sea, 330 kilometers south of Amman. Its strategic location is at the connection of three continents: Asia, Europe and Africa. One of the most diverse aquatic habitats and biodiversity was found in the Gulf of Aqaba, as well as one of the world's greatest coral groups. The Gulf of Aqaba in the Middle East area is one of the most heavily anthropogenically influenced coastlines because the increase in Gulf industrial and commercial activities has led to the degradation of naturally stressed coastal and marine ecosystems. Anthropogenic activities have led to environmental impacts on unique marine communities such as household sewage, industrial waste, ports, dredging and reclamation works, and coastal development (Al-Taani et al, 2020, Al-Najjar, 2008).

Coastal environments across the world are progressively being affected by a variety of human activities, many of which result in high quantities of trace metals (Abu Hilal and Ismail, 2008). In recent years,

industrialization has resulted in an increase in water, air and soil pollution. Heavy Metal poisoning has become a problem in recent years due to its potential influence on human health as well as on the whole ecosystem (Siddiqui, 2015). Heavy metal bioaccumulation occurs at many structural levels in plants (cells, tissues and organs) (Chaplygin et al, 2022). Since the beginning of the industrial revolution, the quantity of metals produced by humans has increased. Metals can originate from a variety of sources in an urban area, including vehicle emissions, industrial discharges and weathered particles (Al-Khashman, 2007). These metals are harmful not only to the health of fish, turtles and other marine animals that feed on polluted seagrass tissues but also to human health because trace metals are ingested through contaminated seafood (Mohammadi et al, 2019).

The negative impacts of marine pollution on the environment influence coral reefs and seagrass ecosystems, human health, food security, and livelihoods (Riechers et al, 2021). The direct interaction of pollutants with DNA causes genotoxic consequences and genome instability (Theodorakis et al, 2001).

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Heavy metals can also interfere with DNA repair enzymes by modifying their protein structures or reducing their expression at the transcriptional level, potentially leading to chromosomal abnormalities (Whiteside et al, 2010). Therefore, bioindicators and biomonitoring systems are promising tools for studying the influence of external factors on ecosystem health and for pollution assessment. Genotoxicity is the term for the ability of chemical substances to change a cell's genetic information, possibly leading to genetic alterations. Genotoxicity is related to mutagenicity, with the exception that genotoxic impacts are often not linked to mutations.

Oxidative stress is a complicated process that involves chemical and physiological responses and occurs in all organisms including higher plants in response to excessive production and accumulation of reactive oxygen species. It occurs as a result of excess production and accumulation of reactive oxygen species (ROS). Genotoxicity refers to the capacity of certain substances to destroy genetic material. Genotoxicity refers to the ability of certain substances to harm genetic material. This does not only mean direct damage to DNA—it can also affect the proteins and structures that help DNA function properly inside the cell. For example, proteins involved in repairing DNA or regulating chromosomes condensation and separation are essential for healthy cell division. Likewise, structures such as the mitotic spindle can also be disrupted (Kumar et al, 2021).

A straightforward, popular, sensitive, adaptable and reliable technique for determining DNA damage is comet assay. The assay can be used to assess genotoxicity and detect the presence of genotoxins in the environment. Many researchers have used this technique to assess DNA damage since 1985. The genotoxic potential of heavy metals has been investigated using the plant comet assay. Plant comet assay is considered an extremely significant method used in ecotoxicology (Mishra, 2020). The comet test involves examining the electrophoretic mobility in a thin coating of agarose of extracted DNA from nucleoids following cell lysis. Sriussadaporn et al. (2003) evaluated DNA damage in Ginkgo trees. They found that environmental stress, increasing exposure time and roadside air pollutants lead to high degrees of DNA damage. In another study, Gómez-Arroyo et al. (2021) found that heavy metals lead to DNA damage in Moss (*Hypnum Amabile*). Liu et al. (2009) determined endosulfan's genotoxicity in Earthworms (*Eisenia foetida*) and White Clover (*Trifolium repens* L.) by using a comet assay. They found that exposure to different doses of endosulfan produces DNA strand breaks that were significantly higher than those in the control group. Al Khateeb (2018) used Comet assay to evaluate the level of DNA damage in a group of wild plants (*Citrullus colocynthis*, *Anabasis setifera*, *Prosopis juliflora*, *Prosopis farcta*, *Suaeda vermiculata*, *Ziziphus Spina-Christi*, *Capparis spinosa* and *Salsola vermiculata*) collected from different areas in Jordan. Results indicated that plants from the back roads of Aqaba and Ghour Assaal showed higher DNA damage levels. On the other hand, samples collected from Wadi Rum, Al Naqab Heights and Alshoneh showed low DNA damage levels. Although comet assay is widely applied in genetic toxicology for detecting DNA damage at the single-cell level, it does not provide information on long-term genetic effects or repair kinetics. Also, comet

assay lack specificity, as it cannot distinguish between different types of DNA damage, such as single-strand breaks, double-strand breaks, or alkali-labile sites, without additional modifications (Olive & Banáth, 2006).

Pollution causes DNA damage in many plants, including *Nicotiana tabacum*, *Allium cepa*, *Vicia faba* and *Tradescantia*. It has been shown that growing *Tradescantia* plants in heavy metals contaminated soil results in a 10–15 fold increase in DNA damage levels in comparison to plants grown in uncontaminated soil (Jaskulak et al, 2019). Radziemska et al. (2021) used *Pinus sylvestris* L. as a pollution bioindicator for railway transport in Poland. They found that rail transport is a source of pollution that inhibits seed germination and root growth. Lumberras et al. (2013) studied regional climate influence on the distribution of aquatic *Ranunculus* communities such as *R. peltatus* Schrank, *R. penicillatus* (Dumort.) Bab., *R. aquatilis* L. They found that Atlantic *ranunculus* species may be less adapted to habitat desiccation. Gomes et al. (2021) evaluated the effects of fluoride emissions on three plant species in Brazil: *Myrcogenia alpigena*, *Byrsonima variabilis* and *Eremanthus erythropappus*. They found that the phytotoxicity of fluoride depends on the stage of leaf development, genetic susceptibility and the concentration of the pollutant in the atmosphere. It has been shown that *Halophila stipulacea* (Forsk.) Aschers. grown on the phosphate mine dump in Aqaba had a substantial change in cell organization that resulted in leaf necrosis, swelling in the outer epidermal wall and chloroplast degradation (Wahsha et al, 2016).

Halophila stipulacea (Forsk.) Aschers. is a tiny tropical seagrass, native to the Red Sea. Seagrasses provide vital ecological functions such as the generation and storage of organic carbon, coastal protection through the stability of sediment and the creation of a necessary habitat for commercially valuable fish and crustaceans (Beca-Carretero et al, 2020). However, the loss of seagrass has been associated with a variety of negative outcomes such as decreased local fishing grounds, accelerated coastal erosion, destruction of related ecological populations and loss of primary productivity (Winters et al, 2020). The aim of this study was to evaluate using *Halophila stipulacea* (Forsk.) Aschers. as a biosensor for a potentially contaminated area of the Gulf of Aqaba by measuring DNA damage using a comet assay.

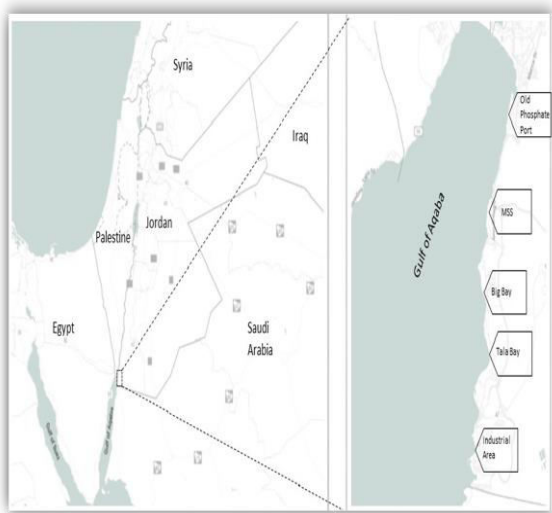
2. Materials and methods

2.1. Study area and sampling

Halophila stipulacea (Forsk.) Aschers. plants were collected from various regions in the Gulf of Aqaba (table 1) in March 2024. Samples were collected from Tala Bay, Old Phosphate Port, Marine Science Station (MSS), Big Bay and the Industrial area (figure 1). Plants with homogeneous sizes were chosen, and only plants without any apparent disease symptoms were collected. Before tissue examination, samples were washed with distilled water to get rid of unwanted debris. The youngest two leaves from each plant were used for comet assay analysis and the remaining leaves were used for heavy metals analysis and lipid peroxidation assay.

Table. 1: Collection sites and coordination for samples used in this study.

Sample number	Collection site	Coordinates
1	Tala Bay	Latitude: 29°31'45.67"N Longitude: 34°59'45.42"E
2	Old Phosphate Port	Latitude: 29°30'10.51"N Longitude: 34°59'30.02"E
3	MSS	Latitude: 29°27'31.51"N Longitude: 34°58'32.79"E
4	Big Bay	Latitude: 29°24'18.74"N Longitude: 34°58'26.86"E
5	Industrial area	Latitude: 29°22'22.26"N Longitude: 34°57'53.93"E

**Figure 1:** Samples collection sites in Gulf of Aqaba/Jordan.

2.2. Alkaline Comet assay

Plant samples were chopped with a razor blade in 1 ml of ice-cold Tris-MgCl₂ (pH=7.5). Then filtered and precipitated by centrifugation at 200 g for 5 min at 4°C to get the nuclei. The pellet was resuspended in 200 µl Tris-MgCl₂ buffer and kept on ice until used in the comet assay (Yıldız et al, 2009). To detect DNA damage in the plant cell, Oxiselect Comet Assay kit (cell Biolabs, Inc, san Diego, USA) was used. Lysis buffer, alkaline solution, and electrophoresis running solution were prepared and chilled at 4°C before performing the assay. Agarose was heated for 20 minutes at 90–95 °C before being cooled to 37 °C. Plant cell samples were prepared by centrifugation at 700 g, then 10 µl of cells were mixed with 90 µl of molten agarose, and then the mixture was placed onto the comet slide. Slides were set in the dark at 4°C for 15 min, then transferred to a basin containing pre-chilled lysis buffer (25ml/slide) and incubated in the dark at 4°C for 30–60 min, then the lysis solution was replaced with an alkaline solution for 30 min in the dark.

Prepared slides were transferred to the electrophoresis chamber and covered with cold electrophoresis buffer that was freshly prepared (300 mM NaOH, 1 mM EDTA, pH < 13). The run was started at 1 Volt/cm for 15–30 min, then slides were transferred carefully to the basin and washed twice with pre-chilled deionized water (DI) for 2 min for

each wash, then left to dry. After the addition of 100µL / well Vista green DNA dye, slides were incubated at room temperature for 15 minutes. After that, slides were examined under a fluorescence microscope (EVOS ®) at 100x magnification (GFP filter, 470 nm excitation, 525 nm emission).

To evaluate DNA damage index, cells were grouped into five classes depending on tail length (0: no tail, 25%: short tail, 50%: medium tail, 75%: long tail, and 100% very long tail length), and then the average of all cells from each site was calculated.

$$\text{DNA damage index} = \frac{\text{Number of cells in each group}}{\text{Number of group}}$$

Damage frequency (%) was calculated by dividing number of cells that were grouped as 25, 50, 75, or 100% by the total number of cells.

$$\text{DNA damage frequency (\%)} = \frac{\text{Number of cells in 25, 50, 75 and 100}}{\text{Total number of cells}} \times 100\%$$

Total number of cells

The experiment was repeated three times.

2.3. Heavy metal analysis

Samples of *Halophila stipulacea* (Forsk.) Aschers. were cleaned of debris by washing with distilled water before being dried at 104°C for 24 hours. Samples were heated at 520°C for 5 hours to assess ash content. In a conical flask, one gram of each powdered sample was weighed. For samples digestion, 8 ml of HNO₃ and 2 ml of perchloric acid (HClO₄) were mixed with samples (Arora et al., 2008). The samples were then placed in a water bath at 80°C for two to three hours, or until the solution was clear. Samples were digested, allowed to cool to room temperature, and then filtered using Whatman filter paper. Spectrophotometer was used for Heavy metals analysis depending on the atomic absorption. The experiment was repeated three times.

2.4. Lipid peroxidation content

To evaluate lipid peroxidation, malondialdehyde (MDA) content was measured following Yagi (1968). About 50 mg of fresh tissue was gently ground in a mortar and pestle in a solution of 0.5% 2-thiobarbituric acid (TBA) and 0.1% trichloroacetic acid (TCA). The mixture was then centrifuged at 4000 g for 15 minutes at 4 °C. The clear supernatant was heated in boiling water for 10 minutes and quickly cooled on ice bath. Then, the absorbance of the supernatant was measured at 532 and 600 nm using a UV-visible spectrophotometer. 0.1% TCA and 0.5% TBA were used as blank.

MDA content (expressed as nmol per gram of fresh tissue) was calculated using the formula:

$$\text{MDA content} = \frac{[(A_{532} - A_{600}) \times v] \times 1000}{(\epsilon \times M)}$$

where ϵ is the extinction coefficient (155 mM⁻¹ cm⁻¹), v is the volume of the extraction solution, M is the sample's fresh weight, and A_{532} and A_{600} are the absorbance readings at 532 and 600 nm, respectively.

2.5. Statistical analysis

SPSS version 16 for Windows was used in the analysis of the data. Data were presented as mean ± standard error from five or more biological replicates. Analysis of variance (ANOVA) and Tukey's test were used to evaluate

the significant differences among the studied sites. P value of less than 0.05 was considered statistically significant.

3. Results

3.1. DNA damage

Comet assay, also known as single-cell gel electrophoresis, was used to evaluate DNA damage in collected samples. A range of DNA damage levels (0 - 4) was used for DNA damage score, where score 0 represents a cell with no damage, while score 4 represents cells that are severely damaged. The score of each sample was calculated using the following equation:

$$\text{Score of DNA damage} = \frac{0 * N_0 + 1 * N_1 + 2 * N_2 + 3 * N_3 + 4 * N_4}{\text{Total number of cells}}$$

The letter N represents the number of cells in each level. Significant differences in DNA damage score ($p < 0.05$) were observed between *Halophila stipulacea* (Forsk.) Aschers. samples collected from different sites in the Gulf of Aqaba. Old Phosphate Port showed the highest score among other groups (1.78). Samples collected from Industrial area showed 1.62 damage score. On the other hand, Marine Science Station (MSS), Big Bay, and Tala Bay showed the lowest score (0.62, 0.72 and 0.73 respectively) (figure 2 and 3).

Results showed significant differences in damage frequency and damage index ($p < 0.05$) between *Halophila stipulacea* (Forsk.) Aschers. samples collected from different sites in Gulf of Aqaba (figure 4 and 5). The Old Phosphate Port showed the highest DNA damage index among other groups (49.7). On the other hand, (MSS), Big Bay, Tala Bay and Industrial area showed similar values (41.5, 39.7, 42.3 and 43.7 respectively). Samples collected from Old Phosphate Port and Industrial area showed the highest levels of DNA damage frequency (82.3% and 82.9% respectively). On the other hand, samples collected from MSS showed the lowest DNA damage frequency (47.9%).

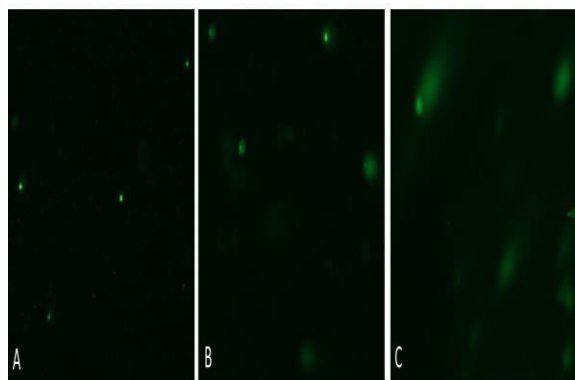


Figure 2: Comet assay for assessing DNA damage levels in *Halophila stipulacea* (Forsk.) Aschers. samples collected from the Gulf of Aqaba (No damage (A) intermediate damage (B) and high damage (C)).

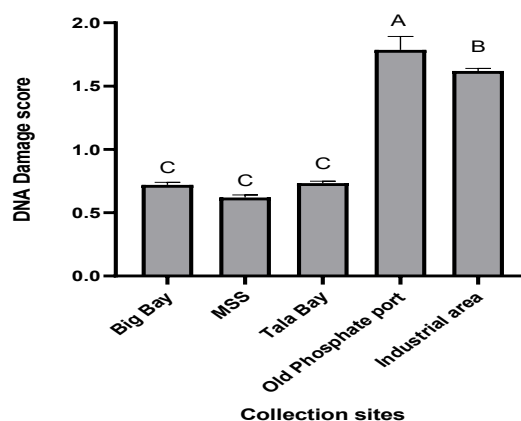


Figure 3. DNA damage score of *Halophila stipulacea* (Forsk.) Aschers. samples collected from different locations in the Gulf of Aqaba. Means with different letters are significantly different at ($p < 0.05$). Error bars represent standard error.

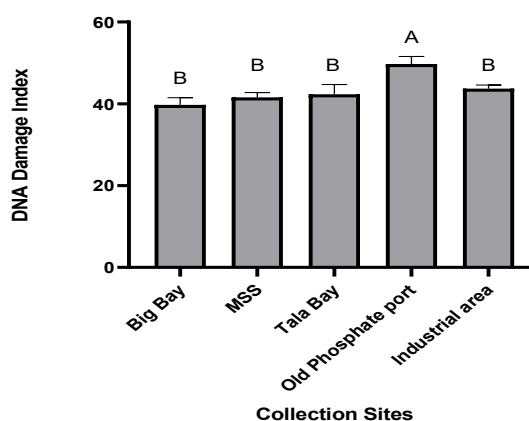


Figure 4. DNA Damage Index of *Halophila stipulacea* (Forsk.) Aschers. samples collected from different locations in the Gulf of Aqaba. Means with different letters are significantly different at ($p < 0.05$). Error bars represent standard error.

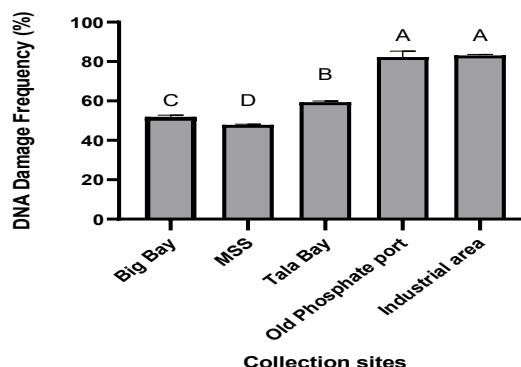


Figure 5. DNA Damage Frequency of *Halophila stipulacea* (Forsk.) Aschers. samples collected from different locations in the Gulf of Aqaba. Means with different letters are significantly different at ($p < 0.05$). Error bars represent standard error.

3.2. Lipid peroxidation level

Significant differences ($p < 0.05$) in Malondialdehyde (MDA) content were observed between *Halophila stipulacea* (Forsk.) Aschers. samples collected from different regions in Gulf of Aqaba (Figure 6). The

Industrial area showed the highest MDA content among other groups (0.277 nmole g⁻¹ FM). On the other hand, samples collected from Marine science station (MSS) showed the lowest MDA content (0.158 nmole g⁻¹ FM). Tala Bay, Old Phosphate Port and Big Bay samples showed intermediate values (0.217, 0.261 and 0.183 nmole g⁻¹ FM, respectively).

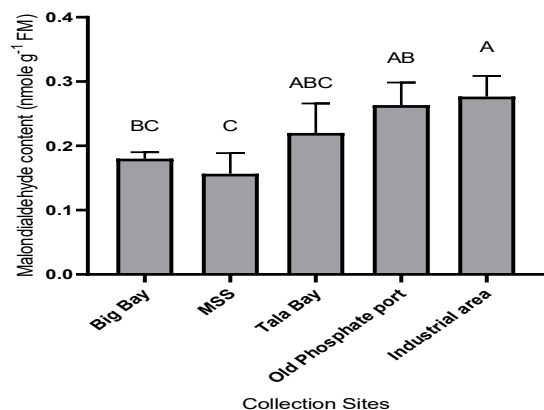


Figure 6. Total Malondialdehyde (MDA) content (nmole g⁻¹ FM) of *Halophila stipulacea* (Forsk.) Aschers. samples collected from different sites in the Gulf of Aqaba. Means with different letters are significantly different at ($p < 0.05$). Error bars represent standard error.

3.3. Heavy metals analysis

Significant differences ($p < 0.05$) in heavy metals content were observed between *Halophila stipulacea* (Forsk.) Aschers. samples collected from different sites in the Gulf of Aqaba (Figure 7). A high level of (Co) concentration in *Halophila stipulacea* (Forsk.) Aschers. was found in Old Phosphate Port samples (20.5 ppb), unlike Big Bay and MSS that showed the lowest concentrations (17.5 and 16.6 ppb respectively). Similar levels were found in Tala Bay and the Industrial Area samples (18.3 and 19.6 ppb respectively). A high level of (Cu) concentration in *Halophila stipulacea* (Forsk.) Aschers. was found in industrial areas (250.6 ppb), unlike in Big Bay and MSS that showed the lowest concentrations (136.9 and 104.4 ppb respectively). Similar content was observed in samples collected from Tala Bay and Old Phosphate Port (196.7 and 159.9 ppb respectively). A high level of (Fe) concentration in *Halophila stipulacea* (Forsk.) Aschers. was found in Old Phosphate Port, Tala Bay and the Industrial Area (186.2, 170.8 and 189.7 ppb respectively), whereas Big Bay and MSS were the lowest (136.2 and 129.7 ppb respectively). A high level of (Cr) was found in Old Phosphate Port and Industrial area samples (286.7 and 267.1 ppb respectively), samples collected from MSS and Big Bay showed the lowest content (103.3 and 97 ppb respectively). A high level of (Mn) was found in Tala Bay, Industrial area and Old Phosphate Port (375.1, 387 and 409.9 ppb respectively). On the other hand, low levels were found in MSS and Big Bay (304 and 317 ppb respectively). High levels of (Ni) were found in Old Phosphate Port and industrial area (128.6 and 117.1 ppb). Big Bay and MSS showed the lowest concentrations (66.5 and 64.4 ppb respectively). The highest level of (Th) was found in Old Phosphate Port (9.8 ppb), and the lowest concentration was found in MSS and Big Bay (3.3 and 3.5 ppb respectively).

High levels of (Zn) were found in Old Phosphate Port, Tala Bay, and the Industrial Area (148.2, 148 and 146.4 ppb respectively), the lowest concentrations were found in MSS and Big Bay (96.2 and 113.2 ppb respectively). Similar Li levels were found in Big Bay, MSS, Tala Bay, Old Phosphate Port and Industrial area (39.2, 34.6, 41.2, 44.3 and 45.7 ppb respectively). A high level of (Mg) was found in Tala Bay and Industrial area (132206 and 137168.2 ppb respectively), and the lowest concentration was found in MSS (100371.1 ppb).

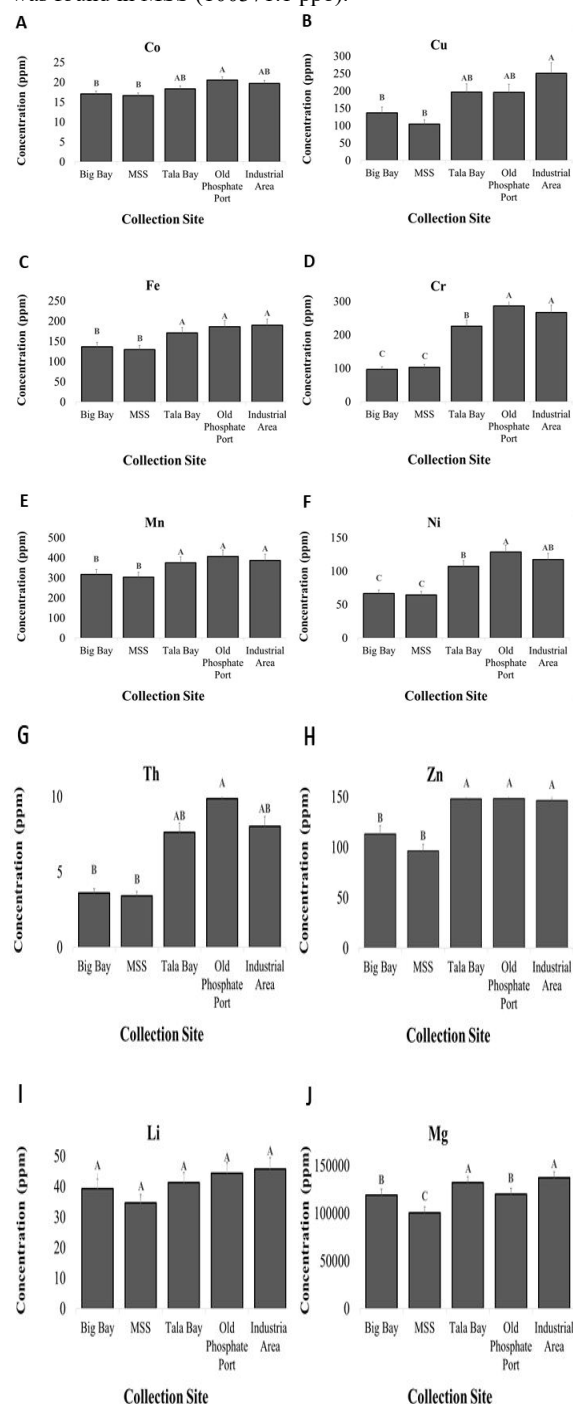


Figure 7. Heavy metals (Co, Cu, Fe, Cr, Mn, Ni, Th, Zn, Li and Mg) concentration (ppb) in *Halophila stipulacea* (Forsk.) Aschers. collected from different sites in the Gulf of Aqaba (A-J). Means with different letters are significantly different at ($p < 0.05$). Error bars represent standard error.

4. Discussion

The aim of this study was to evaluate genotoxicity in *Halophila stipulacea* (Forsk.) Aschers. found in Gulf of Aqaba as a pollution bioindicator using DNA damage, heavy metals and the level of lipid peroxidation

Worldwide, levels of pollutants in the environment are rising alarmingly due to urbanization and industrial growth, which has a negative impact on ecosystem sustainability, organisms and humans (El-Gendy et al, 2021). Harmful environmental stressors can cause genotoxicity and apoptotic cell death, which can lead to a variety of pathological disorders. These stressors include cytotoxic agents, pollutants, and toxicants (Cortés-Eslava et al, 2018, Paulo Cardoso et al., 2024). Marine water makes up the majority of the planet's water supply, yet it must be processed by people in order to be used. Water pollution is defined as any alteration of water's natural qualities by anthropogenic contaminants to the level where it is either toxic to humans or unsuitable for supporting biotic organisms, such as fish, and those that depend on it. Since it causes the development of many fatal diseases, water pollution is a major cause of concern on a global scale (Anju et al, 2010). Because of the large size (local and global) and complex interactions of trace elements in the marine environment, contamination with heavy metals in such areas is a growing problem for ecosystem managers. Trace elements persist indefinitely in the environment. Accumulating in sediments, they enter the food chain and increase in concentration at higher trophic levels, ultimately posing risks to both human and wildlife health.

Environmental exposure to heavy metals (notably arsenic, cadmium, lead, mercury, chromium and nickel) is consistently associated with molecular and cellular markers of genotoxicity (Balali-Mood et al., 2021). Heavy metals generate genotoxicity by at least three interacting processes. First, many metals increase cellular reactive oxygen species (ROS) production and disturb redox homeostasis, yielding oxidative base lesions (e.g., 8-oxo-dG), single-strand breaks (SSBs) and, at higher or persistent exposures, double-strand breaks (DSBs) (Jomova et al., 2025). Second, several metals directly impair DNA-repair pathways. Cadmium and other metals can displace essential metal cofactors in repair enzymes, suppress repair-gene expression, or destabilize repair proteins, reducing capacity for base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR) and DSB repair — an effect that amplifies the mutational consequences of otherwise reparable damage (Morales et al., 2016). Third, certain metals (notably hexavalent chromium and some nickel compounds) form direct adducts or crosslinks with DNA and DNA-binding proteins or perturb chromatin structure, creating bulky lesions and replication blocks that are intrinsically mutagenic and cytotoxic (Balali-Mood et al., 2021).

Significant differences in DNA damage score, DNA damage index and frequency were observed among *H. stipulacea* samples collected from the collection sites. Results indicate that the highest DNA damage (DNA damage score 1.78, DNA damage Index 49.7 and DNA damage Frequency 82.3%) and the highest lipid peroxidation rate (0.261 nmole g⁻¹ FM) were observed in

Old Phosphate Port. Similarly, heavy metal analysis showed high level of (Co) concentration (20.5 ppb), (Cu) concentration (159.9 ppb), (Fe) concentration (186.2 ppb), (Cr) concentration (286.7 ppb), (Ni) concentration (128.6 ppb), (Zn) concentration (148.2 ppb), (Mn) concentration (409.9 ppb), (Th) concentration (9.8 ppb), (Mg) concentration (120006.6 ppb) and (Li) concentration (44.3 ppb) in Old Phosphate Port. This could be explained by ship movement, oil and chemical leakage from ships in Development Corporation (ADC) and Aqaba Container Port. In addition, it could be due to the weak movement of water currents that lead to the accumulation of heavy metals in these regions.

Halophila stipulacea (Forsk.) Aschers. samples collected from the Industrial area showed DNA damage score of 1.62, DNA damage index of 43.7 and DNA damage frequency of 82.9%. These high values of DNA damage were accompanied with the highest lipid peroxidation rate (0.277 nmole g⁻¹ FM), high levels of (Co) (19.6 ppb), (Cu) (250.6 ppb), (Fe) (189.7 ppb), (Cr) (267.1 ppb), (Ni) (117.4 ppb), (Zn) (146.4 ppb), (Mn) (387ppb), (Th) (8 ppb), (Mg) (132206 ppb) and (Li) (45.7 ppb). This could be due to the industrial activity such as container transportation, building activity and vehicle exhaust from chemical factories in this area such as KEMAPCO® Arab Fertilizers and others. In contrast, the lowest DNA damage levels were observed in *Halophila stipulacea* (Forsk.) Aschers. collected from MSS and Big Bay, these areas are far away from tourist and located away from industrial and ship traffic.

Bonanno and Raccuia (2018) used seagrass *Halophila stipulacea* as bioindicator, and found that *Halophila stipulacea* is considered as good bioindicator for a variety of heavy metals. Also, Lemos et al. (2005) used comet assay for the genotoxic assessment of water pollution and found significantly higher levels of DNA damage in *T. rendalli* in Lake Igapo II compared to the control group. Another study done by Al Khateeb (2018) used comet assay to assess pollution in different regions in Jordan; results showed that plants obtained from the back road/ Aqaba and Ghour Assaal had the highest DNA damage amount and high Al, Cr, Fe, Cu, Zn, Cd, and Pb levels, compared to plants obtained from Wadi Rum, Al Naqab Heights, Swaimah/Deadsea, and Alshoneh Aljanobyeh.

Trace elements such as Cu, Zn, Mn, and Ni are considered as micronutrients for plants, but others, such as As, Cd, Cr, Hg, and Pb, are not necessary and may be toxic to many organisms even at low amounts. Li et al., (2016) assessed the relationship between levels of DNA damage of *Leymus chinensis* and heavy metals (Pb, Cd, Cr, Hg) content in China using Comet assay. Results showed that genetic damage (Tail DNA% and Tail Moment) in *L. chinensis* leaf cells under heavy metals was 77% compared to 35% in control samples. In addition, presence of trace elements in large amounts such as Cu and Zn can be hazardous. For example, plants require copper for proper plant development and growth, but a high level of Cu contamination leads to leaf chlorosis, oxidative stress and ROS, and it impacts different pigment secretions during photosynthesis and reduces plant growth. Also, zinc contributes in production of chlorophyll in plants, but high Zn concentrations prevent development of metabolic processes, limit root and shoot development, chlorosis in young leaves, decrease germination,

chlorophyll, carotenoid, sugar, amino acid and growth. In addition, Cadmium causes chlorosis, growth inhibition, browning of the root tips, abnormal membrane function and eventually death. Chromium is well-known for being poisonous and capable of harming both plants and animals (Bonanno and Raccuia, 2018). Chromium damages plant cell membranes and breakdown photosynthetic pigments, which leads to growth reduction. Numerous physiological changes, including chlorosis and necrosis, are driven by excessive Ni in the soil. Finally, plants utilize iron for a primary function in photosynthesis. An excess of iron causes a reduction in plant photosynthesis, and increase in oxidative stress leads to the creation of free radicals, which permanently alter the cellular structure and destroy proteins, DNA and membranes (Bonanno and Raccuia, 2018). Anbuselvan and Sridharan (2018) determined the distribution of heavy metals (Fe, Cd, Co, Cr, Cu, Ni, Zn, and Pb) in the Bay of Bengal as a sign of marine pollution and found that the area was extremely polluted with Cd and Pb contributions from anthropogenic activities in the nearby area. Furthermore, Zhang et al. (2020) studied heavy metals pollution in coastal areas of the East China Sea and found that Cd caused significant pollution and possible ecological hazards due to anthropogenic activities.

Table. 2: The Similarity coefficient matrix between studied parameter.

	frequencu	index	score	co	cu	fe	cr	mn	ni	th	zn	li	mg	lpo
frequencu	1													
index	0.790274	1												
score	0.975253	0.841248	1											
co	0.963261	0.853449	0.941556	1										
cu	0.865616	0.465421	0.737272	0.816707	1									
fe	0.938688	0.72011	0.847019	0.933949	0.94564	1								
cr	0.922737	0.800612	0.843996	0.933546	0.884787	0.986817	1							
mn	0.907135	0.802662	0.830663	0.953698	0.870624	0.978383	0.988204	1						
ni	0.914438	0.814102	0.838828	0.945285	0.870346	0.981955	0.996993	0.99663	1					
th	0.893597	0.846299	0.827048	0.944678	0.826507	0.961947	0.987777	0.994903	0.996011	1				
zn	0.807088	0.61143	0.681993	0.866599	0.909595	0.949465	0.931649	0.958881	0.943696	0.934073	1			
li	0.930786	0.608143	0.853875	0.931024	0.945734	0.944577	0.889957	0.909791	0.893932	0.867462	0.909549	1		
mg	0.61847	0.152018	0.448738	0.611374	0.911725	0.775268	0.680731	0.703555	0.679749	0.63478	0.852318	0.839785	1	
lpo	0.979052	0.696377	0.913025	0.949863	0.945722	0.975268	0.94097	0.933658	0.934113	0.906531	0.889697	0.977876	0.764218	1

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In this study, similarity coefficient matrix indicates a strong positive correlation between DNA damage index, DNA damage score, DNA damage frequency, LPO and heavy metals (table 2). The highest similarity coefficient value was observed between LPO and DNA damage frequency (0.979). Also, similarity coefficient value between LPO and Cu was (0.945), whereas similarity coefficient value between DNA damage frequency and Fe was (0.938) and similarity coefficient value between DNA damage score and LPO was (0.913), which indicates that heavy metal cause direct effect on DNA and lipid. However, the similarity coefficient value between the DNA damage index and Mg was (0.152), possibly because Mg is regarded as macronutrient for plants. Lukatkin et al. (2021) studied the effect of heavy metals on biochemical and physiological responses of *Amaranthus retroflexus*, they found a strong correlation between the accumulation rate of HMs and (MDA) content in *A. retroflexus* roots, stems and leaves. Our results indicate that heavy metals could be the main source of pollution in the gulf of Aqaba. In conclusion, our results indicate that plant comet assay is a reliable method for measuring genotoxicity in marine environment in general and specifically in the Gulf of Aqaba.

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Clove Extract Reverses Hematological Deficits, Hormonal Imbalances and Dyslipidemia in Streptozotocin-Diabetic Rats

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Abstract

This research explores the therapeutic potential of a water-soluble extract from clove (*Syzygium aromaticum*) to alleviate multiple diabetic complications in a rat model. **Materials and Methods:** Over 21 days, we administered clove extract (500 mg/kg/day, orally) to a group of streptozotocin-induced diabetic male Wistar rats, comparing them to untreated diabetic and healthy control groups (n=7 per group). We evaluated the complete blood count (CBC), serum concentrations of reproductive hormones (LH, FSH, testosterone), and key metabolic indicators such as blood glucose and lipid profiles. **Results:** Clove extract treatment prompted a significant recuperation of erythrocyte counts, hemoglobin, hematocrit and platelet levels. Furthermore, it markedly increased the circulating levels of luteinizing hormone, follicle-stimulating hormone, and testosterone ($p < 0.05$). We also observed a pronounced (approx. 30%) reduction in blood glucose and a reversal of dyslipidemia, evidenced by decreased triglycerides, total cholesterol, and LDL, and an elevation in HDL by approximately 38%. **Conclusion:** The outcomes demonstrate that clove extract provides multi-faceted protection against diabetes-induced pathologies through the hematological, endocrine, and metabolic systems, an effect likely driven by its antioxidant and hypoglycemic properties. The exact molecular pathways involved require further exploration.

Keywords: clove, diabetes, streptozotocin, hematology, reproductive hormones, lipid profile, antioxidant

1. Introduction

The global burden of diabetes mellitus, a chronic condition marked by persistent hyperglycemia due to insulin deficiency or resistance, continues to grow. Beyond impaired glucose homeostasis, diabetes establishes a systemic environment of elevated oxidative stress and inflammation. This milieu is a key driver of secondary complications, including dyslipidemia, hormonal axis disruption, and damage to cardiovascular, renal, and neurological systems, often extending to reproductive dysfunction (American Diabetes Association, 2023; Boles et al., 2022).

While conventional pharmaceuticals are a cornerstone of diabetes management, their long-term use can be limited by side effects and cost. This has accelerated the investigation of natural products, particularly medicinal plants, which offer a reservoir of bioactive compounds with historically recognized efficacy and safety (Gupta et al., 2021).

Clove (*Syzygium aromaticum*) is a common culinary spice rich in phytochemicals like eugenol, flavonoids, and phenolic acids. These compounds are credited with potent antioxidant, anti-inflammatory, and glucose-regulating activities (Duke et al., 2021; Kumar et al., 2021).

Specifically, eugenol has been implicated in enhancing insulin sensitivity and modulating metabolic pathways related to glucose (Manjamalai et al., 2012; Bhardwaj et al., 2023). Research also indicates clove's beneficial influence on lipid metabolism (Lima et al., 2019) and its potential to shield various tissues, including hematopoietic and endocrine organs, from oxidative damage (Gao et al., 2022; Ahmed et al., 2018). Given this background, our objective was to determine whether a water-based clove extract could restore hematological indices (red and white blood cell counts, hemoglobin, hematocrit, platelet count), reproductive hormone balance (LH, FSH, testosterone), and metabolic profiles (blood glucose, triglycerides, total cholesterol, LDL, HDL) in an experimental model of streptozotocin-induced diabetes.

2. Materials and Methods

2.1. Ethical Statement and Animal Model

All procedures involving the twenty-one adult male Wistar rats in this study were conducted under controlled laboratory conditions and received full approval from the institutional ethics board at Umm Al-Qura University (Approval No: HAPO-02-K-012-2024-I2-123), in strict accordance with international animal welfare standards.

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2.2. Diabetes Induction and Study Groups

To induce diabetes, rats were fasted and then received a single intraperitoneal injection of streptozotocin (STZ) at 50 mg/kg. Seventy two hours post-injection, animals with confirmed hyperglycemia (blood glucose >250 mg/dL) were randomized into three experimental cohorts (n=7 each):

Group I (Control): Non-diabetic, healthy rats receiving no intervention.

Group II (Diabetic Control): Diabetic rats that remained untreated.

Group III (Treatment): Diabetic rats receiving daily oral clove extract (500 mg/kg) for 21 consecutive days.

2.3. Water Extraction of Cloves

Authenticated dried clove buds (**Syzygium aromaticum**) were sourced locally. A 500-gram batch was mechanically pulverized into a fine powder. This powder was then infused in 2 liters of distilled water within a glass vessel, heated to a temperature range of 70–80°C for 50 minutes with constant agitation. After cooling to ambient temperature (~25°C), the solution was filtered through a double layer of cheesecloth (approx. pore size = 150-200 µm). The clarified filtrate was stored in sterile amber glass bottles at 4°C to ensure chemical stability and prevent microbial contamination. To guarantee consistent bioactivity, a fresh batch of extract was prepared weekly for the duration of the study.

2.4. Biochemical and Hematological Sampling

At the conclusion of the 21-day treatment, all the rats were anesthetized for terminal blood collection via cardiac puncture. Blood for hematology was anti-coagulated with EDTA, while samples for serum biochemistry were collected in plain tubes, allowed to clot and centrifuged at 3,000 rpm for 15 minutes to harvest serum.

2.4.1. Analysis of Blood Cells

A full CBC was automatically generated by a hematology analyzer. Parameters assessed included:

Hemoglobin (Hb), Hematocrit (HCT), Red blood cell count (RBC), Total and differential white blood cell count (WBC), Platelet count (PLT), and erythrocyte indices (MCV, MCH, MCHC).

2.4.2. Evaluation of Hormonal Status

Serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were quantified using specific, commercially available enzyme-

linked immunosorbent assay (ELISA) kits, adhering strictly to the manufacturers' protocols.

2.4.3. Assessment of Metabolic Markers

Fasting serum was utilized to determine glucose concentration and lipid profile. The lipid panel comprised measurements of: total cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). All assays were performed with validated commercial enzymatic colorimetric kits.

2.5. Data Analysis

All numerical data are expressed as mean ± standard deviation (SD). To determine statistical significance across the three groups, a one-way analysis of variance (ANOVA) was applied, followed by Tukey's post-hoc test for pairwise comparisons. A p-value of less than 0.05 was deemed statistically significant.

3. Results

Impact on Hematological Parameters: Relative to the healthy control group, the untreated diabetic rats developed significant hematological deficits, including anemia and thrombocytopenia (Table 1). Intervention with clove extract effectively restored these parameters, bringing red blood cell count (a 45% increase from 5.6 to 8.1 $\times 10^6/\mu\text{L}$), hemoglobin (a 38% increase from 10.3 to 14.2 g/dL), hematocrit (a 50% increase from 30% to 45%), and platelet count (a 25% increase from 522 to 652 $\times 10^3/\mu\text{L}$) back to levels statistically indistinguishable from normal ($p < 0.05$).

Impact on Reproductive Hormones: The diabetic condition led to a significant suppression of the hypothalamic-pituitary-gonadal axis, reflected in markedly lower serum levels of FSH, LH and testosterone (Table 2). Oral administration of the clove extract produced a significant upregulation in the concentrations of all three hormones (FSH increased by 38%, LH by 134%, and testosterone by 166%) ($p < 0.05$).

Impact on Metabolic Parameters: The diabetic control group exhibited severe hyperglycemia and an adverse lipid profile (Table 3). Treatment with clove extract resulted in a significant ($p < 0.05$) 30% decrease in fasting blood glucose and a comprehensive improvement in the lipid panel, characterized by a 22% reduction in triglycerides, a 23% reduction in total cholesterol, a 22% reduction in LDL, and a 38% increase in protective HDL cholesterol.

Table 1: The effect of clove on Complete Blood Count (CBC) in both normal and diabetic rats

Parameter	Literature Reference Values	Untreated Rats (Control)	Diabetic Rats Before Treatment	Diabetic Rats After Treatment
White Blood Cells (WBC) / $\mu\text{L} \times 10^3$	6.0 - 10.0	6.0 ± 2.88	8.2 ± 2.16	$7.1 \pm 2.18^*$
Lymphocytes/ $\mu\text{L} \times 10^3$	2.5 - 5.0	2.5 ± 1.68	2.4 ± 1.85	2.6 ± 1.97
Neutrophils/ $\mu\text{L} \times 10^3$	1.5 - 4.0	3.0 ± 1.83	3.1 ± 2.86	3.5 ± 1.88
Monocytes %	1 - 4	2.5 ± 1.2	6.5 ± 2.1	4.0 ± 1.5
Eosinophils%	0.5 - 3	1.0 ± 0.5	1.2 ± 0.7	1.1 ± 0.6
Basophils%	0 - 1.5	0.5 ± 0.3	0.6 ± 0.4	0.5 ± 0.3
Red Blood Cells (RBC) / $\mu\text{L} \times 10^6$	7.0 - 9.0	7.0 ± 1.66	5.6 ± 1.60	$8.1 \pm 1.75^*$
Hemoglobin (Hb) g/dL	12.0 - 15.0	13.0 ± 2.98	10.3 ± 2.41	$14.2 \pm 1.44^*$
Hematocrit (HCT) %	38 - 48	40 ± 3.82	30 ± 1.47	$45 \pm 2.65^*$
Mean Corpuscular Volume (MCV) fL	50 - 60	55 ± 4.44	51 ± 2.32	$60 \pm 2.46^*$
Mean Corpuscular Hemoglobin (MCH) pg	17 - 21	18 ± 2.55	16 ± 1.65	19 ± 2.35
Mean Corpuscular Hemoglobin Conc.(MCHC)%	30 - 35	30 ± 1.96	24 ± 3.18	31 ± 2.13
Platelets (PLT) / $\mu\text{L} \times 10^3$	550 - 800	600 ± 5.37	522 ± 2.35	$652 \pm 4.39^*$

Note: *Significant difference at $p < 0.05$ level.

Table 2: The effect of clove on Sex Hormones in normal and diabetic rats

Parameter	Literature Reference Values	Untreated Rats (Control)	Diabetic Rats Before Treatment	Diabetic Rats After treatment
FSH (IU/mL)	12.0 - 15.0	13.57 ± 0.07	11.27 ± 0.01	$15.53 \pm 0.20^*$
LH (IU/mL)	1.5 - 2.0	1.76 ± 0.01	1.07 ± 0.08	$2.50 \pm 0.16^*$
Testosterone (ng/mL)	3.0 - 5.0	3.64 ± 0.03	2.23 ± 0.09	$5.94 \pm 0.66^{**}$

Note: *Significant difference at $p < 0.05$ level. **Testosterone showed a highly significant increase beyond control levels ($p < 0.01$).

Table 3: The effect of clove on Glucose and Lipid Level in normal and diabetic rats

Parameter	Literature Reference Values	Normal Rats (mg/dL)	Diabetic Rats Before treatment (mg/dL)	Diabetic Rats After Treated with clove (mg/dL)
Glucose	80 - 110	85.6 ± 5.3	198.8 ± 8.5	$139.6 \pm 6.7^*$
Triglycerides	100 - 150	138.7 ± 6.3	190.6 ± 11.5	$148.4 \pm 8.13^*$
Cholesterol	130 - 170	150.2 ± 9.5	200.2 ± 13.40	$155 \pm 9.18^*$
LDL	70 - 110	94.3 ± 4.2	129.5 ± 16.10	$101.5 \pm 8.60^*$
HDL	45 - 60	50.9 ± 2.9	41.6 ± 3.2	$57.3 \pm 3.22^*$

Note: *Significant difference at $p < 0.05$ level.

4. Discussion

Our findings reveal that diabetic rats developed significant hematological and endocrine disturbances, which were substantially reversed by clove extract supplementation.

The observed anemia and low platelet count in the diabetic group align with the known suppressive effects of chronic hyperglycemia and associated oxidative stress on bone marrow activity and red blood cell longevity (Hussain et al., 2020). The ability of clove extract to normalize these parameters underscores its hematoprotective potential, most plausibly attributable to its high concentration of antioxidant compounds like eugenol, which mitigate oxidative damage to cellular components, including erythrocyte membranes (Srinivasan, 2014; Abdel-Mawgoud et al., 2019).

The suppression of FSH, LH, and testosterone in diabetic rats indicates a dysfunction at the level of the hypothalamic-pituitary-gonadal (HPG) axis. The

successful restoration of these hormones by clove extract points to a central mechanism of action. We posit that the extract alleviates the oxidative stress and inflammation that disrupt the pulsatile secretion of Gonadotropin-Releasing Hormone (GnRH) from the hypothalamus. This restoration of central signaling would subsequently normalize pituitary release of LH and FSH, thereby reactivating testosterone production in the Leydig cells of the testes, a process known to be impaired by diabetes (Ahmed et al., 2018; Gianatti & Grossmann, 2020). The fact that hormone levels were not just normalized but elevated beyond control values suggests a potent, possibly stimulatory, effect of clove's bioactive components on the HPG axis, potentially through enhanced steroidogenic enzyme activity or receptor sensitivity (Huang et al., 2014; Elgharib et al., 2025). The potent antioxidant activity of eugenol, the primary bioactive molecule in clove, is a likely cornerstone of this restorative effect. By scavenging the excess reactive oxygen species (ROS) generated in the diabetic state, eugenol protects sensitive neuroendocrine and testicular tissues from damage, thereby permitting the

resumption of normal hormonal synthesis and regulation (Khosravi Z et al., 2021). Finally, the significant amelioration of hyperglycemia and dyslipidemia in the treated group confirms the extract's metabolic benefits. The glucose-lowering action is consistent with proposed mechanisms of improved insulin sensitivity and peripheral glucose uptake (Manjamalai et al., 2012; Bhardwaj et al., 2023). The corrected lipid profile--reduced triglycerides, cholesterol, and LDL, and increased HDL--likely results from a dual action: the suppression of hepatic cholesterol and fatty acid synthesis, coupled with the promotion of lipid catabolism and clearance (Lima et al., 2019; Hamri et al., 2022).

5. Conclusion

This study provides strong evidence that a water-based extract of clove effectively counteracts diabetes-induced hematological deficits, hormonal imbalances, and dyslipidemia in rats. These findings position clove as a promising candidate for adjunct therapy, potentially offering a natural means to improve quality of life by addressing multiple diabetic complications simultaneously.

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The clinical relevance of *Helicobacter pylori* virulence genes (*VacA*, *OipA*, *IceA1*, *IceA2*, and *BabA*) in relation to clinicopathological findings among Jordanian patients with gastric complaints

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Abstract

Helicobacter pylori (*H. pylori*) establishes chronic gastric infection through key virulence genes- *BabA*, *VacA*, *IceA*, and *OipA*- that enable adhesion, immune evasion, and mucosal damage. Despite extensive research, it remains a major health concern globally due to its complex virulence profile, varied disease outcomes, and possible oral transmission. Emerging evidence suggests that the oral cavity, particularly dental plaque, may serve as a reservoir for *H. pylori*, underscoring the need to understand oral-gastric transmission pathways for effective prevention and management. This study investigated the prevalence, distribution of virulence genes, and oral–gastric transmission patterns of *H. pylori* among 125 Jordanian patients presenting with gastric complaints. A total of 250 gastric biopsies were characterized by endoscopy and assessed using histopathology and polymerase chain reaction (PCR), while 67 subgingival samples were analyzed using PCR. *H. pylori* was detected in 71.2% of biopsies by histopathology and in 66.4% by PCR. Uniplex and multiplex PCR showed high prevalence of *BabA* (95.2%), *IceA2* (81.9%), and *VacA* (74.7%) gene, while *OipA* (43.3%) and *IceA1* (21.7%) were less common. The notably high prevalence of *BabA* and *IceA2* suggests improved strain persistence, enhanced mucosal adhesion, and a greater tendency toward chronic infection. Significant associations were identified between virulence genotypes and the severity of histopathological changes, providing the first detailed genotype- disease severity map for Jordan. Subgingival samples showed *H. pylori* positivity in 83.6% of patients, with strong agreement between oral and gastric PCR results. The highest oral detection occurred in patients with gastritis (69.6%), supporting the role of the oral cavity as a potential reservoir and source of reinfection. Future research in this extent can contribute to improved prevention strategies and innovative clinical approaches for managing infection. The findings of the current study may have provided additional regional data on *H. pylori* virulence patterns and their clinicopathological significance, emphasizing the importance of integrating gastrointestinal and oral health management.

Keywords: *Helicobacter pylori*; Dental Plaque; Histopathology; Endoscopy; PCR.

1. Introduction

H. pylori is a very common human pathogen that adheres to and colonizes the gastric epithelium's luminal surface in about half of the world's population. Once acquired, *H. pylori* can establish a chronic infection leading to a range of gastroduodenal disorders, including chronic gastritis, peptic ulcer disease, gastric adenocarcinoma, and MALT lymphoma (Boden *et al.*, 2021; Rendón-Huerta *et al.*, 2021). Globally, infection rates exceed 50% of the population (Ansari *et al.*, 2017), with transmission primarily occurring through ingestion of contaminated food or water, or via direct person-to-person contact. The prevalence increases with age and is particularly high in low-resource settings, with a notable

regional rise in children under five years old (Kayali *et al.*, 2024; Rostam *et al.*, 2024). The World Health Organization designates *H. pylori* as a Class I carcinogen (Kotilea *et al.*, 2019).

H. pylori is a Gram-negative, spiral-shaped bacterium approximately 3 µm in length, equipped with multiple flagella and characterized by microaerophilic growth, as well as urease, catalase, and oxidase activities. Under adverse environmental conditions, it may convert from a culturable spiral form to a non-culturable coccoid state. This transformation can be triggered by nutrient limitation, prolonged incubation, temperature extremes, alkaline pH, shifts from nutrient-rich media to water, CO₂ deficiency, or exposure to antimicrobial agents (Gladyshev *et al.*, 2020; Jung *et al.*, 2024; Rendón-Huerta *et al.*, 2021).

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The virulence factors of *H. pylori* include blood group antigen-binding adhesin (*BabA*), Sialic-acid binding adhesin (*SabA*), Outer inflammatory protein A (*OipA*), *H. pylori* outer membrane protein Q and Z (*HopQ* and *HopZ*), which enable colonization and pathogenesis (Rendón-Huerta *et al.*, 2021; Odenbreit *et al.*, 2009). Key virulence factors, including *VacA* (vacuolating cytotoxin A), *CagA* (cytotoxin-associated gene A), *IceA* (induced by contact with epithelium gene A), *HtrA* (high temperature requirement A), *Hsps* (heat shock proteins), catalase, urease, phospholipases, and mucinase, contribute to tissue damage by inducing cytotoxicity and inflammatory responses (Baj *et al.*, 2021). Subtyping of strains, typically based on *VacA* and *CagA* expression, provides important information on disease outcomes and may inform both diagnostic and therapeutic approaches (Bordin *et al.*, 2021; Cho *et al.*, 2021). Al-Hyassat *et al.* (2025) reported that *VacA* genotyping identified s2/m2 allelic variants as predominantly associated with mild gastritis, while s1/m1 and s1/m2 were linked to more severe gastric pathology.

Urease is a key virulence factor of *H. pylori*, enabling gastric colonization by locally buffering acidity through the production of ammonia and carbon dioxide. This process maintains a near-neutral cytoplasmic pH, supports motility, nutrient acquisition, and proton motive force, and prevents self-toxicity. After penetrating the mucus layer, the bacteria attach to epithelial cells via *AlpA*, *AlpB*, and *OipA*, promoting immune evasion and effector protein delivery through the type IV secretion system (Cáceres-Delpiano *et al.*, 2015). The expression of *SabA* occurs during gastric disease progression together with outer membrane proteins (OMPs) such as *OipA* and *BabA*, mediating gastric atrophy and neutrophil infiltration (Baj *et al.*, 2021). Concurrently, these proteins, along with lipopolysaccharides (LPS), trigger host immune responses by stimulating cytokines such as IL-10, IL-12, IL-18, IFN- γ , and TNF- α , which attract immune cells that generate oxidative stress. *H. pylori* also exploits trefoil factor family protein 1 (TFF1), a protective mucus-associated peptide, to strengthen its attachment to the gastric lining. The infection stimulates TFF1 expression, which enhances mucin and pepsinogen secretion and disrupts normal gastric function (Baj *et al.*, 2020). Meanwhile, virulence factors such as *CagA* and *VacA* create a proinflammatory environment that activates the JAK/STAT pathway, driving epithelial cell proliferation, inflammation, and apoptosis, thereby contributing to severe gastritis and potentially gastric cancer (Järvå *et al.*, 2020).

Evidence indicates that the degree of bacterial adherence is determined by *BabA* binding to the ABO/Lewis^xb (Leb) and H-type-1 antigens present in stomach tissue (Doohan *et al.*, 2021). Granulocyte infiltration, the release of pro-inflammatory mediators, the production of IL-8, and the expression of *VacA* are all consequences of this process. Alzahrani *et al.* (2014) claim that the simultaneous activation of many mechanisms accelerates the development of cancer and the degradation of mucosal tissue. In addition, *BabA* and *SabA* promote *H. pylori* adherence to the spasmolytic polypeptide-expressing metaplasia (SPeM), a metaplastic linked to the initiation of carcinogenesis (Sáenz *et al.* 2019). **Moreover**, the primary survival strategy utilized by *H. pylori* to persist in the stomach is its ability to produce ammonia through either encoding the urease enzyme from the *ureA*

gene or the urea-independent pathway by hydrolyzing amide. Both pathways result in a neutral cytoplasmic and periplasmic pH. After penetration of the mucus layer, the bacteria attached to the gastric epithelial cells through lipoproteins *AlpA* and *AlpB*, and *OipA*, which allows it to escape immune clearance and inject bacterial proteins into host cells using type IV secretion system. In particular, *OipA* assist in colonization, adherence, and progression of gastrointestinal diseases. Binding of *OipA* to the gastric epithelial cells activates the apoptotic pathways like Bcl-2 and increased secretion of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-11 IL-17, matrix metalloproteinase 1 (MMP-1), and tumor necrosis factor α (TNF- α). It also downregulates the cluster of differentiation 40 (CD40), the cluster of differentiation (CD86), and major histocompatibility complex-II (MHC-II) expression on the cell surface, leading to the suppression of dendritic cells' maturation.

In recent years, the incidence of *H. pylori* infection in Jordan has shown a notable increase. A cross-sectional study by Obaidat and Roess (2019) reported that 88% of Jordanians were seropositive, indicating a substantially high burden of infection. Prevalence rates tend to be higher in developing countries than in developed nations, a pattern often attributed to inadequate hygiene practices within facilities and infrastructure, as well as limited public health awareness (Alaridah *et al.*, 2023). The clinical implications of helicobacteriosis are significant, with documented complications including iron deficiency, peptic ulcer disease, and gastric neoplasms (Cho *et al.*, 2021). According to reports from the Jordanian Ministry of Health (2017), gastric cancer is the ninth most commonly diagnosed cancer in the country and is considered the seventh to tenth most prevalent cancer among Jordanians overall (Alaridah *et al.*, 2023). Efforts aimed at improving preventive and public health policies must consider the prevalence rate and the factors influencing infection rates.

H. pylori is a globally prevalent pathogen transmitted through multiple routes including fecal-oral and oral-oral (Zhang *et al.*, 2022; Burucoa and Axon, 2017). Its distribution varies widely across geographic regions and population groups, with higher rates in developing countries. Although its transmission pathways remain unclear, evidence suggests that the oral cavity may serve as an additional reservoir linked to gastric infection. Non-invasive oral detection methods show promise for improving diagnosis and reducing re-infection. Advancing research in this area could lead to better prevention strategies and innovative clinical approaches for managing *H. pylori* infection. The close relationship between the oral and gastric microbiota suggests that the oral cavity may play a significant role in the movement and persistence of *H. pylori*. Growing interest has focused on the presence of *H. pylori* in the oral cavity, as the mouth has been proposed as a potential extra-gastric reservoir. Although the exact role of *H. pylori* within the oral microbiome remains uncertain, its presence may disrupt microbial homeostasis, influencing the oral-intestinal axis and contributing to both oral and systemic disease. Evidence to date is mixed, with recent reviews reporting inconsistent links between oral *H. pylori* and oral health outcomes (Cuba *et al.*, 2025). Nonetheless, several studies suggest that the oral cavity may support *H. pylori* colonization and transmission, complicating eradication, particularly in the

presence of poor oral hygiene and dental disease (Liang *et al.*, 2021; Popovska *et al.*, 2022; Author *et al.*, 2022).

Studying both gastric and oral *H. pylori* isolates is crucial to understand the dynamics of infection and transmission. The coexistence of the bacterium in both sites may contribute to reinfection, persistence, and treatment failure. Furthermore, the presence of virulence-associated genotypes in oral isolates may influence the severity of gastric disease and facilitate bacterial spread within populations, particularly in regions with high prevalence, such as Jordan (Abu-Lubad *et al.*, 2018). Given the inconsistencies and methodological differences across studies, including variable detection techniques and sampling methods, integrated analysis of gastric and oral isolates is essential for defining transmission dynamics and optimizing clinical management of *H. pylori* infection. Understanding the behavior and virulence mechanisms of *H. pylori* is critical for both clinical practice and public health, especially in regions where the infection is highly prevalent. Such studies are essential because of their significant clinical and epidemiological implications. This study examines the clinical relevance of *H. pylori* virulence genes (*VacA*, *OipA*, *IceA1*, *IceA2*, and *BabA*) in gastric isolates in relation to endoscopic and histopathological findings. It also evaluates the prevalence of *H. pylori* in the oral cavity to better understand infection dynamics, guide eradication strategies, and help reduce the burden of *H. pylori*-associated diseases.

2. Materials and Methods

2.1. Gastric Mucosal Biopsy

Two hundred and fifty gastric mucosal biopsy specimens were taken from 125 patients undergoing white-light upper endoscopy. The Institutional Review Board of Al-Bashir Hospital provided ethical approval for sample collection and study procedures (MBA/IRB/3822). Before specimens were collected, each patient signed a written informed consent form. Every patient completed a prepared questionnaire. Clinical data, including age, gender, occupation, smoking status, history and length of illness, prior or current gastritis complaints, treatments or antimicrobial therapies received within the last three months, and proton pump inhibitor use within the last 15 days, were recorded. For molecular analysis, biopsy specimens were placed in 2 mL of sterile normal saline and transported on dry ice in a cooled container within four hours. Biopsy specimens intended for histopathological examination were fixed in 10% buffered formalin and transported to the histopathology laboratory.

2.2. Isolation of *H. pylori* from subgingival

To investigate the potential of the subgingival region as a reservoir for *H. pylori*, 67 dental samples were obtained from patients who underwent upper endoscopy. Only those patients who provided informed consent for dental sample collection were included in the study. Subgingival plaque samples were collected using sterile curettes. Samples were placed in Eppendorf tubes containing 2 mL of normal saline and transported on dry ice to the laboratory for analysis within four hours (Gebara *et al.*, 2004).

2.3. Molecular Identification of *H. pylori* using (PCR)

2.3.1. 2.3.1. Extracting DNA from Gastric Biopsy and Subgingival Samples

Gastric biopsy samples were collected in sterile containers containing sterile normal saline. Genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, tissue samples were placed in 1.5 mL Eppendorf tubes containing 180 µL of cell lysis buffer, followed by the addition of 20 µL of proteinase K to inactivate nucleases and remove contaminants. The mixture was vortexed and incubated at 56 °C until complete lysis was achieved. After incubation, samples were vortexed again, and 200 µL of AL buffer (lysis buffer) was added and mixed thoroughly using a vortex mixer (Labnet International, USA). Subsequently, 200 µL of ethanol (96–100%) was added and mixed by vortexing. The mixture was then centrifuged at 8,000 rpm for 1 min using a QIAamp Mini spin column placed in a 2 mL collection tube. The flow-through and collection tube were discarded, and the spin column was transferred to a new 2 mL collection tube. DNA was washed with 500 µL of each wash buffer in two successive centrifugation steps. Finally, DNA was eluted by adding 200 µL of elution buffer.

Genomic DNA was extracted from subgingival plaque samples using the Omega DNA extraction kit (Omega, USA) following the manufacturer's instructions (Abu-Lubad *et al.*, 2018). Briefly, samples were centrifuged to obtain a pellet, resuspended in TE buffer, and subjected to enzymatic lysis using lysozyme and proteinase K. RNase A was then added, and the lysate was purified using HiBind DNA Mini Columns. The extracted DNA was eluted in 50–100 µL of elution buffer. The concentration and purity of extracted DNA were determined using a NanoDrop spectrophotometer (Nabi UV/Vis Nano Spectrophotometer; Daejeon, Republic of Korea).

2.3.2. 2.3.2. DNA Amplification

Twenty-base oligonucleotide primers designated were used (Table 1) (Sedaghat *et al.*, 2014). The 16S rRNA gene was amplified using; F- (5'-ATCCTGGCTCAGAGTGAACG-3') and R- (5'-GCAGGTTCACCTACGGTTACC-3') primers. A volume of 20 µL of PCR reaction mixture comprising 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5% (v/v) Triton X-100), 1.5 mM MgCl₂, 200 µM of each dNTP, 10 pmol of each primer, 1 unit of Taq DNA polymerase, and 18 µL of template DNA was used. This reaction yielded a 395-base-pair amplified fragment. The initial denaturation was carried out at 96 °C for 5 minutes. The mixtures were then exposed to 40 cycles, each cycle consisting of three steps: denaturing at 94 °C for 1 minute, primer annealing at 62 °C for 1 minute, and extension of two minutes at 72 °C. This final extension is to make up for the complete elongation of the PCR products, together with an additional ten-minute extension time after the last cycle. A negative control prepared by adding all the master mix reagents, excluding the template, was used and executed in each batch of the PCR assay (Han and Ge, 2010).

Table 1. Primers of virulence genes used to characterize *Helicobacter pylori*

Target gene	Primer sequence (5'-3')	Product (bp)	Size	Annealing Temp. (°C)
<i>VacA</i> gene	F- CAATCGTGTGGTTCTGGAGC R- GCCGATATGCAAATGAGCCGC	678		63.5
<i>BabA</i> gene	F- AATCCAAAAGGAGAAAAAG R- TATGAAA R- TGTTAGTGATTTCGGTGTAG GACA	832		56.8
<i>OipA</i> gene	F- GTTTTTGATGCATGGGATTT R- GTGCATCTCTTATGGCTTT	401		50.3
<i>IceA1</i> gene	F- GTGTTTTTAACCAAAGTATC R- CTATAGCCATTATCTTTGCA	247		52.5
<i>IceA2</i> gene	F- GTGTGCGTTGTTTAAATGAA R- GTCTTAAACCCACGATTAAA	120		48.7

2.3.3. Analysis of PCR Products

PCR-amplified products were analyzed by agarose gel electrophoresis. A total of 10 µL of each PCR product was mixed with 3 µL of loading buffer, which was prepared from 20 mL of 50% glycerol, 25 mg bromophenol blue, and 30 µL of 1 N NaOH, and then loaded onto a 1% agarose gel.

Following electrophoresis, the amplified DNA fragments were verified by staining the gel with ethidium bromide and examined under a UV transilluminator and photographed with a gel documentation system (Gel Doc 2000, Bio-Rad, USA) (Han and Ge, 2010).

2.3.4. Detection of Virulence Genes Using uniplex and multiplex PCR

For PCR amplification of the *VacA*, *BabA*, *OipA*, *IceA1*, and *IceA2* genes, a total of 0.6 µL (0.5 µg) of template genomic DNA was added to a 25 µL reaction mixture containing 2.0 µL of 10× PCR buffer, 0.9 µL of 50 mM MgCl₂, 0.5 µL of 10 mM dNTPs, 1.2 µL of each primer, 0.7 µL of Taq DNA polymerase (5 U/µL), and 4.4 µL of nuclease-free water. Amplification was done in a thermal cycler at the following conditions: initial denaturation for 4 minutes at 95 °C, 31 cycles with denaturation for 44 seconds at 95 °C, annealing for 45 seconds at 51 °C, extension for 62 seconds at 72 °C, and final extension at 72 °C for 5 minutes. The products were further visualized by electrophoresis in 1.5% agarose gels stained with Gel Red as described by Oktem-Okullu *et al*, (2015).

3. Statistical Analysis

The statistical packages of social science program (IBM SPSS Modeler, New York, US) version 19.0 was used to evaluate the data. In this regard, Chi-square and Pearson's correlation analyses were performed with the view to comparing the variables.

4. Results

The study population consisted of 125 biopsies collected from patients attending gastroenterology clinics at Al-Hussein Hospital/Al-Salt and Al-Bashir

Hospital/Amman with different gastric complaints including 87 females and 38 males. The mean age was 42.29 ± 17.75 years for females and 44.95 ± 19.12 years for males. Patients from Amman exhibited a mean age of 40.45 ± 18.46 years, and from Al Salt, 45.54 ± 17.63 years (Figure 1). Geographic data revealed that 60% of the examined cases were from Amman and 40% from Al-Salt city.

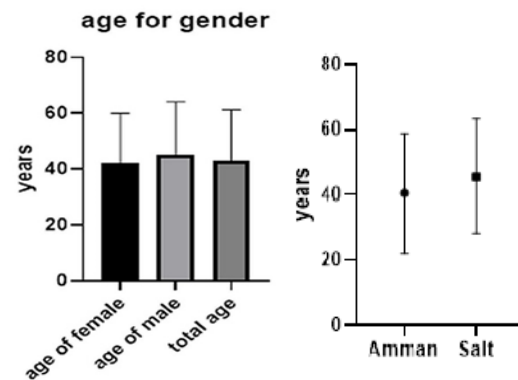


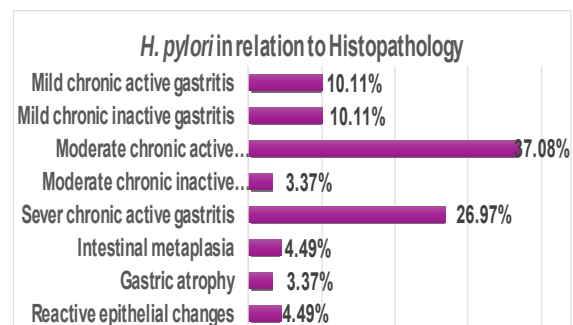
Figure 1. Mean age of the study group in relation to gender (A) and geographic distribution (B)

4.1. Characteristics of gastric biopsy by endoscopy

The endoscopic findings (in relation to the total number of cases) were categorized into normal gastric mucosa (30%), gastritis (65%), and gastric ulcer and carcinoma (5%).

4.2. *Helicobacter pylori* in relation to histopathology

Corresponding to histopathology, *H. pylori* was detected in 89 samples, and the results showed mild chronic active gastritis (10.11%), mild chronic inactive gastritis (10.11%), moderate chronic active gastritis (37.08%), moderate chronic inactive gastritis (3.37%), severe chronic active gastritis (26.97%), intestinal metaplasia (4.49%), gastric atrophy (3.37%), and reactive



epithelial changes (4.49%) (Figure 2).

Figure 2. Percentage of *H. pylori* detected in gastric biopsy in relation to various Histopathology results

4.3. Diagnosis of *Helicobacter pylori* using 16S rRNA

For the detection of *H. pylori*, the 16S rRNA gene was targeted by PCR and the amplified product was loaded on 1.5% agarose gel electrophoresis and photographed under UV light. The amplified gene gives a single band at 1471 bp. In total, 83 (66.4%) biopsy samples were *H. pylori* positive (Figure 3).

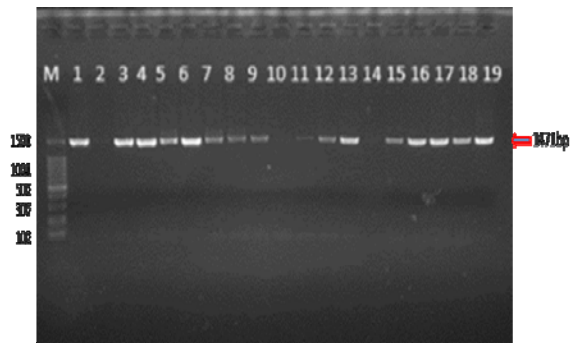


Figure 3. PCR amplification of 16SrRNA (1471 bp) gene. Lane M, 1500 bp DNA ladder; lane 1, positive control; lane 2, negative control; lane (3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 15, 16, 17, 18, 19), positive *VacA* results, lane (10 and 14) negative *VacA* results

The results demonstrated that females developed infections more often than males (62.5% vs. 37.5%), and participants aged 50 years or older showed a persistently elevated risk, indicating greater susceptibility to disease (Figure 4).

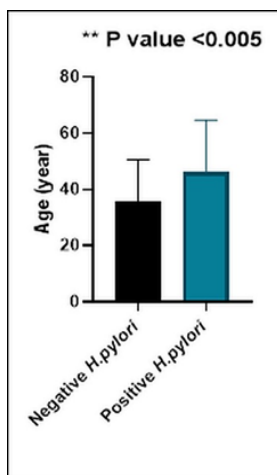


Figure 4. The prevalence of *H. pylori* in relation to age

4.4. Genotype characterization of *H. pylori* using uniplex and multiplex PCR

Using PCR, the presence of genes associated with pathogenicity was sought. The *VacA* gene was present in 62 (74.7%) of *H. pylori* isolates and shows a specific product with 678 bp (Figure 5).

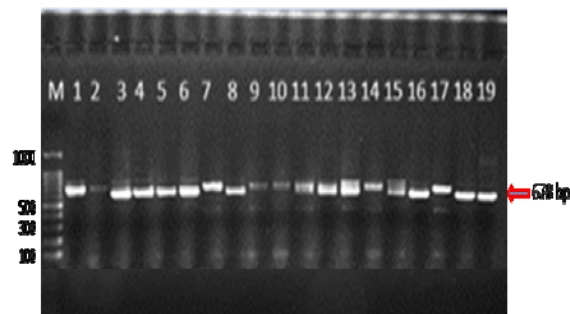


Figure 5. PCR amplification of *VacA* (678 bp) gene. Lane M, 1000 bp DNA ladder; lane 1, positive control; lane 2, negative control; lanes (3-19), positive *VacA* results

OipA is a crucial gene involved in *H. pylori* colonization of the stomach mucosa. A total of 36 (43.3%) isolates had the *OipA* gene. The allelic variants of *IceA1*

gene were also amplified by multiplex PCR (Figure 6). Of the isolates, 68 (81.9%) had the second allele form of *IceA2*, whereas 18 (21.7%) had a positive result for the *IceA1* (Figure 7).

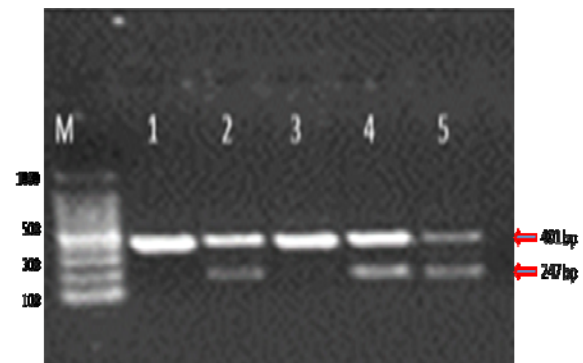


Figure 6. Amplification of *OipA* gene (401 bp) and *IceA1* gene (247 bp) applying Multiplex PCR. Lane M, 1000 bp DNA ladder; Lanes 2, 4, 5 positive results for the two genes; Lanes 1, 3 positive results for *OipA* gene only.



Figure 7. PCR amplification of *IceA2* (120 bp) gene. Lane M, 1000 bp DNA ladder; lane 1, positive control; lane 2, negative control; lanes (3-19) positive *IceA2* results.

To emphasize the adherence ability of *H. pylori* to the gastric mucosa, *BabA* gene was amplified with a 271 bp product. The gene was detected in 79 (95.2%) of the isolates (Figure 8).

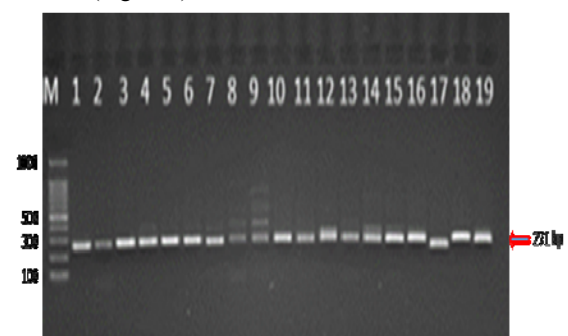


Figure 8. PCR amplification of *BabA* (271 bp) gene. Lane M, 1000 bp DNA ladder; lane 1, positive control; lanes (2-19) positive *BabA* gene results.

4.5. Association of virulence factors with histopathology results

Variable percentages for *H. pylori* virulence genes using PCR showed that *BabA*, *IceA2*, *IceA2*, *VacA* and *OipA* genes are increased in cases with severe chronic active gastritis and moderate chronic active gastritis in relation to other cases (Figure 9). Statistical analysis also showed that there is a significant relation between the

VacA gene and moderate chronic inactive gastritis ($p = 0.002$), severe chronic active gastritis ($p = 0.0009$). Besides, *OipA* gene was significantly related with moderate chronic inactive gastritis ($p = 0.008$), *IceA2* gene with mild chronic inactive gastritis ($p = 0.005$), and *IceA1* gene with severe chronic active gastritis ($p = 0.033$). Only *BabA* gene was

found to be associated with reactive epithelial change with P-value of 0.005.

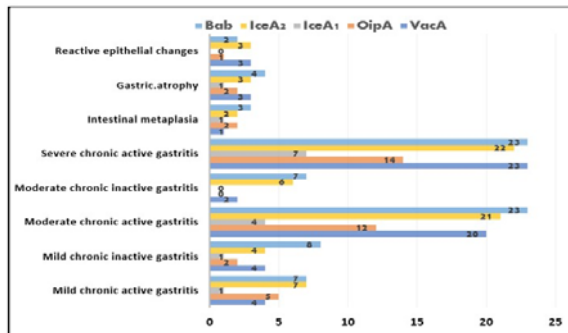


Figure 9. Histopathology results in relation to *H. pylori* genotype.

4.6. Dental samples result in relationship to gastric biopsy results

To investigate the potential role of human dental plaque as a reservoir for *H. pylori*, dental plaque samples were collected from 67 subjects who had undergone endoscopy. Only participants who provided informed consent for dental sample collection were included in the study. Fifty-six samples (83.58%) were positive for *H. pylori*. The prevalence of *H. pylori* in dental plaque was highest in patients with gastritis, $n = 39$ (69.6%), followed by the normal gastric mucosa, $n = 16$ (28.6%), and gastric ulcer & cancer, $n = 1$ (1.8%) (Figure 10A).

Considering the histopathology results of the 56 samples, a high percentage of *H. pylori* was detected in the dental plaque of patients diagnosed as mild chronic gastritis, $n = 18$ (32.1%), moderate chronic gastritis, $n = 22$ (39.3%). However, the lowest prevalence of dental *H. pylori* was detected in patients who had gastric atrophy, $n = 4$ (7.1%) and intestinal metaplasia, $n = 3$ (5.4%). In patients with severe chronic gastritis, *H. pylori* was identified in 9 (16.1%) samples obtained from dental plaque (Figure 10B).

The Chi-square test showed a significant relationship between dental PCR results and the findings by endoscopy (P -value < 0.05). The results revealed that 56 (83.58%) patients who were positive for *H. pylori* by PCR in gastric biopsy were also positive in dental plaque, whereas 11 (16.42%) dental plaque samples were negative for *H. pylori* and positive in gastric biopsy. Only 4 (7.14%) of the dental *H. pylori* positive patients were negative in gastric biopsy.

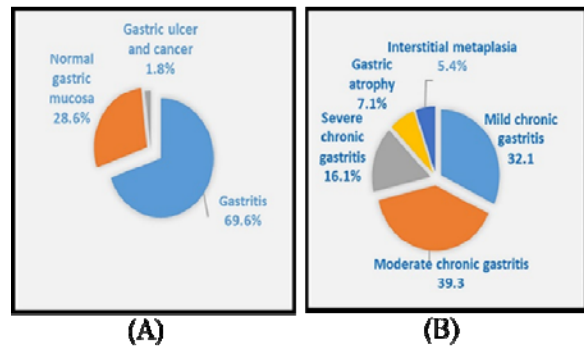


Figure 10. Prevalence of *Helicobacter pylori* in dental plaque in relation to gastric endoscopic (A) and histopathological findings (B)

5. Discussion

Infection with *H. pylori* increases the risk of severe clinical problems which is principally caused by the presence of distinct virulence factors that collectively contribute to the disease severity. Gastric biopsies obtained by endoscopy were utilized for histological examination and molecular analysis to ensure an accurate diagnosis of *H. pylori* infection. Endoscopy reveals gastritis as the primary diagnosis which affects 65% of patients, thus requiring fast detection of gastrointestinal disorders (Ayana *et al.*, 2014; Ho and Chiou, 2023). Histopathological analysis revealed that 71.2% of patients exhibited features associated with *H. pylori* infection, while PCR targeting 16S rRNA detected the bacterium in 66.4% of biopsies, indicating a high prevalence among patients undergoing upper endoscopy. A recent study by Al-Hyassat *et al.* (2025) employed real-time PCR targeting the 16S rRNA gene to detect *H. pylori* in 106 clinical samples, revealing a positivity rate of 74.5% in gastric biopsies, which aligns with our findings. In Jordan, seroprevalence studies reported that *H. pylori* affects 88% of the population nationwide (Obaidat and Roess, 2019).

These findings are comparable to previous research indicating a high global prevalence of *H. pylori*, particularly in the Middle East and North Africa, underscoring the need for improved diagnostic approaches and highlighting the bacterium's pivotal role in gastrointestinal disease progression (Alsulaimany *et al.* 2020).

The results revealed a predominance of female patients, consistent with earlier studies reporting that women are more likely to seek medical care for stomach issues and are more susceptible to specific gastrointestinal conditions (Al-Hyassat *et al.*, 2025; Feyisa & Woldeamanuel, 2021). Similarly, Qiao *et al.* (2024) observed a positive linear correlation between *H. pylori* infection rate and increasing age among women, with older females showing higher infection rates, lower eradication success, and greater recurrence. These findings highlight the complex interplay between age, gender, and *H. pylori* persistence. Bharucha and Lacy (2020) emphasized that digestive system disorders affect individuals across the lifespan, with negligible age disparities between sexes. Consistent with this, the current study found broadly comparable age distributions among patients, with those from Amman showing a slightly lower mean age than those from Al-

Salt; however, the difference was not statistically significant.

Transmission predominantly occurs through oral–oral or fecal–oral routes, most often in early childhood, with infections frequently remaining asymptomatic into adulthood in the absence of targeted antimicrobial intervention. The observed high prevalence may partly reflect insufficient public awareness of transmission ways and preventive health behaviors (Alaridah *et al.*, 2023; Kotilea *et al.*, 2019). However, identification of these fundamental elements enables healthcare professionals to create specific treatment plans (Cardos *et al.*, 2021; Shah *et al.*, 2024).

Histopathological examination revealed gastric impairment in all infected biopsy specimens, demonstrating the method's effectiveness in assessing tissue deterioration and detecting bacterial presence. Histological observations support the role of *H. pylori* infection in a wide range of gastrointestinal disorders, emphasizing its central involvement in disease pathophysiology. The findings further highlight the substantial contribution of *H. pylori* to the development of chronic gastritis and reinforce the diagnostic reliability of histopathological evaluation, consistent with previous reports (Dănilă *et al.*, 2022). Similarly, Santacroce *et al.* (2024) corroborated these results by demonstrating the role of *H. pylori* in chronic gastritis progression, including the development of gastric atrophy and intestinal metaplasia. These findings collectively align with earlier studies confirming that histopathology remains a critical method for confirming *H. pylori* infection in gastric biopsy samples (Al-Hyassat *et al.*, 2025).

The distribution of *H. pylori* virulence genes in positive isolates from the Jordanian cohort demonstrates a distinct regional pathogenic profile, with specific virulence factors significantly associated with the severity of gastric histopathological changes. Previous studies have shown that *H. pylori* infection can lead to chronic gastritis, peptic ulcers, and gastric cancer (Ansari & Yamaoka, 2019; Braga *et al.*, 2019; El-Sayed *et al.*, 2020). Consistent with these findings, our study highlights the role of these virulence genes as biomarkers, demonstrating a strong association with moderate to severe gastritis. Molecular analysis revealed a high prevalence of adhesin genes—*BabA* (95.2%) and *IceA2* (81.9%)—alongside the major cytotoxin *VacA* (74.7%). This pattern suggests that the dominant strains in Jordan are highly adapted for long-term colonization and persistence, facilitated by *BabA* binding to ABO/Lewis^xb (Leb) antigens on gastric epithelium. Chronic colonization underlies subsequent pathology, including the 65% prevalence of chronic gastritis observed in our endoscopic findings.

The *BabA* gene encodes an adhesin that attaches to Leb antigens, promoting bacterial colonization, chronic inflammation, and epithelial changes (Gerhard *et al.*, 1999; Zhao *et al.*, 2020; Singh *et al.*, 2024). Our findings suggest its significant association with reactive epithelial changes in gastric biopsies, highlighting its critical role in disease progression. Critically, the lower prevalence of *OipA* (43.3%) and *IceA1* (21.7%) compared to *BabA* and *VacA* indicates that, within this specific regional population, the most frequent pathogenetic route relies heavily on superior adhesion rather than solely on immediate inflammatory stimulation by *OipA*, or acute damage traditionally linked

to *IceA1*. This specific genotype ratio, therefore, provides the first detailed genotype-prevalence mapping for these five key factors in Jordan, a novel contribution essential for defining local strain pathology.

The research investigates how *H. pylori* virulence factors affect histopathological results by studying genes which produce significant clinical effects. The higher frequency of *BabA*, *IceA2* genes in severe and moderate chronic active gastritis cases was corroborate earlier findings supporting its role in gastric diseases (Phan *et al.*, 2017). The *VacA* gene plays a critical role in the progression of gastric disease. It supports regulate the host's immune response and can influence how severe an infection develops. In our study, higher *VacA* gene expression was significantly associated with both severe gastric disease ($p=0.0009$) and chronic active gastritis ($p=0.002$). Importantly, *VacA* was not detected in normal gastric mucosa, reinforcing its value as a marker specific to disease (Al-Ouqaili *et al.*, 2023). Multiplex PCR showed a lower prevalence of *IceA1* and a higher prevalence of *IceA2* in our cohort, consistent with the findings of Yakoob *et al.* (2015) and contrasting with earlier reports for *IceA1* (Feliciano *et al.*, 2015). These findings support the role of *IceA2* in severe gastrointestinal disorders, including peptic ulcers and gastric cancer through its association with IL-8 overexpression ((Yakoob *et al.*, 2015; Baj *et al.*, 2020). Additionally, our results show a significant association between *IceA1* and *IceA2* alleles and severe chronic active gastritis, consistent with Akeel *et al.* (2019), who reported that these genes correlate with varying degrees of gastric inflammation and pathology.

The ability of *H. pylori* to colonize the oral cavity is becoming more widely acknowledged. Once the stomach is cleared, it may act as a chronic reservoir that leads to reinfection.

This gap limits our understanding of why most patients continue to experience recurrent infection despite successful treatment. Turning to the oral environment, a successfully isolated viable *H. pylori* from infected root canals demonstrated that the bacteria can persist within protected oral tissues and supports the possibility that the oral cavity may act as a reservoir (Elger *et al.*, 2024). The detection of *H. pylori* DNA, including the pathogenic *cagA* gene, was found in dental plaque and saliva (Wongsuwanlert *et al.*, 2023). It has also been found more often in deeper periodontal pockets, particularly when the periodontal pathogen *Porphyromonas gingivalis* is present (Kadota *et al.*, 2020). The likelihood of oral colonization appears to be influenced by lifestyle and environmental factors, such as dietary habits and oral hygiene. Poor oral hygiene has been consistently associated with higher detection rates. Although a link between oral and gastric *H. pylori* has been previously reported (Cai *et al.*, 2014), the relationship between oral infection and gastrointestinal disorders remains unclear. The severity of *H. pylori* infections has not significantly declined, and even after successful eradication, recurrence rates remain high, largely due to oral colonization. In our study, a strong significant correlation was observed between *H. pylori* isolates from dental plaque and *H. pylori*-positive gastric samples, with the highest oral prevalence observed in patients with gastritis (69.6%). Miyabayashi *et al.* (2000) examined patients with *H. pylori* gastritis to evaluate the

relationship between successful gastric eradication and the persistence of oral *H. pylori* after treatment. Their conclusion indicated that oral colonization by *H. pylori* negatively impacts eradication success and may contribute to the recurrence of gastric infection.

Our study indicates that dental plaque acts as a reservoir for *H. pylori*, facilitating recurrent infections after gastric eradication therapy. The high prevalence of infection in gastritis patients (69.6%) highlights the bacterium's involvement in the early stages of gastric disease. These findings align with previous research showing higher *H. pylori* detection rates in non-atrophic gastritis (Cuba *et al.*, 2023). Our results further support the inclusion of oral sites in diagnostic and therapeutic strategies to prevent reinfection (Cuba *et al.*, 2025; Fan *et al.*, 2025; Anand *et al.*, 2025; Scholz *et al.*, 2025). While oral *H. pylori* detection can serve as an early biomarker for gastritis, its clinical utility diminishes as gastric pathology progresses toward precancerous stages, underscoring the critical role of the oral cavity in the bacterium's life cycle.

6. Conclusion

This study shows that *H. pylori* virulence factors *VacA*, *BabA*, and *OipA* play a major role in severe gastric diseases. The findings highlight the importance of early detection, thorough diagnostics, and personalized treatment plans. The high prevalence of *H. pylori* in dental plaque highlights oral hygiene as a critical component of infection. The bacterium's persistence in the gastrointestinal tract can lead to chronic inflammation and increase the risk of cancer. Managing these infections effectively requires better diagnostic tools, tailored therapies, and preventive strategies, which should be priorities in both clinical care and public health. Future research should focus on large-scale, long-term studies to pinpoint the most critical virulence factors, investigate the oral cavity as a potential reservoir for recurrent infection, and refine diagnostic and treatment approaches by comparing gastric and oral bacterial strains.

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Institutional Review Board Statement

The Ministry of Health's Institutional Review Board authorized this study and was carried out in agreement with the Declaration of Helsinki, Amman, Jordan (MBA/IRB/3822).

The research required all participants to give their consent before joining the study.

The research data appears in the article as its primary original contribution. The corresponding author will receive all additional inquiries.

The authors state no conflict of interests in this work.

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