



Hashemite Kingdom of Jordan



Jordan Journal
of



Biological Sciences

An International Peer-Reviewed Scientific Journal

Financed by the Scientific Research and Innovation Support Fund



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المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)

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Jordan Journal of Biological Sciences (JJBS) (ISSN: 1995–6673 (Print); 2307-7166 (Online)); An International Peer- Reviewed Open Access Research Journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research, Jordan and published quarterly by the Deanship of Scientific Research , The Hashemite University, Jordan.

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Jordan Journal of Biological Sciences (JJBS)
ISSN 1995- 6673 (Print), 2307- 7166 (Online)

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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.
March, 2024

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Tolectin-Induced Apoptosis in Human Gastric Adenocarcinoma and Cervical Cancer Cells Through Caspase-Dependent Pathways

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Received: October 1, 2025; Revised: November 23, 2025; Accepted: December 20, 2025

Abstract

The cytotoxic effects of Tolectin on cancer cells have been reported, but its underlying mechanism remains unclear. This study aimed to evaluate the apoptotic effects of Tolectin on gastric (AGS) and cervical (HeLa) cancer cells *in vitro*. AGS and HeLa cells were treated with various concentrations of Tolectin, while untreated cells served as controls. Cell viability was assessed using MTT assay, and the half-maximal inhibitory concentration (IC₅₀) was determined. Expression levels of Bcl-2, BAX, and P53 genes were measured by quantitative RT-PCR. Caspase-3, -8, and -9 activities were evaluated using colorimetric assays. Apoptosis was confirmed by flow cytometry. Data were analyzed using ANOVA and Student's t-test. Tolectin treatment significantly decreased cell viability in both AGS and HeLa cells. The BAX/Bcl-2 ratio increased to 1.557 in AGS and 1.385 in HeLa cells at IC₅₀ concentrations. No significant changes in P53 expression were observed. Caspase-3, -8, and -9 activities were significantly elevated in treated cells compared to controls ($p < 0.05$). Flow cytometry confirmed apoptosis induction in both cell lines. Tolectin exerts cytotoxic effects on AGS and HeLa cells through apoptosis, involving both intrinsic and extrinsic pathways.

Keywords: Gastric cancer, Cervical cancer, Apoptosis, Tolectin, AGS cell line, HeLa cell line

1. Introduction

Tolectin® is one of the most important non-steroidal anti-inflammatory drugs (NSAIDs), commonly used to reduce pain, swelling, tenderness, and stiffness in conditions such as osteoarthritis and rheumatoid arthritis (Park *et al.*, 2023). Tolectin, similar to other NSAIDs, may exert anticancer effects through caspase-dependent pathways, yet its effects on gastric and cervical cancer cells remain unexplored. Although its mechanism of action is not fully understood, Tolectin reduces cyclooxygenase activity and, consequently, the production of prostaglandins (Gao W *et al.*, 2024). Beyond their anti-inflammatory effects, NSAIDs have shown significant potential in cancer prevention, including cancers of the breast, uterus, and gastrointestinal tract (Chapelle *et al.*, 2020; Meyerhardt *et al.*, 2021; Bakrim *et al.*, 2025).

Gastric cancer, one of the most common gastrointestinal malignancies, exhibits a higher mortality rate in men compared to women (Aalipour *et al.*, 2022; Thrift *et al.*, 2023). Infection with *Helicobacter pylori* has been implicated in over 60% of gastric cancer cases. Cervical cancer, originating in the cervix, is primarily classified as either epidermoid carcinoma or adenocarcinoma and is the third most common cancer in women worldwide, often associated with human papillomavirus (HPV) infection (Arbyn *et al.*, 2020).

Treatment strategies for gastric and cervical cancers include radical surgery, chemotherapy, immunotherapy, and targeted therapy (Kumar *et al.*, 2024; Ren *et al.*, 2025). Recent studies have explored the anticancer potential of NSAIDs, demonstrating their ability to inhibit cell proliferation and induce apoptosis in various cancer types (Li J *et al.*, 2023; Robledo-Cadena DX 2024). Specifically, NSAIDs such as diclofenac have exhibited anti-proliferative effects on colorectal, ovarian, and esophageal cancer cells (Wang *et al.*, 2024; Tabaku *et al.*, 2023; Fletcher *et al.*, 2021). *In vitro* studies have also highlighted the role of NSAIDs in apoptosis induction through activation of caspase pathways, including Caspase-3, Caspase-8, and Caspase-9, as well as modulation of anti-apoptotic (*BCL2*) and pro-apoptotic (*BAX*) gene expression, and upregulation of the tumor suppressor gene *TP53* (Zhang *et al.*, 2020; Azarshab *et al.*, 2021; Wong, 2019; Nagy *et al.*, 2021). Nevertheless, contradictory findings exist, as some studies report that NSAIDs may not induce apoptosis in certain cancer cells or could even promote proliferation (Kazberuk *et al.*, 2020). Therefore, the present study was designed to comprehensively investigate the cytotoxic and apoptosis-inducing effects of Tolectin on gastric (AGS) and cervical (HeLa) cancer cells *in vitro*. Specifically, this study focused on elucidating the involvement of caspase-dependent pathways and examining the regulation of critical apoptotic genes, including *TP53*, *BAX*, and *BCL2*. Although numerous studies have demonstrated the

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anticancer potential of various non-steroidal anti-inflammatory drugs (NSAIDs) in different cancer models, the specific effects of Tolectin on gastric and cervical cancer cells remain largely unexplored. Addressing this gap, the current research aims not only to evaluate Tolectin-induced cytotoxicity and apoptotic responses but also to provide mechanistic insights into its modulation of key molecular regulators of programmed cell death, thereby contributing to the understanding of its potential as a novel therapeutic agent for gastrointestinal and gynecological malignancies.

2. Materials and Methods

2.1. Ethics Statement

This study was reviewed and approved by the International Association of Scientists (IAS) Ethics Committee (Ref: IASSP.22.1CL), and all experimental procedures were conducted in accordance with institutional guidelines for *in vitro* research.

2.2. Cell Lines and Cell Culture

Human gastric adenocarcinoma (AGS) and cervical cancer (HeLa) cell lines were obtained from the National Cell Bank of Iran (NCBI). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin under standard culture conditions (37°C, 5% CO₂, humidified atmosphere). When cultures reached 70–80% confluency, cells were washed with phosphate-buffered saline (PBS), detached using trypsin–EDTA for 3–4 min at 37°C, neutralized with culture medium containing 10% FBS, and collected by centrifugation. The resulting cell pellet was resuspended in fresh culture medium for subsequent experiments.

2.3. Tolectin Preparation and Rationale

Tolectin powder was dissolved in PBS and DMEM to prepare stock solutions, followed by serial dilutions to yield final concentrations of 0.0012, 0.0023, 0.0049, 0.0097, 0.0194, and 0.0389 mmol/mL. These concentrations were selected based on preliminary pilot studies conducted in our laboratory, designed to identify both sub-toxic and cytotoxic doses for AGS and HeLa cells. Fresh Tolectin solutions were prepared prior to each experiment to ensure stability and reproducibility (Ghasemi *et al.*, 2023; Rao *et al.*, 2023).

2.4. Cell Viability Assay (MTT)

Cell viability was assessed using the MTT assay. AGS and HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere for 24 hours. Cells were then treated with indicated concentrations of Tolectin for 24 hours. After treatment, cells were washed with PBS, and 0.05 mg/well MTT solution (DO BIO Biotech, Shanghai, China) was added, followed by incubation for 3 hours at 37°C in darkness. Formazan crystals were solubilized in 100 μ L DMSO per well, and absorbance was measured at 570 nm using a microplate reader (BioTek, USA). Cell viability (%) was calculated as $[\text{OD}(\text{sample})/\text{OD}(\text{control}) \times 100]$. Experiments were performed in eight replicates. The half-maximal inhibitory concentration (IC₅₀) of Tolectin was calculated using a linear regression model ($Y = aX + b$), where Y represents

the fraction of viable cells, X is the Tolectin concentration, a is the slope, and b is the Y-intercept. IC₅₀ was determined as $\text{IC}_{50} = (0.5 - b)/a$.

2.5. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

AGS and HeLa cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated for 24 hours. Cells were treated with the effective concentration of Tolectin (0.0131 mmol/mL) for 24 hours. Total RNA was extracted using the RNA-isolation kit (Transgene Biotech, ER101-01) according to the manufacturer's instructions. The concentration and purity of RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA), and samples with an A260/A280 ratio between 1.8–2.0 were used for cDNA synthesis. First-strand cDNA was synthesized using the PrimeScript™ First-Strand cDNA Synthesis Kit (Transgene Biotech, AE301-02).

qRT-PCR was performed using 10 μ L Power SYBR Green PCR Master Mix (2X), 1 μ L of each specific primer (Table 1), 1 μ L cDNA (100 ng), and 7 μ L double-distilled water. Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was performed to confirm primer specificity. Gene expression of *TP53*, *BAX*, and *BCL2* was normalized to GAPDH and calculated using the $2^{-\Delta\Delta\text{CT}}$ method. All reactions were performed in triplicate, and results are presented as mean $\pm$ standard deviation (SD).

Table 1. Primer sequences used in qRT-PCR

Gene	Primer Sequences
Bcl-2	Forward: 5'-GGTGCCGGTTCAGGTA CTCA-3'
	Reverse: 5'-TTGTGGCCTTCTTGA GTTCG-3'
BAX	Forward: 5'-CGGCAACTTCAACTGGGG-3'
	Reverse: 5'-TCCAGCCCAACAGCCG-3'
P53	Forward: 5'-CATCTACAAGCAGTCACAGCACAT-3'
	Reverse: 5'-CAACCTCAGGCGGCTCATAG-3'
GAPDH	Forward: 5'-CCCACTCTCCACCTTTGAC-3'
	Reverse: 5'-CATACCAGGAAATGAGCTTGACAA-3'

2.6. Caspase-3, -8, and -9 Activity Assay

Caspase activity was measured using colorimetric assay kits for caspase-3, caspase-8, and caspase-9 (Novex). AGS and HeLa cells were treated with the effective concentration of Tolectin (0.0131 mmol/mL) and incubated at 37°C for 24 h in a humidified atmosphere with 5% CO₂. The cells were then trypsinized and centrifuged at $600 \times g$ for 5 min. The resulting pellets were lysed in 50 μ L of chilled cell lysis buffer and kept on ice for 10 min. The lysates were centrifuged at $10,000 \times g$ for 1 min, and the supernatants were collected.

For each assay, 200–300 μ g of total protein was mixed with 50 μ L of cell lysis buffer. Subsequently, 50 μ L of $2\times$ reaction buffer containing 10 mM DTT was added to each sample. pNA-conjugated substrates specific for each caspase (final concentration: 200 μ M) were then added, and the mixtures were incubated at 37°C for 2 h.

Absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA) at 400 nm. Caspase activity was calculated according to Equation (1):

Caspase activity (%) = $[(\text{Abs_sample} - \text{Abs_blank}) / \text{Abs_control}] \times 100$ (24)

Protein concentrations were determined using the Bradford assay.

2.7. Apoptosis Quantification by Flow Cytometry

Apoptotic cells were quantified using Annexin-V-FLUOS and propidium iodide (PI) staining. AGS and HeLa cells were seeded in 24-well plates and treated with the effective concentration of tolectin (0.0131 mmol/mL). The cells were incubated at 37°C in a humidified incubator containing 95% air and 5% CO₂ for 48 h.

After treatment, the cells were stained with Annexin-V-FLUOS and PI and incubated for 15 min at 25°C in the dark. Apoptotic and necrotic cells were visualized using a fluorescence microscope with an excitation/emission range of 515–565 nm (Karimi M *et al.*, 2023; Amiri *et al.*, 2025).

2.8. Statistical Analysis

Data were analyzed using SPSS version 21. Normality was assessed with the Kolmogorov–Smirnov test. Statistical comparisons were performed using one-way ANOVA and Student's t-test, with $p < 0.05$ considered statistically significant. Results are presented as mean $\pm$ standard deviation (SD).

3. Results

3.1. Cytotoxic effect of tolectin on AGS and HeLa cells

The MTT assay demonstrated that all tested concentrations of Tolectin (0.0012, 0.0023, 0.0049, 0.0097, 0.0194, 0.0389 mmol/mL) significantly reduced the viability of both AGS and HeLa cells compared to the control group ($p < 0.01$ – 0.001) (Fig. 1 and 2). The calculated IC₅₀ values were 0.0131 mmol/mL for AGS cells and 0.0147 mmol/mL for HeLa cells, indicating potent cytotoxic activity of Tolectin in a concentration-dependent manner.

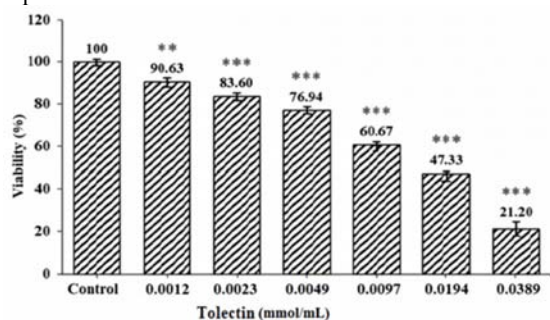


Figure 1. Viability of AGS cells treated with different concentrations of Tolectin. * indicates significant difference compared with control group (**: $p < 0.01$, and ***: $p < 0.001$).

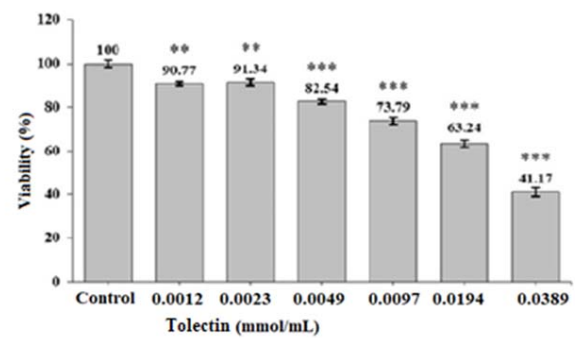


Figure 2. Viability of HeLa cells treated with different concentrations of Tolectin. * indicates significant difference compared with control group (**: $p < 0.01$, and ***: $p < 0.001$).

3.2. Effect of Tolectin on Expression Levels of BCL2, BAX, and TP53 in AGS and HeLa Cells

qRT-PCR analysis revealed that treatment of AGS cells with the effective concentration of Tolectin (IC₅₀) significantly decreased the relative expression of *BCL2* and *BAX* genes compared with the control group ($p < 0.001$ and $p < 0.01$, respectively). Conversely, in HeLa cells, Tolectin treatment led to a significant increase in *BCL2* and *BAX* expression levels ($p < 0.01$ and $p < 0.001$, respectively). The *BAX/BCL2* ratio was calculated as 1.557 and 1.385 in AGS and HeLa cells, respectively, suggesting activation of apoptosis pathways, particularly in AGS cells (Fig. 3–5). The relative expression of *TP53* did not show significant changes in either cell line. These differences between AGS and HeLa cells may reflect cell-type-specific responses to Tolectin, potentially due to inherent differences in apoptotic regulatory mechanisms.

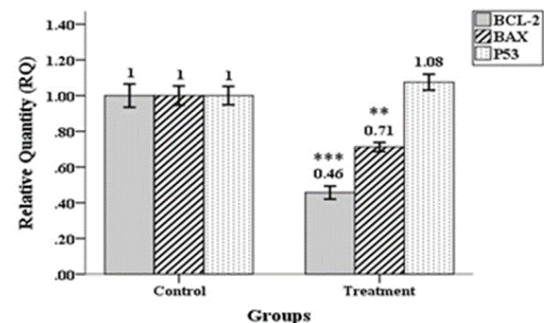


Figure 3. Relative quantitative expression (RQ) of Bcl-2, *BAX* and P53 gene in AGS cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (**: $p < 0.01$, ***: $p < 0.001$).

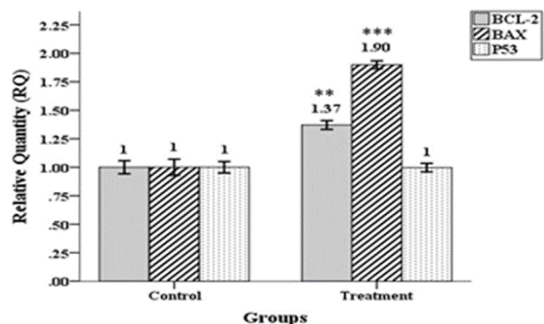


Figure 4. Relative quantitative expression (RQ) of Bcl-2, *BAX* and P53 gene in HeLa cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (**: $p < 0.01$, ***: $p < 0.001$).

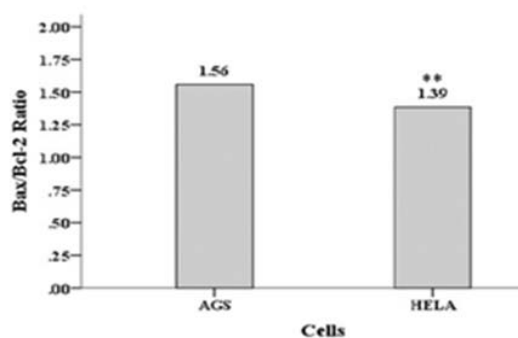


Figure 5. *BAX/BCL2* expression level ratio in AGS and HeLa cells treated with effective concentration of Tolectin.

3.3. Effect of tolectin on caspase-3, -8 and -9 activity level in AGS and HeLa cells

Colorimetric caspase assays indicated that Tolectin treatment significantly increased the activity of caspase-3, -8, and -9 in both AGS and HeLa cells compared to controls ($p < 0.05$ – 0.001) (Fig. 6 and 7). The elevated caspase activity corroborates the induction of caspase-dependent apoptotic pathways by Tolectin.

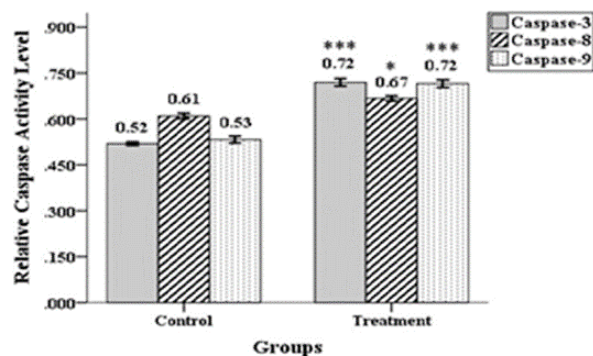


Figure 6. Activity level of caspase-3, -8, and -9 in AGS cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (*: $p < 0.05$, ***: $p < 0.001$).

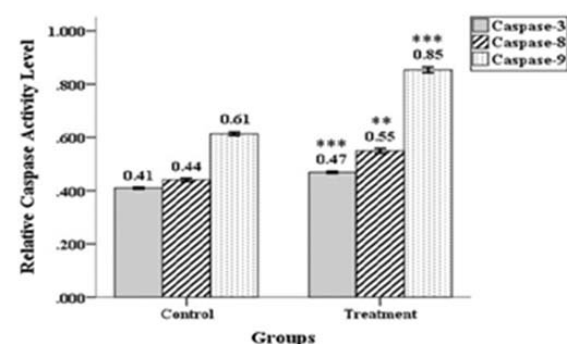


Figure 7. Activity level of caspase-3, -8, and -9 in HeLa cells treated with effective concentration of Tolectin compared with control group. * represents significant difference compared to control group (**: $p < 0.01$, ***: $p < 0.001$).

3.4. Apoptosis Induction Detected by Flow Cytometry

Flow cytometric analysis confirmed that Tolectin induced apoptosis in both cell lines. In AGS cells, primary apoptosis was predominantly observed, whereas in HeLa cells, both early and late apoptosis phases were evident. The percentage of apoptotic cells significantly increased in

treated groups compared to controls ($p < 0.05$ – 0.001) (Fig. 8), supporting the cytotoxic and pro-apoptotic effects of Tolectin.

3.5. Summary

Overall, Tolectin exhibited concentration-dependent cytotoxicity in AGS and HeLa cells, modulated key apoptotic genes (*BCL2* and *BAX*), activated caspases, and induced apoptosis, highlighting its potential as an anti-cancer agent with cell-type-specific effects.

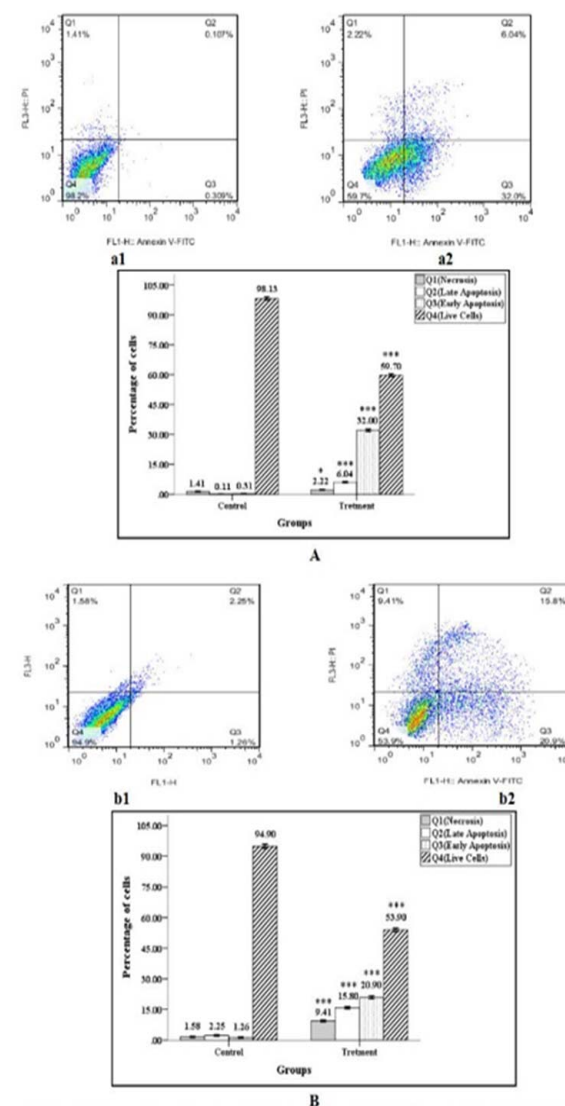


Figure 8. Apoptosis in AGS and HeLa cells induced by effective concentration of Tolectin: Q1: Necrosis; Q2: Late Apoptosis; Q3: Early Apoptosis; Q4: Viable cells. (a1) Control (untreated) AGS cells; (b1) Control (untreated) HeLa cells, (a2) AGS cells treated with effective concentration of tolectin, (b2) HeLa cells treated with effective concentration of Tolectin. A and B indicate apoptosis phases (Q1 – Q4) compared to control groups in AGS and HeLa cells, respectively. The analysis was made by FACSDiva Version 6.1.3. * and *** represent significant difference compared to control group (*: $p < 0.05$, ***: $p < 0.001$).

4. DISCUSSION

This study comprehensively evaluated the cytotoxic and apoptosis-inducing effects of Tolectin on AGS gastric and HeLa cervical cancer cells. The MTT assay results demonstrated a significant, concentration-dependent

decrease in cell viability, with an IC₅₀ of 0.0131 mmol/mL for both cell lines. Apoptotic induction was confirmed through increased activity of caspase-3, -8, and -9, as well as modulation of the *BAX/Bcl-2* ratio, indicating activation of both intrinsic and extrinsic apoptotic pathways (Norouzi *et al.*, 2020a; Norouzi *et al.*, 2020b).

The anticancer potential of NSAIDs has been well documented, primarily through inhibition of cell proliferation and induction of apoptosis via mitochondrial and death-receptor pathways. In line with these findings, Tolectin effectively modulated apoptotic regulators, increasing the *BAX/Bcl-2* ratio and caspase activity, thereby promoting programmed cell death (Ahmadi *et al.*, 2019; Kassab, 2025). Specifically, activation of caspase-9 indicates engagement of the intrinsic mitochondrial pathway, whereas elevated caspase-8 activity supports involvement of the extrinsic death-receptor pathway (Arisan *et al.*, 2019).

Interestingly, *TP53* expression did not show a statistically significant change in either AGS or HeLa cells. This may reflect cell-type-specific responses, basal *p53* status, or the presence of functional mutations in these cell lines (Li Q., 2017; Mishra *et al.*, 2024). Nevertheless, the observed pro-apoptotic effects suggest that Tolectin can induce apoptosis independently of *TP53* modulation, highlighting its potential applicability in tumors with *p53* mutations or functional inactivation. These findings underscore the importance of exploring alternative pathways that contribute to Tolectin-induced cell death (Brandolini *et al.*, 2020; Thiruchenthoooran *et al.*, 2023; Maytalman & Nemutlu Samur, 2025; Kazberuk *et al.*, 2022; Kolawole & Kashfi, 2022).

Although NSAIDs generally exert pro-apoptotic effects, previous reports have shown cell-type-dependent variability or even anti-apoptotic outcomes under certain conditions (Du *et al.*, 2023; Erfani *et al.*, 2018; Bhagavatula *et al.*, 2024). Factors such as drug concentration, exposure duration, and genetic background of the cells likely contribute to these discrepancies. In this study, both AGS and HeLa cells displayed robust apoptotic responses, indicating that Tolectin may possess broadly effective anticancer activity across different tumor types.

This study has several limitations. First, experiments were limited to *in vitro* assays, and apoptosis was assessed using a restricted set of markers. Second, while caspase activation and *BAX/Bcl-2* modulation were evaluated, other critical pathways such as NF- κ B, PI3K/AKT, and mTOR, as well as mitochondrial membrane potential changes and cytochrome-c release, were not investigated (Hesham *et al.*, 2025; Song *et al.*, 2024; Kassab *et al.*, 2021; Zhang *et al.*, 2024). Further studies should include these analyses to clarify the molecular mechanisms underlying Tolectin-induced apoptosis. Moreover, *in vivo* studies, including xenograft models and combination therapy assays with standard chemotherapeutics, are warranted to evaluate potential synergistic effects and to determine whether Tolectin can overcome drug resistance.

In conclusion, Tolectin induces significant apoptosis in AGS gastric and HeLa cervical cancer cells via both intrinsic and extrinsic pathways, accompanied by *BAX/Bcl-2* modulation and caspase activation (Kwatra *et al.*, 2024; Chu *et al.*, 2024; Marei, 2025; Rai *et al.*, 2022;

Jan & Chaudhry, 2019). The compound demonstrates potential as a promising candidate for further preclinical evaluation, particularly for tumors resistant to conventional apoptosis-inducing therapies. Future research should focus on elucidating additional molecular mechanisms and assessing the therapeutic efficacy of Tolectin *in vivo* to validate its translational potential.

5. Conclusions

Our study is the first to examine the cytotoxic and apoptotic effects of Tolectin on gastric (AGS) and cervical (HeLa) cancer cells *in vitro*. The results indicate that the cytotoxic effects of Tolectin are mediated through both intrinsic and extrinsic apoptotic pathways, while the expression of the tumor suppressor gene *TP53* did not significantly change. These findings suggest that Tolectin may have potential as an anticancer agent; however, further studies are needed to investigate its effects *in vivo* (Kan *et al.*, 2025; Al-Qadi *et al.*, 2023).

This study has several limitations. Experiments were conducted only *in vitro* using two cancer cell lines, which may not fully represent the tumor microenvironment or systemic interactions *in vivo* (Darfeuille *et al.*, 2024). Only a limited number of apoptosis markers (*BAX*, *BCL2*, and caspases-3, -8, and -9) were examined, and other molecular pathways involved in Tolectin-induced cytotoxicity were not evaluated (Li *et al.*, 2025). Potential off-target effects and the pharmacological safety of Tolectin were also not assessed. Future research should include *in vivo* studies and additional cancer models to better define the therapeutic potential and safety profile of Tolectin as an anticancer agent (Shafiei *et al.*, 2024).

Funding

This research received no external funding. The work was supported by the International Association of Scientists (IAS) and the Global Research, Education, and Event Network (GREEN). This study was not part of a funded student thesis.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This study did not involve human participants or animals. All experiments were conducted *in vitro* using established human cell lines. The handling and experimentation with these cell lines were performed in accordance with standard ethical guidelines and laboratory safety regulations. The study protocol was reviewed and approved by the International Association of Scientists (IAS) Ethics Committee (Ref: IASSP.22.1CL).

Author Contributions

Rahim Ahmadi: Conceptualization, writing – review and editing, final approval.

Mahsa Kavousi: Conceptualization, writing – review and editing, final approval.

Mirela Tabaku: Project administration, writing – review and editing, final approval.

Sanaz Noroozi: Methodology, formal analysis, writing – original draft preparation, writing – review and editing, final approval.

Masoumeh Aalipour: Writing – original draft preparation, writing – review and editing, final approval.

All authors have read and approved the final manuscript.

Data Availability

The datasets generated and/or analyzed during the current study are openly available at:

<https://drive.google.com/file/d/19QeK2jueVWDImjdH1zUqV9xTVFAOovX5O/view?usp=sharing>

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In Vitro Callus Cultured of *Epimedium Brevicornum* as Sustainable Source of Icariin with Demonstrated Hepatic Protective Activity Against Cadmium Toxicity

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Received: November 7, 2025; Revised: December 16, 2025; Accepted: December 24, 2025

Abstract

In vitro experiment was conducted to develop callus cultures of *Epimedium brevicornum* for sustainable icariin (ICA) production. Callus development was enhanced on Murashige and Skoog (MS) basal medium supported by selected growth regulators. HPLC results showed that callus cultures yielded high ICA content, suggesting tissue culture as an efficient alternative for controlling ICA production. Icariin (ICA) is a naturally plant flavonoid product used for its diverse biological and pharmacological uses. Cadmium (Cd) is a widespread and highly hazardous heavy metal that poses severe toxicity risks to both human and animal health. However, the shielding impact of ICA on hepatic damage caused by Cd has not yet been explored. For this purpose, 40 mice were randomly assigned into four investigational groups: CON, ICA, Cd, and ICA+Cd. Experimental mice were garaged with either ICA (100 mg/kg BW), Cd (5 mg/kg BW), and co-treatment via intragastric administration for 2 weeks. Results indicated that Cd-alone treated mice induced hepatic injury as evidenced by increased liver function (ALP, ALT, AST, and GGT) enzymes and induced histopathological alteration in mice. Moreover, Cd exposure induced oxidative stress by reducing activities and expression of antioxidant-related genes (GSH-Px, T-AOC, GSH, T-SOD, and CAT) and accumulation of MDA content in the liver of mice. The Cd treated group showed increased inflammatory response as evidenced by increased pro-inflammatory (TNF- α , IL-1 β , IL-6) and a decrease in anti-inflammatory (IL-10) mediator. Interestingly, ICA treatment restored the abovementioned alterations and alleviated hepatic injury by reducing oxidative stress and inflammation that was initiated by Cd. Eventually, the present finding indicates that the protective effect of ICA against Cd-inflicted hepatic damage is associated with inhibiting inflammatory response and oxidative stress in mice.

Keywords: Callus, Cadmium chloride, Icariin, Liver, Oxidative stress, Inflammation

1. Introduction

Traditional Chinese Medicine (TCM) has gained popularity and medical importance due to its low toxic effects and multi-targeted treatments (Ma *et al.*, 2019). Within TCM, identifying active medicinal compounds is a major research focus. The herb *Epimedium* (commonly known as Horny Goat Weed or *Yin Yang Huo*) has been used in China since ancient times for the treatment of various disease (Ma *et al.*, 2011). Icariin (ICA), a biologically active flavonoid glycoside extracted from the *Epimedium brevicornum*, is widely recognized for its pharmacological activity. Previous studies have demonstrated that ICA alleviates oxidative stress and lipid peroxidation in multiple models of toxicant-induced injury (shaukat *et al.*, 2022, chung *et al.*, 2008, el-Shitany & Eid, 2019). Moreover, ICA enhances antioxidant enzyme activity in organs such as liver, kidney, spleen, and testes (Ma *et al.*, 2015, Zhao *et al.*, 2020, Sai *et al.*, 2022, Ma *et al.*, 2023). In addition, ICA exhibits anti-inflammatory (Bi *et al.*, 2022), anti-apoptotic (Jia *et al.*, 2019), anti-osteoporosis (Liu *et al.*, 2024), and anti-tumor effects (Zhou *et al.*, 2011). Collectively, these findings suggest

that ICA may offer protection against cadmium (Cd)-induced hepatic damage.

Plant tissue culture has emerged as an effective and environmentally friendly approach for producing secondary metabolites (Prashant & Bhawana, 2024). Through callus induction, plants can biosynthesize flavonoids and phenolics in controlled conditions, independent of season and geography (Yamamoto, *et al.*, 1992). In *Epimedium* species, *in vitro* callus cultures have been shown to enhance icariin yield due to optimized hormonal and nutritional conditions (Mihaljevic, 2009). The application of Plant tissue culture has expanded across numerous medicinal species, serving purposes such as rapid multiplication, germplasm preservation, secondary metabolite production, and genetic advancement (Shatnawi, *et al.* 2011). *In vitro* conservation provides an alternative approach to traditional field conservation (Shahrour, *et al.* 2024). Plant tissue culture, particularly callus culture, offers a sustainable and controlled approach for producing pharmacologically important secondary metabolites with consistent quality and yield. Unlike field-grown plants, callus cultures enable continuous production independent of environmental and seasonal variability, ensuring reproducibility for downstream biological studies

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(Murthy *et al.*, 2014; Espinosa-Leal *et al.*, 2018). In this study, callus culture was optimized to obtain icariin as a standardized source, which was subsequently used to evaluate its hepatoprotective effects in a cadmium-induced toxicity model. Sustainable production of icariin is critical due to increasing demand and ecological concerns related to overharvesting of *Epimedium* species. Tissue culture-based systems provide an environmentally friendly alternative that supports scalability and batch-to-batch consistency, which are essential for pharmacological and toxicological investigations (Verpoorte *et al.*, 2002; Cragg & Newman, 2013).

Cd is a prevalent heavy metal emitted by industrial activities or naturally occurring in the environment. It has significant toxicity and represents a risk to both human and animal health (Mishra *et al.*, 2019). The primary ways that Cd enters the human body are through cigarette smoke, polluted water and food, and industrial exposure (Suhani *et al.*, 2021). Studies have shown that Cd accumulates in the body, leading to pathological damage in the kidneys, liver, testes, spleen, bones, lungs, and neurological system (Fang *et al.*, 2021, Genchi *et al.*, 2020, Davidova *et al.*, 2024). The International Agency for Research on Cancer has classified Cd as a potent carcinogen because of its widespread environment occurrence and cumulative toxic effect in humans (Joseph, 2009). The liver is among the most severely affected organs and is particularly vulnerable both acute and chronic Cd-induced injury (Yang *et al.*, 2021). Epidemiological and experimental investigations have established that Cd exposure leads to hepatic oxidative damage, inflammation, fibrosis, and impaired liver function (Yang *et al.*, 2021, Fang *et al.*, 2021). Oxidative stress has been identified as a key contributor to Cd-induced liver injury. Cd may indirectly cause cellular damage towards oxidative conditions by producing free radicals, reactive oxygen species (ROS) and reduces the concentration of primary antioxidant compound inside cells through deactivation of enzymes and other antioxidant compounds, thus leading to lipid peroxidation (LPO) (Matovic *et al.*, 2015, Cuypers *et al.*, 2010). Numerous research has shown that Cd exposure could disrupt cell-mediated oxidative stress and lead to the impairment of cell membrane, potentially causing secondary liver injuries such as inflammatory response, cell death, and liver failure (Yang *et al.*, 2021, Zhang *et al.*, 2019). Enhancing antioxidant capacity in organisms is crucial for alleviating the pathogen alterations induced by Cd exposure through the reduction of oxidative stress. Current knowledge indicates that exposure to Cd may induce oxidative damage and led to hepatotoxicity.

ICA is known for its potential antioxidant and anti-inflammatory properties, yet its protective role against Cd-induced hepatic injury has not yet been fully explored. This study was conducted to evaluate whether ICA supplementation can attenuate Cd-induced oxidative stress and inflammation in the liver of mice. Additionally, an *in vitro* component was included to establish a callus culture system of *E. brevicornum* for sustainable ICA production. The findings of this research may provide a scientific basis for developing novel therapeutic and biotechnological strategies to manage heavy metal-induced hepatic toxicity. Despite extensive reports on the antioxidant and anti-inflammatory properties of icariin, limited information is available on the efficacy of callus-derived icariin against

heavy metal-induced liver injury. Therefore, this study aimed to optimize callus culture for sustainable icariin production and to assess its protective effects against cadmium-induced hepatic toxicity. We hypothesized that callus-derived icariin would mitigate cadmium-induced liver damage by reducing oxidative stress and inflammatory responses.

2. Materials and Methods

2.1. Chemicals and reagents

Cadmium chloride (CdCl_2) was obtained from Sigma Aldrich (city, country). *Epimedium brevicornum*, and measurement kits for IL-10, IL-1 β , IL-6, TNF- α , T-AOC, GST, GSH-Px, T-SOD, CAT, and MDA were obtained from Novus Biologicals, Thermofisher, and sigma Aldrich. Commercial suppliers were used to obtain additional chemicals and analytical grades.

2.2. Plant material, extraction, and analysis of ICA

2.2.1. Plant materials and culture conditions:

Epimedium brevicornum plants and seeds, *in vitro* cultured, followed a previously published procedure (Al Zoubi, 2019). Micro-shoot segments (2-3mm) were cultured in 9 cm Petri dishes containing 15 mL of MS (Murashige & Skoog, 1962) supplement with serial concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹) of 6-Benzyladenine (BA), and 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (NAA). Cultures were kept in darkness inside a growth chamber adjusted at 25 $\pm$ 2°C to evaluate the most effective Auxin/Cytokinin combination for callus induction. Each treatment included ten replicates arranged in a completely randomized design. After weeks the percentage of callus induction, color and texture were documented. Fresh weights were determined from three randomly selected samples, which were subsequently dried in oven at 80°C until the weight was constant to get the dry weight measurement.

2.2.2. Extraction, and analysis of ICA

The extraction method was followed as a previously published procedure (De Naeyer *et al.*, 2005). High-Performance Liquid Chromatography determined icariin (ICA) following a previously published method (Shaukat *et al.*, 2022). The purity of ICA was determined using HPLC (Waldbronn, Germany) connected to a Hypersil C18 column.

2.3. Animals, experimental design and treatment

Male C57BL/6 mice were obtained from animal house and maintained under controlled conditions at 23 $\pm$ 2 °C in a twelve-hour light/ twelve-hour dark cycle, with water and food supplies provided upon request *ad libitum*. Euthanasia and all the procedures used in this study were done based on the standards for Animal Ethical and Welfare Committee. Following a 7-day acclimation period, the animals were randomly allotted to four experimental treatment groups (12 mice per group): 1) Control, received corn oil; 2) ICA, received 100 mg/kg BW icariin; 3) Cd, received 5 mg/kg BW cadmium chloride, and 4) ICA + Cd, received 100 mg/kg BW icariin and 5 mg/kg BW cadmium chloride. All mice received a daily oral gavage of either ICA dissolved in corn oil or Cd dissolved in fresh distilled water. The dosages, administration routes, and

durations for ICA and Cd were calculated from prior studies (Li *et al.*, 2021, Yang *et al.*, 2021). The liver coefficient was estimated depending on this equation:

$$\text{Liver index (\%)} = \text{Liver weight (g)} / \text{body weight (g)} \times 100\%$$

2.4. Liver function enzymes analysis

The blood was obtained via the ocular regions of mice. Following serum collection, the activity of the enzymes AST, ALT, GGT, and ALP was assessed using an automatic biochemistry analyzer based on the manufacturer's protocol.

2.5. Histopathological observation

Livers of experimental animals were collected and then kept in paraformaldehyde at a concentration of 4%. Subsequently, for the dehydration of liver tissues, alcohol was used and then embedded in paraffin, segmented to a 5 μm , and finally prepared for staining with hematoxylin and eosin. The liver section from all groups was microscopically examined.

2.6. Analysis of inflammatory cytokines

Liver samples were homogenized, centrifuged and supernatants were stored and kept at -20°C for later investigation. The inflammatory response was influenced by measuring the levels of IL-6, IL-1 β , IL-10, and TNF- α in the supernatants of mice. The cytokines were assessed by employing an ELISA kit following the procedure recommendations.

2.7. Assessment of oxidative stress in the liver

According to a prior study, the liver's tissue homogenates were produced (Rajput *et al.*, 2021b). The liver tissues were briefly homogenized at 4°C with a Potter homogenizer in an ice-cold 0.9% NaCl solution. Following the centrifugation of homogenates, the supernatants were collected. GSH-Px, GSH, CAT, T-AOC, T-SOD, MDA, and total protein in the liver tissue were determined based on the manufacturer-recommended procedure.

2.8. Analysis of quantitative real-time PCR (qRT-PCR)

The whole RNA was isolated from liver tissue by Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following a previously published method (Rajput *et al.*, 2021a). PrimeScript™ RT reagent Kit was utilized to perform reverse transcription for cDNA synthesis based on the manufacturer's instructions. Successively, quantitative real-time PCR (qRT-PCR) was achieved on a real-time PCR system by SYBR Green PCR Master Mix (Applied Biosynthesis, Waltham, MA, USA) (Rajput *et al.*, 2019). In the current research, the sequence of primers is listed in Table S1. The β -actin was used as a standard gene. The data was quantified using the $2^{-\Delta\Delta\text{CT}}$ method as previously described (Gao *et al.*, 2018).

2.9. Statistical analysis

Data was analyzed by SPSS software V. 22 (IBM Corporation, Armonk, NY, USA). Statistically Significant ($P < 0.05$) was determined using one-way ANOVA followed by an LSD test. Data were displayed as mean $\pm$ SD.

3. Results

3.1. The role of Plant Growth Regulators on Callus Induction from *E. brevicornum*

Callus initiation started within 10–12 days, forming friable yellow-green tissue. The effect of different concentrations of BA, NAA, 2,4-D and their mixtures were assessed for their impact on callus induction, fresh and dry weight *in vitro* after twelve weeks were summarized in Tables 1–4.

Table 1: *In vitro* growth response of *E. brevicornum* to different BA concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹) BA	Fresh weight (mg) $\pm$ SD	Dry weight (mg) $\pm$ SD
0.0	687 $\pm$ 152 ^b	52.71 $\pm$ 6.70 ^b
0.5	2891 $\pm$ 136 ^a	125.3 $\pm$ 54.6 ^a
1	3011 $\pm$ 557 ^a	161.5 $\pm$ 62.9 ^a
1.5	3452 $\pm$ 323 ^a	185.3 $\pm$ 53.3 ^a
2	3558 $\pm$ 254 ^a	196.6 $\pm$ 56.7 ^a
2.5	3803 $\pm$ 341 ^a	222.4 $\pm$ 76.2 ^a

*Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

Table 2: *In vitro* growth response of *E. brevicornum* to different 2,4D concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹)) 2,4D	Fresh weight (mg) $\pm$ SD	Dry weight (mg) $\pm$ SD
0.0	889 $\pm$ 255.0 ^c	53.6 $\pm$ 8.90 ^c
0.5	2542 $\pm$ 389.0 ^b	141.3 $\pm$ 35.4 ^{ba}
1	3245 $\pm$ 335.0 ^b	157.4 $\pm$ 44.29 ^{ba}
1.5	4001 $\pm$ 846.0 ^a	223.6 $\pm$ 61.23 ^{ba}
2	4178 $\pm$ 876.1 ^a	243.4 $\pm$ 82.09 ^{ba}
2.5	4298 $\pm$ 897.0 ^a	266.7 $\pm$ 81.96 ^a

* Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

Table 3: *In vitro* growth response of *E. brevicornum* to different NAA concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹) NAA	Fresh weight (mg) $\pm$ SD	Dry weight (mg) $\pm$ SD
0.0	816 $\pm$ 215.0 ^c	54.7 $\pm$ 10.70 ^c
0.5	2265 $\pm$ 145.3 ^b	181.7 $\pm$ 35.76 ^b
1	2654 $\pm$ 791.2 ^{ba}	197.8 $\pm$ 46.50 ^{ba}
1.5	3212 $\pm$ 1028.3 ^a	264.3 $\pm$ 56.10 ^{ba}
2	3819 $\pm$ 657.1 ^a	287.9 $\pm$ 76.90 ^a
2.5	3936 $\pm$ 787.6 ^a	309.3 $\pm$ 73.40 ^a

* Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

Table 4: *In vitro* growth response of *E. brevicornum* to different combinations of 2,4D and BA concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹) 2,4D	Concentration (mg L ⁻¹) BA	Fresh weight (mg) ±SD	Dry weight (mg) ±SD
0.0	0.0	823±226 ^c	72.7±19.11 ^d
2	0.5	5357±123 ^a	357.8±54.8 ^a
2	1	2315±406.82 ^{bc}	143.4±41.05 ^{bc}
2	1.5	2534±326.3 ^b	146.6±32.71 ^{bc}
2	2	2650±276.1 ^b	149.8±42.3 ^{bc}
2.5	0.5	2101±204 ^c	131.6±31.7 ^{bc}
2.5	1	1667±251 ^d	103.1±34.4 ^d
2.5	1.5	1611±263 ^d	102.6±24.8 ^d
2.5	2	1247±233 ^d	74.7±44.3 ^d

* Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

All growth regulators significantly enhanced callus induction compared with the control. Increasing BA concentrations led to a steady rise in callus biomass, with the highest fresh (3803 mg) and dry (222.4 mg) weights obtained at 2.5 mg L⁻¹ BA. Similarly, 2,4-D treatments showed a marked stimulatory effect, where callus mass increased from 898 mg (control) to 4298 mg at 2.5 mg L⁻¹ 2,4-D, accompanied by a dry weight of 266.7 mg. NAA also promoted callus growth in a concentration-dependent manner, reaching maximum values of 3936 mg fresh and 309.3 mg dry weight at 2.5 mg L⁻¹ NAA. Among the combinations, the interaction between auxin and cytokinin significantly influenced callus proliferation. The mixture of 2 mg L⁻¹ 2,4-D with 0.5 mg L⁻¹ BA resulted in the highest callus biomass (5357 mg fresh and 357.8 mg dry weight), while higher BA concentrations caused a gradual decline in growth. Overall, auxin-based treatments, particularly 2,4-D and NAA, were more effective than BA alone, and the combination of 2 mg L⁻¹ 2,4-D with a low BA concentration provided the most favorable conditions for callus induction and growth in *E. brevicornum*.

3.2. ICA alleviates liver injury induced by Cd

As shown in Fig. 1, the ALP, AST, ALT, and GGT concentrations were markedly raised after Cd exposure in comparison to the untreated-CON group. However, in comparing the Cd alone treated group, the ALP, AST, ALT, and GGT levels decreased in the liver of the ICA + Cd group ($P < 0.05$). Compared to untreated group (CON), the liver index was increased under Cd exposure while recovering to the normal values under the co-treatment of ICA and Cd ($P < 0.05$) (Fig. 2B).

Based on the histopathological observations from the experimental groups presented in Fig. 2A, the CON and ICA groups showed normal liver architecture with intact hepatic cords and hepatic sinusoids. Moreover, the central vein has intact endothelium and the hepatic triad consisting of portal vein, bile duct, and hepatic artery were also normal. There were no obvious microscopic changes in the hepatocytic nucleus in the CON and ICA-treated groups. Contrastingly, mice exposed with Cd revealed significant pathological changes including, central vein was filled with proteinaceous fluid along with inflammatory cells. The endothelium of central vein was distorted.

Degenerative changes such as ballooning of hepatocytes with peripheral nucleus due to fatty change were detected. Furthermore, necrotic changes including karyolysis were observed in hepatocytes which indicate mild to moderate coagulative necrosis of liver. Strikingly, ICA treatment significantly ameliorated and restored liver injury caused by Cd (Fig. 3A).

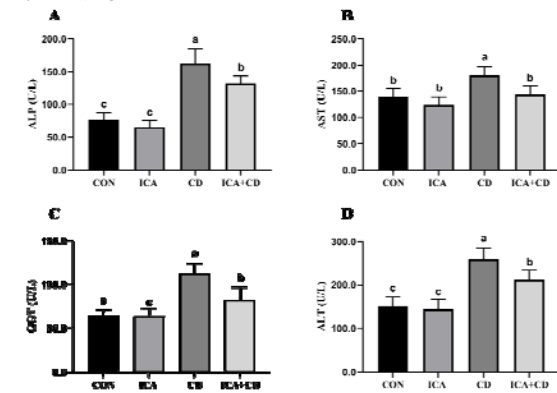


Figure 1. Effect of ICA on liver function enzymes under Cd-exposed mice. The results are stated as mean ± standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. AST: Aspartate aminotransferase; ALP: Alkaline phosphate; ALT: Alanine aminotransferase and GGT: Gamma-glutamyl transferase.

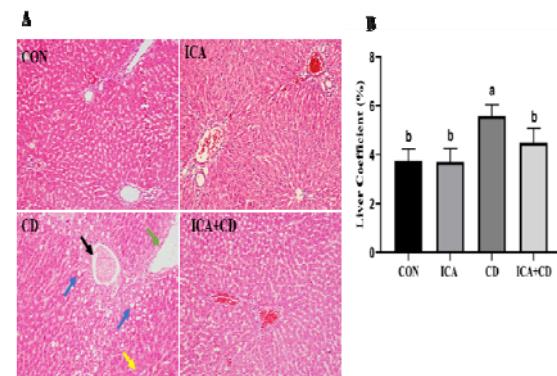


Figure 2. (A) Effect of ICA and Cd on Histopathological observations. (B) Liver coefficient. The results are shown as mean ± standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns.

3.3. ICA alleviates inflammatory response induced by Cd

The effects of ICA and Cd on the inflammatory cytokines are presented in Fig. 3A, B. Compared with the experimental groups, the TNF- α , IL-1 β , and IL-6 levels, as well as gene expression were enhanced in response to Cd treatment ($P < 0.05$). On the other hand, co-treatment of ICA and Cd exhibited significantly decreased expressions of these pro-inflammatory cytokines in comparison to the Cd group. However, the ICA + Cd administered group showed an up-regulation in the concentration and transcriptional abundance of anti-inflammatory cytokines (IL-10) in comparison to the Cd alone treated group.

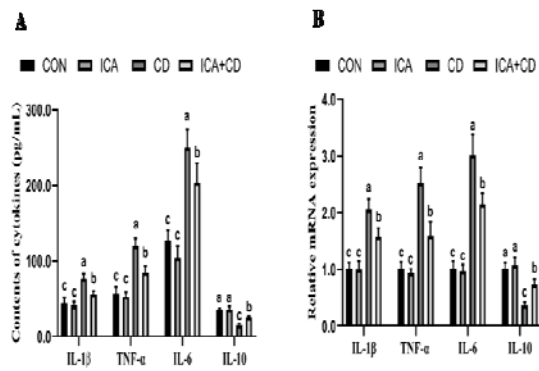


Figure 3. Effect of ICA on inflammatory response under Cd-exposed mice. (A) Levels of inflammatory cytokines. (B) The transcriptional expression of inflammatory cytokines. The results are presented as mean $\pm$ standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed, followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. IL-10: Interleukin-10; TNF- α : Tumor necrosis factor-alpha; IL-6: Interleukin-6, and IL-1 β : Interleukin 1 beta.

3.4. ICA mitigates hepatic oxidative damage induced by Cd

To examine the mitigation effect of ICA on oxidative stress caused by Cd in the liver of mice, T-AOC, CAT, T-SOD, GSH-Px, GSH, and MDA content or activities were determined. As shown in Fig 4, the activities of T-AOC, CAT, T-SOD, GSH-Px, and GSH markedly reduced in the Cd alone treated group as compared with the other experimental groups. However, Cd-exposed displayed a significant increase in MDA levels when compared to the experimental groups. Contrastingly, the antioxidant and oxidative stress markers alterations were ($P < 0.05$) markedly attenuated by ICA treatment under Cd exposure.

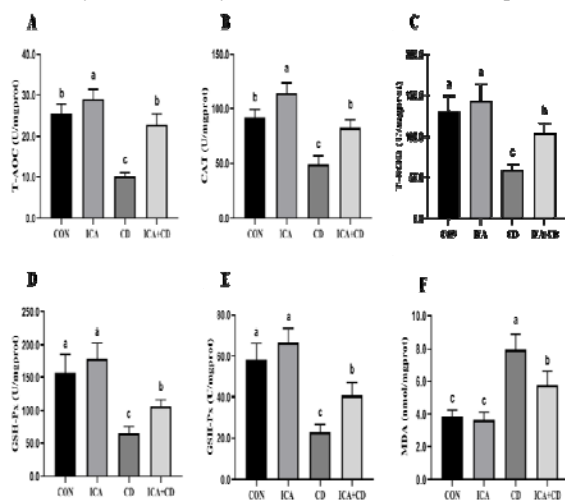


Figure 4. Effect of ICA on oxidative stress under Cd-exposed mice. Activities of antioxidant enzymes. The results are shown as mean $\pm$ standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed, followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. MDA: Malondialdehyde; CAT: Catalase; T-SOD: Total superoxide dismutase; GSH-Px: Glutathione peroxidase; GSH: Glutathione.

The transcriptional expression of antioxidants-related genes was further detected to confirm the antioxidant capability of ICA. Compared with experimental groups, the transcriptional levels of antioxidant genes were

markedly ($P < 0.05$) down-regulated in the Cd-treated group. However, the expression of these genes was significantly regulated in the ICA + Cd group (Fig. 5).

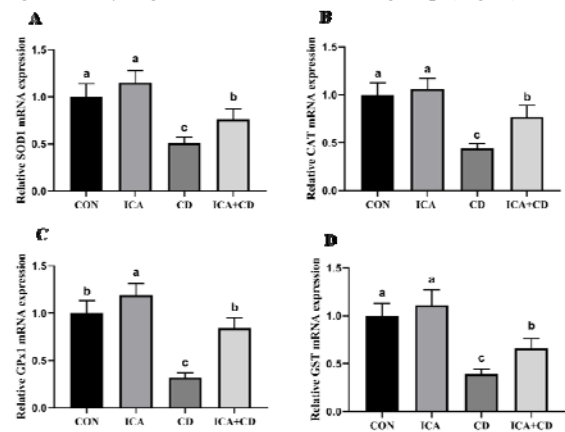


Figure 5. Effect of ICA on antioxidant-related transcriptional levels in Cd-exposed mice. The results are shown as mean $\pm$ standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed, followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. SOD1: Superoxide dismutase 1; CAT: Catalase; GPx1: Glutathione peroxidase 1; and GST: Glutathione S-transferase.

4. Discussion

The present study shows that plant growth regulators (PGRs) strongly influenced callus induction and biomass accumulation in *E. brevicornum* after 12 weeks. Cytokinin (BA) promoted callus formation in a concentration-dependent manner, with fresh and dry weights increasing steadily from the control to 2.5 mg L⁻¹ BA. Auxins (2,4-D and NAA) produced even greater biomass accumulation than BA alone at higher concentrations, indicating that callogenesis in *E. brevicornum* is largely auxin-responsive. Notably, the combination treatment 2 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BA gave the highest biomass (fresh weight 5357 mg, dry weight 357.8 mg), showing a synergistic auxin-cytokinin interaction where moderate auxin with low cytokinin favored maximal callus proliferation; higher BA levels in the presence of auxin reduced biomass, consistent with cytokinin-induced shift away from unorganized proliferation toward differentiation.

Previous studies have confirmed that *Epimedium* callus cultures can produce characteristic flavonoid glycosides (Yamamoto *et al.*, 1992). The high biomass obtained under auxin-dominant conditions, which may mimic mild stress states appears advantageous for secondary metabolites biosynthesis. However, since both drying and PGR regimes can affect metabolite composition, a trade-off exists between maximizing callus biomass and optimizing the yield of specific secondary metabolites such as icariin and epimedesides. According to our findings, the following recommendations can be made for practical applications-metabolite production or mid-term storage:- (1) use auxin-rich media (e.g., 2–2.5 mg L⁻¹ 2,4-D or NAA) to maximize callus biomass; (2) maintain low BA levels (≈ 0.5 mg L⁻¹) in auxin-cytokinin combination with sustain proliferation; and (3) carefully control drying and storage condition, as temperature and drying rate strongly influence flavonoid stability. These recommendations are

consistent with micropropagation, and callus-culture protocols previously developed for other *Epimedium* species (Al-Matar *et al.*, 1999) and support the potential of callus-based systems for controlled flavonoid production in the genus.

The improved icariin (ICA) yield in callus cultures indicates that *in vitro* conditions stimulate secondary metabolism, likely through activation of phenylpropanoid pathway regulated by 2,4-D and BAP (Niazian & Sabbatini, 2021). Such culture systems provide a sustainable and controlled alternative to field cultivation, ensuring consistent ICA production irrespective of environmental fluctuations (Alamgir, 2017). Integrating tissue culture-based biosynthesis with pharmacological investigation, as demonstrated in this study, could therefore advance commercial-scale icariin production for hepatoprotective and therapeutic applications (Kiran & Tiwari, 2024).

Cadmium (Cd) is considered one of the most noxious heavy metals in the environment. The liver is known to be a major target of Cd toxicity and accumulation, leading to liver damage (Imed *et al.*, 2009, He *et al.*, 2022). The current findings demonstrated that Cd exposure significantly raised liver index in mice. The increased liver weight can be explained by the fact that Cd can accumulate lipids in the liver, resulting in hepatomegaly. The present study results agreed with the earlier studies, which reported that Cd could cause alterations in liver index in experimental animals (Chen *et al.*, 2023). The detection of AST, ALT, GGT, and AST, can directly indicate the damage and dysfunction of liver caused by Cd. Histopathological changes are a direct parameter used to judge the impairment of tissue structure (Fang *et al.*, 2021). The current observations revealed that Cd caused pathological and clinical symptoms of hepatotoxicity in mice. This is evidenced by increased ALT, AST, GGT, and ALP concentration along with degenerative changes in the liver of mice. The current outcomes indicate that Cd caused liver injury, in agreement with the previous studies (He *et al.*, 2022, Souza-Arroyo *et al.*, 2022, Yang *et al.*, 2021). ICA administration ameliorated Cd-induced growth retardation and mitigated harmful impacts on liver function enzymes and pathological alterations in the liver of mice. The findings align with previous studies that revealed ICA-protected thioacetamide-induced liver fibrosis (Algandaby *et al.*, 2017) and CCl₄-induced acute liver injury in mice (Xiao *et al.*, 2024). The current results suggested that ICA could alleviate liver injury in Cd-induced mice.

Inflammation is a well-established area of Cd-induced poisonousness (Yang *et al.*, 2021). After the initiation of Cd toxicity, macrophages and Kupffer cells are activated, generating an intricate system of inflammatory mediators and releasing detrimental cytokines, including IL-6, IL-1 β , and TNF- α (Renu *et al.*, 2021). These cytokines are essential in the progression of inflammation. TNF- α secreted by T-lymphocytes, macrophages, and monocytes as well as proved to have a crucial function in releasing and helping the other pro-inflammatory cytokines and activating neutrophils (Burger & DAYER, 2002). Moreover, IL-6 is released during infections and stress, and its major function is maintaining tissue injury induced by internal and external stimuli. Furthermore, IL-1 β secreted by macrophages and modulates the inflammatory

response caused by toxicants (Megha *et al.*, 2021). Therefore, pro- and anti-inflammatory mediators by ELISA and qRT-PCR techniques were detected. The results showed that ICA treatment decreased all the validated pro-inflammatory cytokines and raised the anti-inflammatory cytokines induced by Cd. These results are consistent with earlier research (Luo *et al.*, 2022, Shaikat *et al.*, 2022, Yang *et al.*, 2021) and, therefore, highlighted the mitigation impact of ICA on Cd-induced liver inflammation.

Oxidative damage is categorized by the accumulation of free radicals and reactive oxygen species, which cause lipid peroxidation and the breakdown of cell membranes (Recknagel *et al.*, 2020). Various antioxidant defense reactions reduce oxidative damage and regulated the status of free radicals in the body, such as (GSH, CAT, T-SOD, and GSH-Px) (Davies, 2000). Furthermore, MDA is a hallmark of lipid peroxidation and is frequently used to detect the state of endogenous oxidative damage (Rajput *et al.*, 2017). Therefore, detecting MDA, CAT, T-SOD, GSH-Px and GSH can measure an organism's oxidative status. The present study findings revealed that Cd exposure increased MDA content and decreased the GSH, GSH-Px, CAT, T-AOC, and T-SOD activities. The results showed that Cd could induce oxidative stress in the liver of mice, which aligns with prior studies (Fang *et al.*, 2021, Cuypers *et al.*, 2010). Notably, the administration of ICA restored the antioxidant defense capacity, which was reduced by Cd. Our results are in agreement with studies that showed supplementation of ICA alleviates oxidative damage in the liver induced by Cd (Shaikat *et al.*, 2022, Wang *et al.*, 2019). These results verified that ICA has the capability to rescue Cd-induced hepatic oxidative stress.

In summary, this study demonstrates: (1) optimized *in vitro* callus culture provides a reliable strategy for sustainable icariin production from *E. brevicornum*, and (2) icariin exerts a potent hepatoprotective effect against Cd-induced injury by enhancing antioxidant defense and suppressing inflammatory responses. These findings bridge plant biotechnology and pharmacological application, offering a promising framework for future studies and commercial-icariin-based therapeutics.

5. Conclusion

Auxin-dominated media and an optimized auxin-cytokinin balance produce the highest callus biomass in *E. brevicornum*. Future studies should focus on quantifying key flavonoids such as icariin and epimedinol under the most effective PGR combination and standardized drying condition to determine the optimal balance between biomass yield and metabolite quality.

Additionally, this study demonstrated that ICA exerts marked hepatoprotective effects against cadmium (Cd) – induced liver injury by restoring liver function enzymes, improving histopathological integrity, reducing oxidative stress, and suppressing inflammatory response. These findings provide new insights into the therapeutic potential of ICA in mitigation Cd-induced hepatic toxicity and establish a novel knowledge for its future development as natural histopathological agents.

In conclusion, the present study demonstrates that callus-derived icariin effectively attenuates cadmium-induced hepatic injury in mice. The protective effects are

associated with the restoration of antioxidant gene expression and the reduction of pro-inflammatory cytokines. These findings support the potential of sustainably produced icariin as a hepatoprotective agent against heavy metal-induced toxicity. Further studies are warranted to investigate protein-level mechanisms and to confirm these effects in additional experimental models.

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Supplementary material:**Table S1.** Sequence of the primers used for quantitative real-time PCR assay.

Target gene	Primer	Primer sequence (5'→3')	Accession No.
β-Actin	Forward	GTTGGAGCAAACATCCCCCA	NM_007397.5
	Reverse	ACGCGACCATCCTCCTCTTA	
SOD1	Forward	GGTCTCCAACATGCCTCTCT	NM_011434.2
	Reverse	AACCATCCACTTCGAGCAGA	
CAT	Forward	CACTGACGAGATGGCACACT	NM_009804.2
	Reverse	TGTGGAGAATCGAACGGCAA	
GPx1	Forward	GTACTTGGGGTCGGTCATGA	NM_001329527.1
	Reverse	GGTTTCCCGTGCAATCAGTT	
GST	Forward	TGGCCGGAAGCACAAACCTG	BC031818
	Reverse	CCCCACCAACACCGGCAC	

Antioxidant Activity of β -Carotene Derived from *Spirulina platensis*: An *in vitro* and *in silico* Study

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Received: September 25, 2025; Revised: December 14, 2025; Accepted: January 4, 2026

Abstract

The search for natural antioxidants has intensified due to concerns regarding the side effects of synthetic compounds. *Spirulina (Arthrospira) platensis*, a cyanobacterium, is recognized for its rich content of bioactive compounds, particularly β -carotene, a powerful antioxidant pigment. This study aimed to isolate and identify β -carotene from *S. platensis* and evaluate its antioxidant activity using both *in vitro* and *in silico* approaches. The extraction of β -carotene was performed using a methanol–acetone solvent system, followed by fractionation with diethyl ether and isolation through preparative thin-layer chromatography (TLC). The identity of β -carotene was confirmed via TLC, UV-Vis spectrophotometry (λ_{max} at 452 and 478 nm), and High-performance liquid chromatography (HPLC) (retention time 30.65 min). The *in vitro* antioxidant activity was measured using the DPPH assay, which indicated an IC_{50} of 476 ± 0.32 mg/L for the β -carotene isolate, demonstrating greater potency than the crude fraction. *In silico* molecular docking against catalase, glutathione reductase, and superoxide dismutase (SOD) revealed that β -carotene exhibited stronger binding affinities (ΔG up to -9.34 kcal/mol) than the reference compound quercetin. Absorption–distribution–metabolism–excretion–toxicity (ADMET) predictions indicated high intestinal absorption and blood-brain barrier permeability, but poor water solubility and oral bioavailability based on Lipinski's criteria. The compound was found to be non-mutagenic, non-hepatotoxic, and exhibited no cardiotoxicity via human ether-à-go-go-related gene (hERG) I inhibition. These results affirm the antioxidant potential of β -carotene from *S. platensis* and open its potential application in pharmaceutical and nutraceutical formulations, particularly for non-oral delivery systems.

Keywords: *Spirulina platensis*; β -Carotene; Antioxidant activity; DPPH assay; Molecular docking; ADMET prediction

1. Introduction

The quest for effective antioxidants has gained momentum due to the increasing awareness of the detrimental effects of free radicals in promoting oxidative stress and their role in various chronic diseases (Alamoudi, 2019). Free radicals, distinguished by their unpaired electrons, can trigger chain reactions that harm cellular components, such as lipids, proteins, and DNA, potentially leading to pathological conditions (Girgis *et al.*, 2024). Oxidative stress occurs when the production of free radicals surpasses the body's antioxidant defences, underscoring the significance of exogenous antioxidants in maintaining cellular homeostasis (Lobo *et al.* 2010). Antioxidants counteract oxidative damage by neutralizing free radicals through electron donation or scavenging mechanisms (Sayed *et al.*, 2022). Although synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone are widely used, concerns regarding their toxicity and carcinogenic potential have fuelled the demand for safer, natural alternatives (Ayoub and Mehta, 2018; Neethu *et al.*, 2017).

Natural antioxidants, which are abundant in plant-based foods and microorganisms, are increasingly favoured because of their safety profiles and synergistic effects of multiple bioactive compounds within complex extracts (Mohan and Kakkar, 2020; Pourreza, 2013). These synergistic interactions often enhance the antioxidant activity beyond that of isolated synthetic compounds (Fajriah *et al.*, 2021). However, the effectiveness of natural antioxidants can vary based on factors such as their stability, bioavailability, and interactions with other dietary components (Batinić-Haberle *et al.*, 2010). Among natural sources, *Spirulina (Arthrospira) platensis*, a cyanobacterium rich in nutrients and pigments, has attracted significant interest because of its antioxidant potential (Kumar *et al.*, 2022).

The antioxidant activity of *S. platensis* is largely due to its wide range of bioactive components, including chlorophylls, carotenoids, phycocyanin, and phenolic acids (Gurugubelli *et al.*, 2023). Among these, β -carotene is a significant carotenoid with strong antioxidant capabilities. It effectively neutralizes free radicals and prevents lipid peroxidation, particularly by quenching singlet oxygen, a highly reactive species (Genç *et al.*, 2020). In addition to

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β -carotene, other carotenoids, such as zeaxanthin and lutein, also enhance the antioxidant capacity of *Spirulina*. Carotenoids derived from algae offer distinct benefits over terrestrial sources, including reduced production costs, simpler extraction methods, and improved environmental sustainability (Christaki *et al.*, 2012; Galasso *et al.*, 2017).

While the general antioxidant activity of *S. platensis* is well-documented, there is a scarcity of studies specifically focusing on β -carotene or examining its molecular interactions in detail. To address this research gap, the present study aims to isolate and identify β -carotene from *S. platensis* extracts, assess its antioxidant activity using *in vitro* DPPH assays, and elucidate its mechanism of action through *in silico* molecular docking with key antioxidant-related enzymes. By integrating experimental and computational methodologies, this study seeks to provide a comprehensive understanding of the antioxidant potential of β -carotene from *S. platensis* and support its targeted development for nutraceutical or pharmaceutical applications.

2. Materials and Methods

2.1. Samples and Materials

The *S. platensis* samples were taxonomically identified by E. Siahaan at the Marine Bioindustry Centre, Indonesian Institute of Sciences (LIPI), under reference number B-167/III/DI.02/9/2021. Before analysis, the microalgae powder was stored at -20°C . All the solvent and reagent used in this study were analytical-grade obtained from Merck (Germany) and Sigma-Aldrich (USA).

2.2. Extraction

To extract the compounds, 50 g of *S. platensis* powder was immersed in 200 mL of methanol–acetone solution (7:3, v/v) at ambient temperature for 10 min, with occasional stirring to enhance solvent absorption. This extraction process was repeated thrice under identical conditions to optimize metabolite recovery, and the resulting extracts were filtered using Whatman No. 1 paper. The combined filtrates were concentrated under reduced pressure using a rotary evaporator (Heidolph, Germany) at a temperature below 30°C to prevent the degradation of heat-sensitive substances. The concentrated crude extract was partitioned with diethyl ether in a separating funnel at a 1:1 (v/v) ratio. The organic phase was collected and rinsed with distilled water to remove the polar impurities. The solvent from the diethyl ether fraction was evaporated under a gentle nitrogen stream until completely dry. The dried fractions were stored in airtight containers, shielded from light, and maintained at -20°C until further analysis (Kusmita *et al.* 2015).

2.3. Identification and Isolation of β -Carotene

The presence of β -carotene in the diethyl ether fraction was confirmed by thin-layer chromatography (TLC) using silica gel GF254 as the stationary phase. Chromatographic separation was performed using a mobile phase composed of n-hexane, acetone, and diethyl ether in a ratio of 6:3:2 (v/v/v). The resulting chromatograms were visualized under visible light, where β -carotene was identified by its characteristic yellow-orange bands. For further purification, preparative thin-layer chromatography (TLC)

was performed using the same solvent system. The distinct yellow β -carotene band was carefully scraped from the silica gel plate, eluted with an appropriate solvent, and collected for subsequent analyses (Wang *et al.*, 1995).

2.4. UV-Vis Spectrophotometry and High-performance liquid chromatography (HPLC) Analysis

The isolated β -carotene fraction was dissolved in acetone and analysed using a UV–Vis spectrophotometer (Shimadzu UV-1700, Japan) over a wavelength range of 350–800 nm. The resulting absorption spectrum was compared with that of a genuine β -carotene standard and reference values from the literature to confirm the identity of the compound. For additional verification and measurement, high-performance liquid chromatography (HPLC) was performed using a Shimadzu system with a photodiode array (PDA) detector and a column temperature controller. The separation process was performed using a Shim-pack VP-ODS C18 column (250×4.6 mm i.d.) under isocratic conditions with methanol and acetonitrile as the mobile phase (70:30, v/v). The flow rate was set to 1.0 mL/min, and the column temperature was maintained at 25°C . β -Carotene was detected at 420 nm, with chromatographic peaks identified by comparing retention times and absorption spectra to those of a β -carotene standard.

2.5. *In Vitro* Antioxidant Activity

The antioxidant properties of the fractions and isolated β -carotene were assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, as described by (Samanta *et al.* 2016) with minor adjustments. In summary, 1 mL of the test sample solution at different concentrations was combined with 3 mL of a 0.08 mM DPPH solution in methanol. The mixtures were thoroughly mixed using a vortex mixer to ensure uniformity and then incubated in the dark at room temperature for 45 min to complete the reaction. The reduction in absorbance was recorded at 517 nm using a UV–Vis spectrophotometer (Shimadzu UV-1700, Japan). A control solution was prepared by mixing 3 mL of DPPH solution with 1 mL of methanol under identical conditions. Methanol served as a blank. Antioxidant activity was quantified as the percentage of DPPH radical inhibition, calculated using the following equation:

$$\% \text{ Inhibitory} = \frac{[DPPH]_0 - [DPPH]_s}{[DPPH]_0} \times 100\%$$

$[DPPH]_0$ = Initial DPPH concentration

$[DPPH]_s$ = Final DPPH concentration remaining

2.6. *In Silico* Antioxidant Activity

2.6.1. Molecular Docking Simulation

The interaction between β -carotene and antioxidant-associated enzymes was assessed using *in silico* molecular docking. AutoDockTools 1.5.6 was used to predict the binding affinity and interaction patterns of β -carotene within the active sites of specific enzymes. The proteins targeted in this study included catalase (PDB ID: 1DGH), glutathione reductase (PDB ID: 1XAN), and superoxide dismutase (SOD; PDB ID: 4A7G), all sourced from the RCSB Protein Data Bank (<https://www.rcsb.org/>). Protein

structures were prepared using BIOVIA Discovery Studio 2021 by eliminating water molecules and heteroatoms, incorporating hydrogen atoms, and saving the modified receptors in the *pdb format. The 3D structure of β -carotene was acquired from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in *sdf format, converted to *pdb format, and optimized through energy minimization with the addition of Gasteiger charges. Docking simulations were conducted to position the ligand within each enzyme's catalytic pocket, and the binding affinities were determined based on the binding energy (kcal/mol). The docking protocol was validated by redocking the native ligand to its respective receptor-binding site. The method was deemed valid when the root mean square deviation (RMSD) between the docked and crystallographic poses of the native ligand was less than 2 Å. Lower RMSD values indicate greater reliability of the docking process, ensuring that the predicted binding poses of β -carotene closely match biologically relevant interactions (Pandey *et al.*, 2019; Ijoma *et al.*, 2024).

2.6.2. Drug-Likeness and Absorption–distribution–metabolism–excretion–toxicity (ADMET) Prediction

To evaluate the drug-likeness of β -carotene, Lipinski's Rule of Five was utilized, which is a widely recognized guideline in drug discovery for estimating oral bioavailability. According to this rule, a compound may exhibit poor absorption or permeability if it satisfies any of the following criteria: a molecular weight exceeding 500 Da, more than 10 hydrogen bond donors, or a calculated log P (ClogP) greater than 5 (or or MlogP >4.15). These physicochemical properties serve as an initial screening tool to determine the suitability of molecules for drug development. Additionally, Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) predictions were performed to further assess the pharmacokinetic and safety profiles of β -carotene. The evaluated parameters included intestinal absorption (Caco-2 cell permeability), blood–brain barrier (BBB) penetration, metabolic stability concerning cytochrome P450 (CYP2D6) interactions, total clearance (excretion), and potential toxicity risks. Toxicological assessments also considered Ames

mutagenicity and inhibition of human ether-à-go-go (hERG) potassium channel types I and II, which are critical indicators of cardiotoxicity. Collectively, these analyses provide a comprehensive prediction of the potential of β -carotene as a bioactive candidate (Rahardhian *et al.*, 2025).

2.6.3. Software and Computational

The preparation of these proteins, which involved the elimination of water molecules, addition of hydrogen atoms, and optimization of receptor structures, was conducted using BIOVIA Discovery Studio (v21.1.0.2.20298, Dassault Systèmes). Molecular docking simulations were performed using AutoDock Tools (version 1.5.6, Sep_17_14 release), which facilitated ligand preparation, assignment of Gasteiger charges, grid box setup, and docking process. Binding affinities, docking poses, and interaction profiles were analysed on the same platform, and ligand–protein complex visualizations were created using BIOVIA Discovery Studio to depict hydrogen bonding, hydrophobic interactions, and spatial orientation within the active site (Abdulkareem *et al.*, 2025).

3. Results

3.1. Isolation and Identification of β -carotene

The pigment composition of the diethyl ether fraction of *S. platensis* was examined using thin-layer chromatography (TLC). The separation profile is depicted in Figure 1A, and the corresponding pigment identification is detailed in Table 1. To obtain purified β -carotene, preparative TLC was performed using silica gel GF254 as the stationary phase and a solvent mixture of hexane, ether, and acetone (6:3:2, v/v/v) as the mobile phase (Figure 1B). The distinct yellow band corresponding to β -carotene was carefully scraped, eluted, and collected for further analysis. The identity of the isolated compound was confirmed using analytical TLC against an authentic β -carotene standard. Both the isolate and the standard exhibited identical R_f values (0.98), providing compelling evidence that the isolated pigment was β -carotene (Figure 1C).

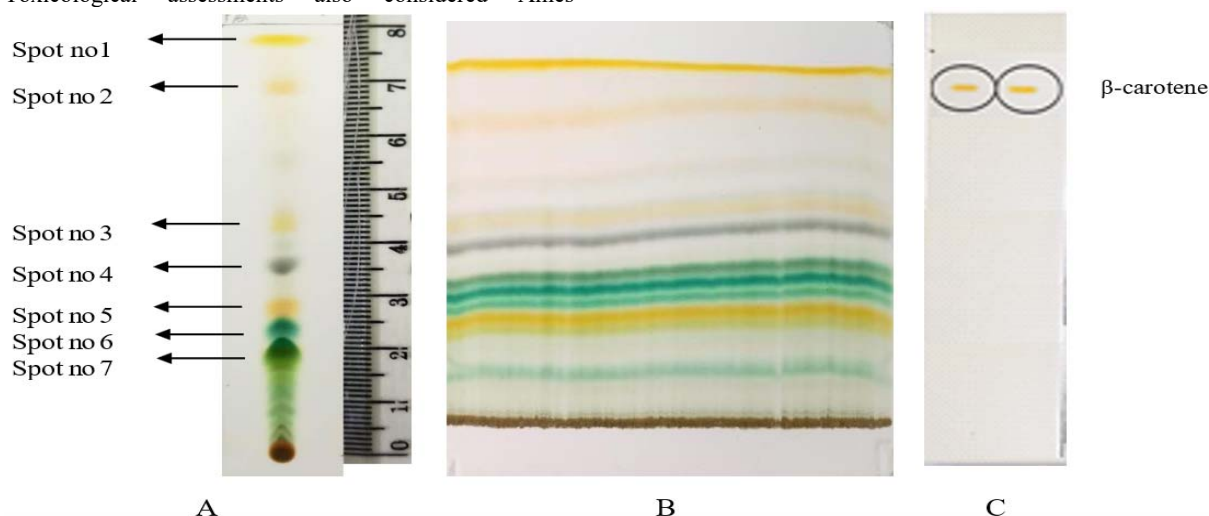
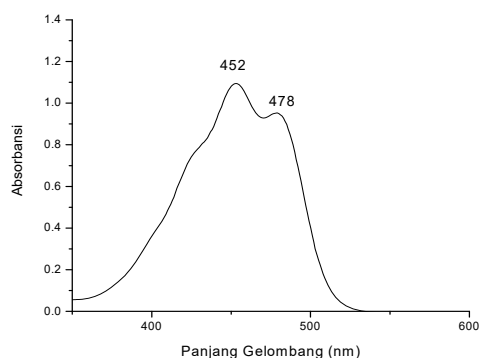


Figure 1. Separation Pattern of Diethyl Ether Fraction Stain of *S. platensis* extract (A), Isolation of β -carotene using preparative-TLC, with a stationary phase of silica gel GF 254 and a mobile phase composed of hexane, ether, and acetone in a 6:3:2 ratio (B), TLC identification of β -carotene isolate (left) and β -carotene standard (right) (C)

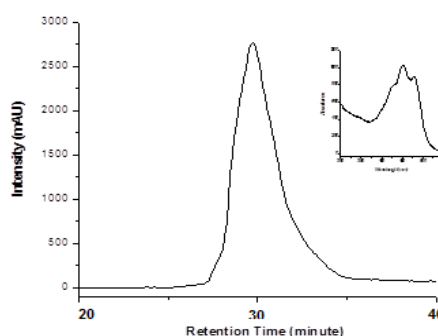
Table 1. Results of Pigment Identification in Diethyl Ether Fraction of *S. platensis*

No	Colour	Rf	Identification Ref
1	Yellow	0.98	β -carotene (Britton, 1985)
2	Orange	0.86	Carotene (Britton, 1985)
3	Yellow	0.55	Carotene (Britton, 1985)
4	Gray	0.45	Pheophytin <i>a</i> (Kusmita, 2015)
5	Orange	0.35	Xanthophyll (Kusmita, 2015)
6	Bluish green	0.30	Chlorophyll <i>a</i> (Kusmita, 2015)
7	Dark green	0.24	Chlorophyll <i>b</i> (Kusmita, 2015)

*Rf: the retention factor



A

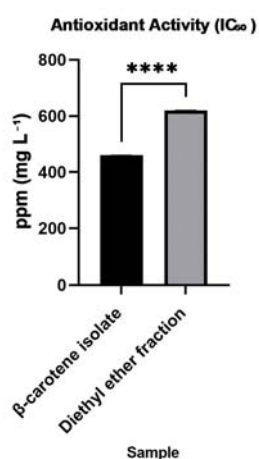


B

Figure 2. Spectrum of β -carotene isolate from the *S. platensis* (A) and HPLC profile β -carotene isolate. The detection was conducted at a wavelength of 420 nm, with a flow rate of 1 mL per min⁻¹ (B).

3.2. *In vitro* antioxidant activity

The antioxidant capacity of the β -carotene isolate was assessed using the DPPH radical-scavenging assay, and the results are shown in Figure 3. This assay is extensively utilized to evaluate the free radical scavenging potential of natural compounds because of its simplicity, sensitivity, and reproducibility.

**Figure 3.** Antioxidant activity diethyl ether fraction and β -carotene isolate from *S. platensis* using DPPH scavenging assay (****= significant, p value <0.0001, n=5)

The structure of the isolated pigment was confirmed using UV-Vis spectrophotometry (Figure 2A). To further substantiate these findings, the β -carotene isolate was subjected to high-performance liquid chromatography (HPLC) analysis, employing detection at 420 nm with a flow rate of 1 mL min⁻¹ (Figure 2B).

3.3. *In silico* antioxidant activity

The interaction between β -carotene and the target proteins catalase, glutathione reductase (GR), and superoxide dismutase (SOD) plays a crucial role in strengthening the body's antioxidant defense system. β -Carotene, as a lipophilic antioxidant, can bind to the active site or surface of proteins through hydrophobic interactions and non-covalent bonds, thereby helping stabilize the enzyme structure and increasing its efficiency in neutralizing free radicals. The antioxidant activity of β -carotene was further evaluated *in silico* using the molecular docking method, as illustrated in Figure 4.

The validation process yielded RMSD (Root Mean Square Deviation) values, which are instrumental in assessing conformational differences or comparing ligand positions post-redocking with their original positions on the protein. A smaller RMSD value indicates a reduced likelihood of ligand displacement from its original position. An acceptable RMSD value for this redocking process is 3 Å (Syahputra *et al.*, 2022). The RMSD values obtained for redocking with catalase, glutathione reductase, and superoxide dismutase (SOD) proteins were 3.25 Å, 3.16 Å, and 1.41 Å, respectively. The RMSD value that met the criteria was observed in the redocking with superoxide dismutase (SOD). Following the completion of the validation process, molecular docking of the β -carotene compound with the three proteins was conducted to ascertain the optimal docking result or antioxidant activity among them. In this analysis, quercetin served as a reference compound. The binding results were visualized in 2D using the Biovia Discovery Studio application, as depicted in Figures 5a, b, and c.

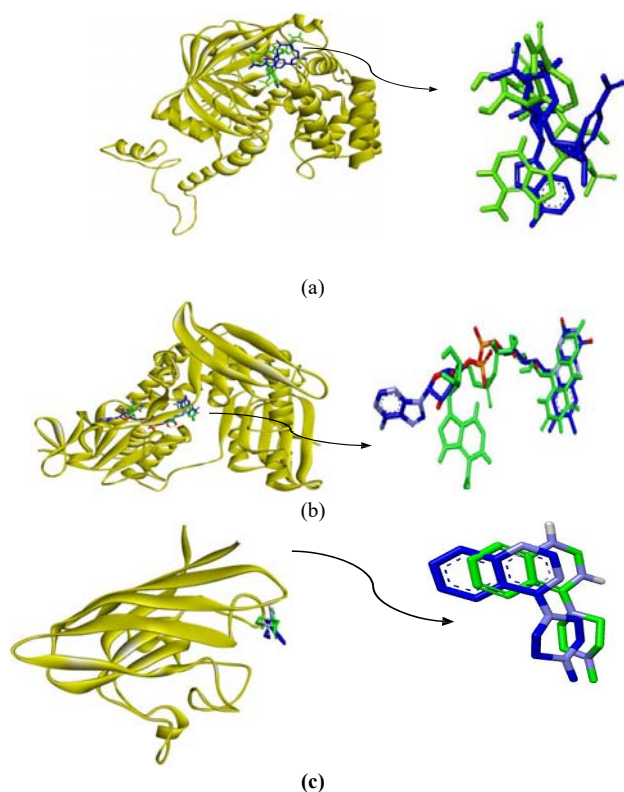
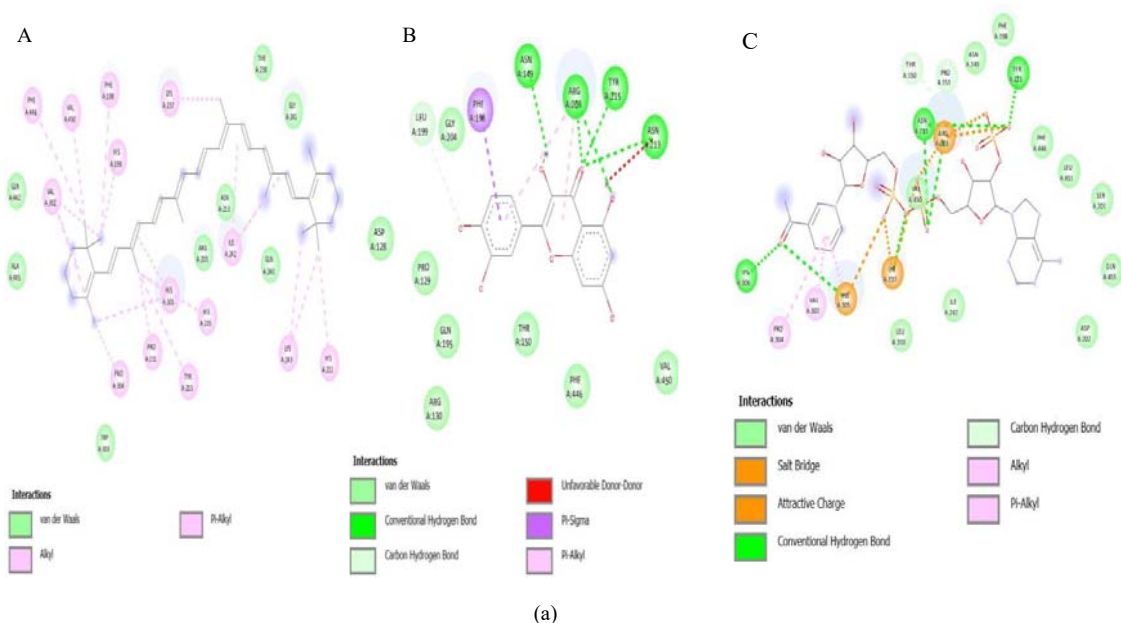


Figure 4. Overlay visualization of the IDGH (a); IXAN (b); and 4A7G (c) complex with natural ligands, depicted in blue before and in green after the redocking process.



(a)

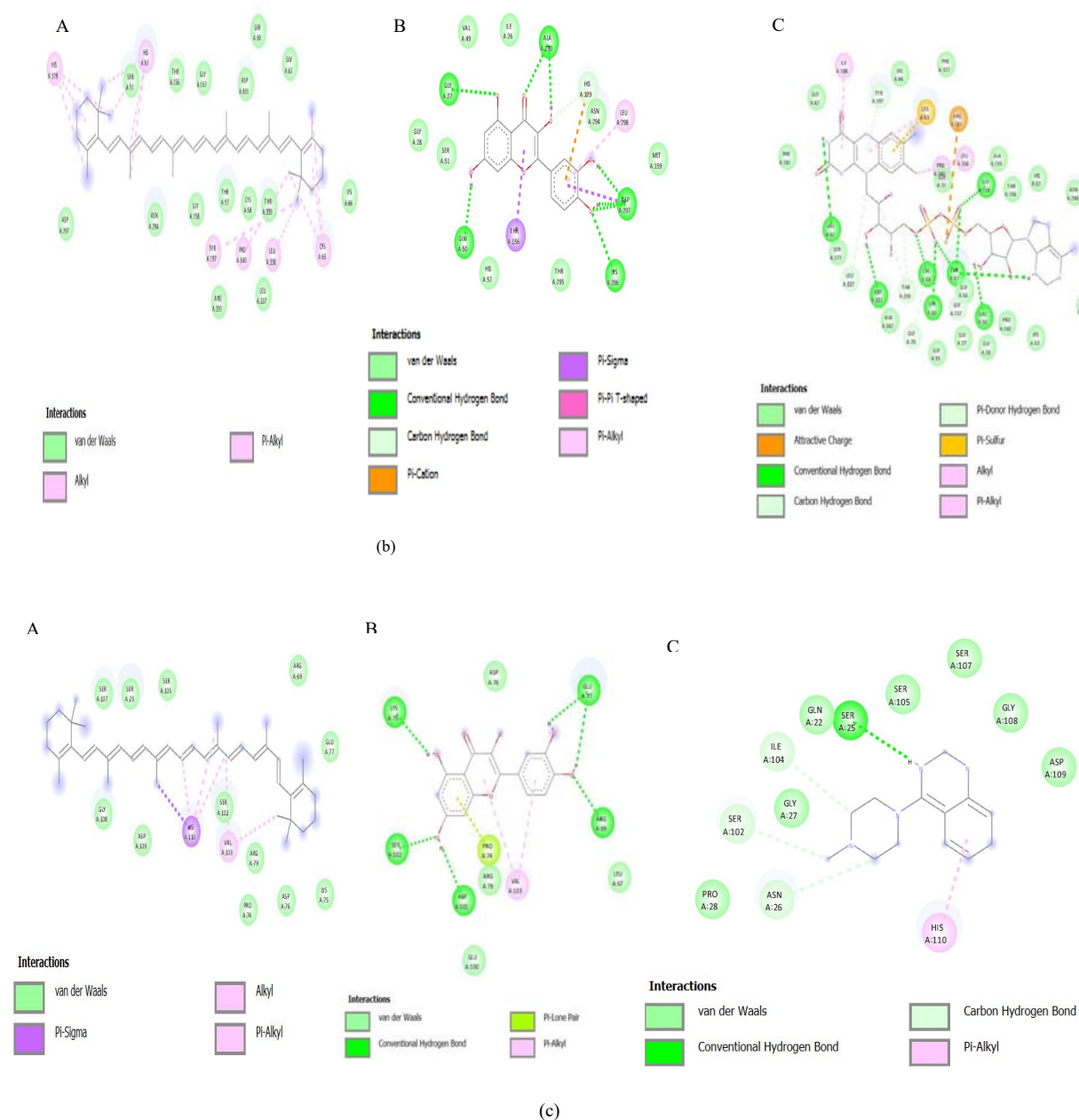


Figure 5. 2D Visualization of molecular docking results β -carotene [A], Quercetin [B], dan Natural ligand [C], PDB ID: 1DGH (a); PDB ID: 1XAN(b); and PDB ID: 1XAN (c).

Table 2. Results of Molecular Docking Analysis of β -carotene and Other Compounds

No.	Coumponds	ΔG (Kkal/mol)	Ki (μM)	Amino acid residue interactions
1DGH (Catalase Enzyme)				
1.	β -carotene	-8.30	0.83	Ala 445, Gln 442, Phe 446, Val 302, Val 450, Phe 198, His 194, Trp 303, Pro 304, Pro 151, Tyr 215, His 305, His 235, Lys 237, Arg 203, Asn 213, Ile 242, Gln 240, Lys 243, His 211, Thr 238, Gly 241
	Quercetin	-7.94	1.50	Asp 128, Pro 129, Gln 195, Arg 130, Thr 150, Phe 446, Val 450, Leu 199, Gly 204, Phe 198, Asn 149, Arg 203, Tyr 215, Asn 213
3.	NADPH dihydro nicotinamide adenine dinucleotide phosphate (Natural ligand)	-7.84	1.79	Lys 306, Pro 304, Val 302, His 305, Lys 237, Val 450, Leu 310, Ile 242, Asn 213, Arg 203, Thr 150, Pro 151, Asn 149, Phe 198, Tyr 215, Phe 446, Leu 451, Ser 201, Gln 455, Asp 202
XAN (Glutathione Reductase Enzyme)				
1.	β -carotene	-9.34	0.14	His 129, Asp 297, Ser 51, His 52, Asn 294, Thr 156, Gly 157, Gly 158, Tyr 197, Pro 340, Leu 338, Arg 291, Leu 337, Thr 57, Cys 58, Thr 339, Asp 331, Ser 30, Gly 62, Cys 63, Lys 66
2.	Quercetin	-8.32	0.79	Gly 28, Ser 51, Glu 50, His 52, Thr 156, Thr 295, Lys 296, Asp 297, Gly 27, Val 49, Ile 26, Ala 130, His 129, Asn 294, Leu 294, Met 159
3.	Flavin adenine dinucleotide (Natural ligand)	-9.65	0.084	Phe 181, Gly 62, Ile 198, Tyr 197, Lys 66, Cys 63, Phe 372, Arg 291, Pro 340, Gly 31, Leu 338, Gly 158, Ala 155, Thr 156, His 52, Asn 294, Val 61, Ser 177, Leu 337, Asp 331, Ala 342, Gly 29, Thr 339, Cys 58, Ser 30, Gly 55, Thr 57, Gly 56, Gly 157, Gly 27, Glu 50, Gly 28, Pro 160, Lys 53, Met 159
Protein 4A7G (Superoxide Dismutase (SOD) Enzyme)				
1.	β -carotene	-9.06	0.23	Ser 107, Ser 25, Ser 105, Gly 108, Asp 109, His 110, Ser 102, Val 103, Arg 79, Pro 74, Asp 76, Lys 75, Glu 77, Arg 69
2.	Quercetin	-8.08	1.20	Lys 75, Ser 102, Asp 101, Pro 74, Arg 79, Val 103, Leu 67, Arg 69, Glu 77, Asp 76, Glu 100
3.	4-(4-methylpiperazin-1-yl) quinazoline (Natural ligand)	-5.21	151.90	Pro 28, Asn 26, Ser 102, Gly 27, Ile 104, Gln 22, Ser 25, Ser 105, His 110, Ser 107, Gly 108, Asp 109

In the development or discovery of new drugs, there are guidelines used to evaluate and select the characteristics of compounds that will be used for drugs so that it can be determined whether the compound can be used for oral drugs or not. These guidelines are known as Lipinski's Rule of Five. This guideline states that if a compound has good oral bioavailability, the following five parameters must be met: the compound must have a molecular weight of no more than 500 Dalton, lipophilicity (Log P) or the tendency of the compound to dissolve in fat <5, have fewer than 5 hydrogen donors, have no more than 10 hydrogen acceptors in the compound, and have a molar refractivity index between 40 and 130 (Lipinski *et al.*,

2001). The Lipinski prediction results for β -carotene isolates are presented in Table 3.

Table 3. Prediction Results of 5 Lipinski Parameters of β -carotene Isolates

No.	Molecular Weight	Log P	Donor Hydrogen Bond	Acceptor Hydrogen Bond	Surface Area
1.	536.888	12.6058	0	0	247.375

After predicting parameter values using Lipinski's rule, ADMET testing using pk-CSM was then performed to predict the pharmacokinetic activity and toxicity of β -carotene (Table 4).

Table 4. ADMET Prediction of β -carotene Compounds as Antioxidants

PharmacokineticModel Name Parameters	Prediction	Unit
Absorption	Water solubility	-7.252
	CaCO ₂	1.239
	Intestinal absorption (human)	90.393
	Skin permeability	-2.739
	P-glycoprotein substrate	No
	P-glycoprotein I inhibitor	No
	P-glycoprotein II inhibitor	Yes
Distribution	VDss (human)	0.202
	Fraction unbound (human)	0
	BBB Permeability	0.92
	CNS permeability	-1.074
Metabolism	CYP2D6 substrate	No
	CYP3A4 substrate	Yes
	CYP1A2 inhibitor	No
	CYP2C19 inhibitor	No
	CYP2C9 inhibitor	No
	CYP2D6 inhibitor	No
	CYP3A4 inhibitor	No
Excretion	Total Clearance	1.061
	Renal OCT2 substrate	No
Toxicity	AMES toxicity	No
	Max. tolerated dose-0.51 (human)	
	hERG I inhibitor	No
	hERG II inhibitor	Yes
	Oral Rat Acute Toxicity (LD50)	2.134
	Oral Rat Chronic Toxicity (LOAEL)	0.624
	Hepatotoxicity	No
	Skin Sensitisation	No
	T.Pyiformis toxicity	0.319
	Minnow toxicity	-3818

4. Discussion

Among the separated pigments, β -carotene was identified as a yellow spot with an R_f value of 0.98 (Spot 1), which aligns with previously reported data (Britton, 1985). Owing to its highly nonpolar nature, β -carotene migrates further in normal-phase chromatography systems, resulting in a high R_f value (Butnariu, 2016). Other identified pigments included additional carotene derivatives, xanthophylls, pheophytin *a*, and chlorophylls

a and *b*, reflecting the broad spectrum of photosynthetic and accessory pigments in *S. platensis*. *S. platensis* contains chlorophyll *a* pigment and its derivatives, which have antioxidant activity (Alotaiby *et al.*, 2024). Maharani *et al.*, (2024), also showed that the pigments in *S. platensis* are carotenoids and chlorophyll and its derivatives.

The successful isolation and confirmation of β -carotene from *S. platensis* highlights its potential as a significant bioactive carotenoid. Given its antioxidant properties and role as a precursor of vitamin A, β -carotene enhances the functional and nutraceutical value of *S. platensis*. Furthermore, the co-occurrence of other pigments, such as chlorophylls and xanthophylls, suggests a synergistic contribution to the antioxidant capacity of the extract, which is consistent with the DPPH assay results presented later in this study. Chlorophyll and its derivatives, namely pheophytin and pheophorbide, have high antioxidant activity. Testing was carried out using the FRAP, TEAC, and DPPH methods (Alotaiby *et al.*, 2024).

The spectrum of the isolate exhibited two distinct absorption maxima within the wavelength range of 400–600 nm, with peak values at 452 and 478 nm. This absorption pattern, characterized by the presence of two well-defined maxima, is consistent with the spectral features of carotenoid. Notably, the recorded absorption maxima closely aligned with the reference values for β -carotene, as reported by Britton (1995) and Rodriguez-Amaya *et al.* (2004), further substantiating the successful isolation of this compound. The appearance of dual absorption peaks can be attributed to the extensive conjugated double-bond system of β -carotene. This conjugation lowers the energy gap between the ground and excited states, allowing the molecule to absorb visible light at longer wavelengths. The fine structure observed in the spectrum arises from electronic transitions within the polyene chain, which are a distinctive feature of carotenoid molecules (Telegina, *et al.*, 2023).

The chromatographic profile from HPLC revealed a predominant peak with a retention time of 30.65 min, which aligned with the elution characteristics of β -carotene documented in the literature under comparable conditions. Additionally, the spectral profile associated with this chromatographic peak exhibited maximum absorbance at 452 and 478 nm, corroborating the identification of the isolate as β -carotene.

The integration of evidence from TLC, UV-Vis spectroscopy, and HPLC strongly supports the identification of the isolated pigment as β -carotene. The concordance between the chromatographic retention time and distinctive spectral characteristics reinforces the validity of this identification. This multi-method verification is particularly crucial, given the structural similarity of carotenoid derivatives, which frequently co-occur in microalgal extracts. The use of complementary analytical approaches, therefore, minimizes the risk of misidentification and increases confidence in distinguishing closely related compounds. By integrating chromatographic behavior, spectral characteristics, and quantitative patterns, the analysis becomes more robust, especially when dealing with complex matrices rich in overlapping metabolites (de Rosso *et al.*, 2007).

The β -carotene isolate demonstrated an IC_{50} value of $460 \pm 0.33 \text{ mg L}^{-1}$, whereas the diethyl ether fraction exhibited a higher IC_{50} of $620 \pm 0.25 \text{ mg L}^{-1}$. Kusmita *et*

al. (2023) research tested the antioxidant activity of β -carotene in *S. polycystum*. The results obtained showed that β -carotene in spirulina had the same activity as β -carotene in *S. polycystum*. Given that lower IC₅₀ values are indicative of stronger antioxidant capacity, the markedly reduced IC₅₀ observed in the purified β -carotene clearly suggests that the isolated pigment possesses substantially greater radical-scavenging activity compared to the crude fraction. This enhanced bioactivity can be explained by the intrinsic structural properties of β -carotene, which is characterized by an extensive conjugated polyene chain composed of alternating double and single bonds. Such a configuration allows the π -electrons to become highly delocalized, enabling efficient stabilization of unpaired electrons through resonance. As a result, β -carotene is able to neutralize a broad range of reactive species, particularly singlet oxygen and peroxy radicals, by acting as an effective electron donor or energy quencher (Young *et al.*, 2018).

Furthermore, the purification process likely removes interfering compounds—such as proteins, carbohydrates, or other pigments—that may compete with or hinder the antioxidant potential of β -carotene in the crude extract. By concentrating the active carotenoid and eliminating non-functional matrix components, the isolate exhibits improved accessibility and reactivity toward free radicals, thereby enhancing its measured antioxidant potency (Stahl, *et al.*, 2003). This relationship between purification and increased bioactivity has also been highlighted in previous carotenoid research, emphasizing that matrix effects can significantly influence antioxidant test outcomes. These structural characteristics enable β -carotene to act as an efficient quencher of free radicals. The findings of this study are consistent with the report by Christiana *et al.* (2008), who demonstrated that β -carotene in *Spirulina platensis* powder exhibits antioxidant activity approximately 5–6 times stronger than that of chlorophyll *a*. Their results reinforce the concept that β -carotene is one of the major contributors to the overall antioxidant profile of *S. platensis* and plays a central role in its protective biochemical functions. The agreement between the present data and earlier literature strengthens the validity of β -carotene as a potent natural antioxidant with high physiological relevance.

Overall, the superior activity of the purified β -carotene relative to the crude pigment fraction underscores the importance of purification strategies for maximizing functional and bioactive properties of carotenoid-rich materials. Enhancing pigment purity not only improves analytical accuracy in antioxidant assays but also holds practical implications for the development of high-value bioproducts, including nutraceuticals, functional foods, and natural antioxidant formulations.

The analysis of β -carotene binding with three proteins revealed that this compound exhibits a more negative binding energy (ΔG) value compared to quercetin, which served as a positive antioxidant control. This value, along with the inhibition constant (K_i), is utilized to assess the strength of a ligand's binding to a specific site on a protein or receptor. In its interaction with the catalase protein (1DGH), β -carotene and quercetin demonstrated binding energy values of -8.30 kcal/mol and -7.94 kcal/mol, respectively (Table 2). When bound to glutathione reductase, β -carotene and quercetin exhibited binding

strengths of -9.34 kcal/mol and -8.32 kcal/mol, respectively. Furthermore, in interaction with the superoxide dismutase (SOD) protein (4A7G), the binding energies of β -carotene and quercetin were -9.06 kcal/mol and -8.08 kcal/mol, respectively (Table 2). The more negative binding energy of β -carotene suggests a stronger binding affinity to the proteins or target receptors, resulting in enhanced bond stability and improved antioxidant activity (Krinsky *et al.*, 2005). The more negative binding energy of β -carotene suggests a stronger and more stable interaction with the target antioxidant enzymes, which in turn indicates its potential to enhance the overall antioxidant mechanism. In this study, β -carotene exhibited varying affinities toward catalase, glutathione reductase, and SOD. Among these proteins, the β -carotene–SOD complex demonstrated the most robust binding, as reflected by the most negative binding energy compared to the other two protein complexes. Interestingly, this strong affinity was achieved despite the involvement of fewer interacting amino acid residues, suggesting that the quality of interaction plays a more critical role than the quantity of residues involved (Meng, *et al.*, 2011).

The interaction of β -carotene with the three antioxidant proteins did not involve hydrogen bonding. Instead, the docking results showed that the binding was predominantly driven by hydrophobic interactions, including π -alkyl, alkyl, and van der Waals forces. Although hydrophobic interactions are typically weaker than hydrogen bonds, they collectively contribute to the stabilization of the ligand–protein complex and support the maintenance of protein conformational integrity (Lohita Sari *et al.*, 2022). The dominance of these interactions is consistent with the physicochemical nature of β -carotene, which is a highly hydrophobic compound characterized by a long-conjugated hydrocarbon chain. This structural feature limits the formation of polar or electrostatic interactions, particularly hydrogen bonds, due to the absence of electronegative functional groups capable of interacting with donor or acceptor atoms on the protein surface.

Overall, the strong hydrophobic-driven binding of β -carotene to SOD, catalase, and glutathione reductase supports its role as a potent antioxidant molecule. The stability of these interactions, especially with SOD, may contribute to the enhanced scavenging of reactive oxygen species, reinforcing the biological relevance of β -carotene in modulating oxidative stress responses.

In binding with the three proteins, β -carotene and quercetin do not form bonds with specific amino acid residues, according to existing literature. The amino acid residues involved in bonding with the catalase protein include Arg112, which forms a salt bridge with the heme carboxylate radical; Tyr358, which regulates reactivity by donating electrons to the catalase, particularly within the iron-heme group; and Arg354, which contributes to oxidative stability by generating an electrostatic field through protonation. Meanwhile, the amino acid residues involved in bonding with the glutathione reductase protein include Tyr407, which plays a protective role in the human protein complex; and His82 along with Cys90, or alternatively His75, Met79, Phe78, and Asp81, which are located at the enzyme's disulfide bond active site (Hossen *et al.*, 2022).

Although quercetin does not bind to specific key amino acid residues, it forms conventional hydrogen bonds during its interaction with the proteins, an important type of bond commonly found in human biological systems. The interaction between quercetin and the catalase protein results in four hydrogen bonds, involving Asn149, Arg203, Tyr215, and Asn213. Its interaction with the glutathione reductase protein forms five hydrogen bonds with Gly27, Ala130, Glu50, Lys296, and Asp297. Meanwhile, its binding with the SOD protein involves five hydrogen bonds formed with Lys75, Ser102, Asp101, Glu77, and Arg69.

The interaction between β -carotene and the three proteins produced several amino acid residues that were the same as those found in the binding between quercetin and proteins with conventional hydrogen bonds. The amino acid residues that were the same in the interaction with catalase protein were Arg 203, Tyr 215, Asn 21, and those in the interaction with glutathione reductase protein were Asp 297. In the binding of β -carotene with SOD protein, the amino acid residues obtained are Lys 75, Ser 102, Glu 77, and Arg 69, which have π -alkyl and alkyl bonds (hydrophobic interactions), but in the binding of quercetin with SOD, these bonds are hydrogen bonds.

The in-silico prediction parameters indicate that β -carotene possesses physicochemical properties that strongly influence its absorption, distribution, and overall pharmacokinetic behavior. Its molecular weight of 536.888 Da exceeds the commonly cited ≤ 500 Da threshold in Lipinski's Rule of Five (Table 3). According to Lipinski, compounds with molecular weights above this limit tend to exhibit reduced membrane permeability through passive diffusion, as larger molecules encounter steric hindrance and require higher energy to traverse phospholipid bilayers (Lipinski *et al.*, 2001). This is consistent with Benet *et al.*, (2016), who emphasized that molecular size plays a critical role in passive transport, where compounds above 500 Da typically demonstrate poor oral absorption.

The log P value of β -carotene is extremely high (log P = 12.6058), far exceeding the recommended limit of ≤ 5 for optimal drug-likeness. Log P represents the balance between hydrophilic and lipophilic characteristics; compounds with very high log P values are excessively lipophilic and tend to partition strongly into lipid membranes. β -Carotene's long conjugated hydrocarbon chain accounts for this high hydrophobicity. While lipophilicity supports membrane association, excessive hydrophobicity is associated with poor aqueous solubility, limited bioavailability, increased retention in lipid bilayers, and broader, non-specific tissue distribution (Hughes *et al.*, 2008). Hakiki *et al.* (2024) further noted that compounds with extremely high lipophilicity are more prone to off-target interactions, potentially increasing toxicity and diminishing target-binding selectivity.

Hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) are also critical parameters influencing drug absorption and permeability. β -Carotene contains zero HBD and HBA, which technically complies with Lipinski's criteria (HBD ≤ 5 , HBA ≤ 10). However, compounds with extremely low polarity and no hydrogen-bonding capability often exhibit very poor aqueous solubility, limiting their ability to dissolve in gastrointestinal fluids and reducing systemic absorption (Zhang *et al.*, 2012). Shofi (2021) emphasizes that

deviations—whether excessive or insufficient—from optimal hydrogen-bonding capacity can hinder absorption because energy is required to desolvate molecules before membrane passage.

Taken together, these properties suggest that although β -carotene can interact strongly with lipid membranes due to its high lipophilicity, its poor solubility, large molecular size, and extreme hydrophobicity reduce its suitability as a drug-like molecule under typical pharmacokinetic criteria. Its biological activity is more likely attributed to membrane-associated antioxidant roles rather than behavior expected of an orally bioavailable small-molecule drug.

In the absorption process, there is a parameter called water solubility. β -carotene compounds have low water solubility because their value is -7.252, which is less than -6 (Table 4). The CaCO_2 parameter is used to determine drug transport in the absorption process through the intestinal epithelium originating from carcinoma cells. A high CaCO_2 value indicates that the compound also has high permeability. The compound β -carotene has a CaCO_2 value of 1.239, indicating sufficient permeability since it exceeds 0.90. The parameter intestinal absorption refers to the absorption of drugs administered orally in the human intestinal tract. The absorption of compounds in the intestine is poor if this parameter value is $< 30\%$, so that β -carotene can be absorbed well by the intestine because the value of 90.393% has a high degree of absorption (Inayah *et al.*, 2024). The Log Kp parameter is an important parameter in identifying or developing drugs administered via the transdermal route. β -carotene has a Log Kp value of -2.739, indicating that this compound has sufficiently high skin permeability, making it suitable for formulation with a transdermal delivery system since its value is < 2.5 (Hakiki *et al.*, 2024).

Parameters in the distribution process are used to determine the distribution of drugs in the body on their way to their target. VDss (Steady state of volume distribution) is used to determine the distribution of drugs in the body in a steady state or equilibrium. β -carotene has a low distribution volume because its value is less than 0.71, namely 0.202 L/kg. Other parameters used to determine the permeability of compounds through the blood-brain barrier (BBB) include the Blood Brain Barrier (BBB). Prediction results indicate that β -carotene has a log BB value of 0.92, meaning that this compound can easily pass through the blood-brain barrier because the log BB value is greater than 0.3. This is also due to β -carotene being a hydrophobic molecule, enabling it to easily penetrate the BBB through the lipid bilayer (Hakiki *et al.*, 2024; Inayah *et al.*, 2024).

Prediction results indicate that β -carotene is metabolized by CYP3A4 because it acts as a substrate for CYP3A4. CYP3A4 belongs to the CYP450 enzyme family. This enzyme plays a role in biotransformation, accounting for 30.20%, and is most abundant in the liver, where it is responsible for eliminating substances that are not needed by the body or detoxification (Hakiki *et al.*, 2024). The metabolism of drug compounds occurs through reduction, oxidation, and other reactions, which increases the polarity of drug compounds, allowing them to be excreted from the body more easily (Dari *et al.*, 2022).

The prediction results at the excretion stage showed a total clearance value of 1.061 ml/min/kg. This value is a

combination of hepatic clearance and renal clearance used to determine the dose required to achieve a steady-state concentration and to determine the rate of drug clearance in the body. Another parameter is OCT2, a transporter in the kidneys that plays a role in the transport and clearance of various substances or drugs. The prediction results indicate that β -carotene does not affect the OCT2 substrate, meaning this compound is not an OCT2 substrate. Therefore, the risk of harmful interactions with OCT2 inhibitors can be minimized (Herdini and Setyawati, 2023).

The AMES toxicity test results indicate that β -carotene does not cause mutations. The hERG (human Ether-à-go-go-Related Gene) inhibition test was performed to assess whether β -carotene has the potential to induce arrhythmia. A positive result in this test suggests that a compound may cause arrhythmia and pose a risk of sudden death, as the hERG channel plays a critical role in regulating heart rhythm. The prediction results show that β -carotene does not inhibit hERG I, indicating it is unlikely to cause cardiac arrhythmia (Hakiki *et al.*, 2024). However, β -carotene shows positive inhibition of hERG II, which is involved in other tissues such as the brain. The LD50 prediction indicates a value of 2.134 mol/kg, equivalent to 1,147,950 mg/kg, placing β -carotene in toxicity class VI (>5,000 mg/kg), which is considered non-toxic. This very high LD50 value means that only at such a high dose would β -carotene cause 50% mortality in mice or other test animals. Furthermore, β -carotene is predicted not to induce liver toxicity (hepatotoxicity) or skin sensitivity.

5. Conclusion

The present work demonstrates the antioxidant relevance of β -carotene derived from *Spirulina platensis*, supported by complementary in vitro assays and molecular docking analysis. These findings underscore its potential as a natural bioactive compound suitable for further development in health-related applications. To advance its practical utilization, future studies should include in vivo validation, assessment of bioavailability, and exploration of optimized delivery systems. Such efforts will help clarify its functional role and broaden its applicability in nutraceutical and therapeutic contexts.

Acknowledgement

This research was supported by the Rumah Program of the Research Organization for Food and Agriculture (OR PP), National Research & Innovation Agency (BRIN), Indonesia, under the Decree of the Head of OR PP BRIN No. 21/III.11/HK/2026

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Construction of Infectious Clones and Confirmation of Pathogenicity of *Tomato leaf curl New Delhi virus* and *Chickpea chlorotic dwarf virus*

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Received: July 25, 2025; Revised: December 17, 2025; Accepted: January 8, 2026

Abstract

Geminiviruses are a group of single stranded DNA viruses that are responsible for severe disease in many crops, especially in tropical and subtropical regions. The development of infectious clones for plant viruses has proven to be a powerful tool to study viral genes, and it facilitates the genetic screening of germplasms for virus resistance. In the present study, rolling circle amplification as well as restriction digestion techniques have been successfully employed to develop tandem repeats and subsequently infectious clones of two economically important geminiviruses, i.e. *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Chickpea chlorotic dwarf virus* (CpCDV). The pathogenicity of these infectious clones has been tested in several plant species. Infected plants were evaluated for symptoms development and infection was confirmed by PCR. PCR data showed that 77.5% and 81.7% of all inoculated plant species were infected with ToLCNDV-JO and CpCDV-JO, respectively.

Keywords: CpCDV-JO, ToLCNDV-JO, Geminiviruses, *Orosius albicinctus*, *Bemisia tabaci*.

1. Introduction

According to the Food and Agriculture Organization, the total share of the agriculture sector in the Gross Domestic Product (GDP) is approximately 5 % (FAO, 2025). In addition, it has an essential role in the economic and social system of rural communities (Hindi, 2009). Given the economic importance of agriculture in Jordan and globally, it is utmost important to study the incidence of plant diseases, particularly those caused by bacteria, fungi, and viruses, that cause severe yield losses to vegetable crops.

Plant diseases caused by viruses are considered among the most important diseases that affect many agricultural crops worldwide (Tatineni and Hein, 2023). In general, these diseases are not easy to control (Gómez *et al.*, 2009). In nature, plant viruses that belong to the *Geminiviridae* family are widely spread and cause great crop damages (Bhattacharjee and Hallan, 2022). Members of this family have monopartite circular ssDNA genomes, except begomoviruses, which have either monopartite (DNA-A) or bipartite (DNA-A and DNA-B) genomes. DNA-A encodes for genes necessary for virus replication and packaging, vector transmission, and viral suppressors (Prasad *et al.*, 2020), while DNA-B encodes genes required for virus movement (Materatski *et al.*, 2021). Since monopartite viruses lack DNA-B, their mobility is controlled by the coat protein and pre-coat protein genes (Prasad *et al.*, 2020).

Begomoviruses are causing economically significant diseases in major vegetable crops such as tomatoes, beans,

cassava, squash, and cotton (Saad *et al.*, 2021). These viruses are considered as the most important limiting factors for vegetable production in most tropical and subtropical regions (Roumagnac *et al.*, 2022). Plants infected with begomoviruses show growth reduction, yellowing, leaf rolling, and necrosis (Varma and Malathi, 2003). In nature, begomoviruses are transmitted by the whiteflies (*Bemisia tabaci*) (Tabein and Behjatnia, 2023). Another group of plant viruses that cause severe yield reduction belongs to the genus *Mastrevirus*. *Chickpea chlorotic dwarf virus* (CpCDV), a member of the genus *Mastrevirus*, is transmitted by leafhoppers such as *Orosius albicinctus* and *O. orientalis* (Akhtar *et al.*, 2011). The virus was reported for the first time in India to affect chickpea plants (Horn *et al.*, 1993). In addition to chickpea, CpCDV infects plants in the *Fabaceae*, *Cucurbitaceae* and *Solanaceae* families (Kraberger *et al.*, 2013). Yield losses due to CpCDV infection was reported to reach 100% (Horn *et al.*, 1995).

The development of infectious clones of plant viruses is crucial for studying virus-virus interactions, functional genomics, replication, host range, expression of viral proteins, as well as understanding host-virus interactions and testing plants germplasm for disease resistance (Brewer *et al.*, 2018; Pasin *et al.*, 2019; Arif *et al.*, 2021).

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2. Materials and Methods

2.1. Virus sources

In a previous work, Jordanian isolates of ToLCNDV (hereafter ToLCDV-JO) and CpCDV (hereafter CpCDV-JO) have been isolated, characterized and cloned into pCambia2300 and pBINPLUS plasmid vectors, respectively (Unpublished data). The sequences of these

two viruses have been deposited in the GenBank under accession numbers (MW407988 for CpCDV) and (OM455497, OM455498 for ToLCNDV-JO).

In this study, the recombinant nature of these plasmids has been confirmed using the alkaline lysis method as described by Green and Sambrook (2016) with minor modifications. After that, plasmids were used as templates in PCR tests using virus-specific primer pairs (Table 1).

Table 1. Oligonucleotides used in this study

Viruses	Primer Name	Primer Sequence (5'-3')	Cyclic Conditions	bp
CpCDV-JO	CpCDVCP527F	AGGGAATCGTTGCAGGTGG	1 cycle:94°C/3min;35 cycles: 94°C/50sec; 60 °C/1min;	619
	CpCDVCP1145R	CGGACTTGAAGTACACTCGGA	72°C/1min; 1 cycle:72°C/10min	
ToLCNDV-JO	ToLCNDVF1	TCTAGAACGTCTCCGTCTTTGTGTCGATG	1 cycle:94°C/3min;30 cycles: 94°C/1min; 55 °C/30sec;	1321
	ToLCNDV R2	GCAGGCCACTTCCGTAAGACTTGATAAC	72°C/2min; 1 cycle:72°C/10min	
	ToLCNDVCP1	CTCCAAGAGAATTGAGAAGTCC	1 cycle:94°C/3min;40 cycles: 94°C/50sec; 55 °C/1min;	1049
DNA-A	ToLCNDVCP2	TCTGGACGGGCTTACGCCCT	72°C/2min; 1 cycle:72°C/10min	
DNA-B	ND-B-BamHI921F	TTTGGATCCGTGAATGCCTGC	1 cycle:94°C/3min;35 cycles: 94°C/30sec; 57 °C/30sec;	498
	ND-B-ClaI1419R	AATCGATCCAGGTCCATCGGCA	72°C/2min; 1 cycle:72°C/10min	
	ND-B-BamHI933R*	CACGGATCCAAACATTTCACAAAAGG	1 cycle:94°C/3min;35 cycles: 94°C/30sec; 57 °C/30sec;	2206
	ND-B-ClaI1410F	TGGATCGATTGCCGAATCATCTAATC	72°C/2min; 1 cycle:72°C/10min	

*Parrella *et al.*, 2018

2.2. Development of Chickpea chlorotic dwarf virus infectious clone

In brief, a recombinant pBINPLUS bearing the full-length genome of CpCDV-JO (pBINPLUS-2.5-CpCDV) was extracted and subjected to PCR analysis using the CpCDV-JO specific primer pair CpCDVCP527F/CpCDVCP1145R (Table 1). After that, pBINPLUS-2.5-CpCDV was subjected to restriction digestion using *EcoRI* enzyme and the obtained ~2.5 kb fragment was self-ligated and subjected to Rolling circle amplification (RCA) using Φ 29 DNA polymerase (Thermo Scientific, USA) according to the manufacturer's instructions. RCA products were partially digested with *KpnI* enzyme to generate a dimer (~5 kb) of virus genome. The fragment of the expected size was gel-eluted and cloned into the binary pBINPLUS cloning vector to develop pBINPLUS-5-CpCDV-JO. As a final step, the GV3101 strain of *A. tumefaciens* was transformed with pBINPLUS-5-CpCDV.

2.3. Development of Tomato leaf curl New Delhi virus infectious clone

The dimeric molecule of viral DNA-A was generated by digestion of the recombinant pCambia2300 carrying the full-length DNA-A with *BamHI*. After self-ligation, DNA-A was subjected to RCA and the reaction products were partially digested with *BamHI* to produce the dimeric molecule. The obtained ~5.4 kb fragment was cloned into the *BamHI* site of pBINPLUS to generate pBINPLUS-5.4-ToLCNDV-DNA-A and transformed into *A. tumefaciens* and stored at -80°C. The dimeric molecule of viral DNA-B was generated by digestion of the recombinant pCambia2300 carrying the full-length DNA-B with *BamHI*. After self-ligation, DNA-B was subjected to RCA and the amplified RCA products were partially digested with *Acc65I* to produce the dimeric molecule. The obtained ~5.4 kb fragment was cloned into the *Acc65I* site of pBINPLUS to generate pBINPLUS-5.4-ToLCNDV-DNA-B and transformed into *A. tumefaciens* and stored at -80°C.

The infectivity of the CpCDV-JO and ToLCNDV-JO clones has been tested on different plants species (Table 2) by infiltrating the lower side of the leaves with the bacterial suspension using a syringe without needle.

Table 2. Response of different plant species inoculated with ToLCNDV-JO and CpCDV-JO clones.

Clones	Plant Species	No. of inoculated plants	No. of symptomatic plants	PCR +ve/ total
ToLCNDV-JO	<i>Solanum lycopersicum</i> cv. Baladiah	20	1	13/20
	<i>Nicotiana benthamiana</i>	10	10	10/10
	<i>Cucurbita pepo</i> cv. Black Beauty	6	6	6/6
	<i>Cucurbita pepo</i> cv. JAZYF1	8	8	8/8
	<i>Citrullus lanatus</i> cv. Sugar Baby	8	1	6/8
	<i>Cucumis melo</i> var cv. Melon Piel de Sapo	7	7	7/7
	<i>Capsicum annuum</i> cv. Peperone Corno di Toro	7	0	4/7
	<i>Capsicum annuum</i> cv. Peperone Yolo Wonder	7	0	2/7
	<i>Solanum melongena</i> cv. Melanzana BIANCA	7	0	6/7
CpCDV-JO	<i>Nicotiana benthamiana</i>	10	10	10/10
	<i>Solanum lycopersicum</i> cv. Baladiah	20	19	19/20
	<i>Cucurbita pepo</i> cv. Black Beauty	6	0	6/6
	<i>Citrullus lanatus</i> cv. Sugar Baby	9	0	8/9
	<i>Cucumis melo</i> var cv. Melon Piel de Sapo	6	6	6/6
	<i>Capsicum annuum</i> cv. Peperone Corno di Toro	20	7	20/20
	<i>Capsicum annuum</i> cv. peperone Yolo Wonder	7	0	7/7
	<i>Solanum melongena</i> cv. Melanzana BIANCA	15	0	0/15

3. Results

3.1. Development of Chickpea chlorotic dwarf virus infectious clone and pathogenicity test

Before plants were inoculated with the recombinant bacterium and to confirm that the dimeric molecule of CpCDV-JO has been correctly transformed into *A. tumefaciens*, pBINPLUS-5-CpCDV-JO has been extracted from *A. tumefaciens* cells and subjected to double digestion with *SacI* and *SmaI* restriction enzymes. The expected size (~5kb) of CpCDV-JO dimer could be released (not shown).

Data presented in Table 2 summarize the response of different plant species to CpCDV-JO infection. Three plant species including pepper, melon and *N. benthamiana* developed disease symptoms 28 days post infection with CpCDV-JO (Fig. 1), and infection has been confirmed by PCR. On the other hand, all tomato plants were symptomless 28 days post inoculation and disease symptoms developed 60 days post inoculation and with time plants recovered. Plant species that were symptomless throughout the experiment were watermelon, zucchini, pepper (Peperone Yolo Wonder) and eggplant. When tested with PCR, the virus could be detected in 8 out of 9 inoculated watermelon plants, and all zucchini and pepper (Peperone Yolo Wonder) plants were found infected with the virus. All attempts to detect CpCDV-JO in inoculated eggplant plants failed. PCR results showed that 81.7% of all inoculated plants were infected with CpCDV-JO.

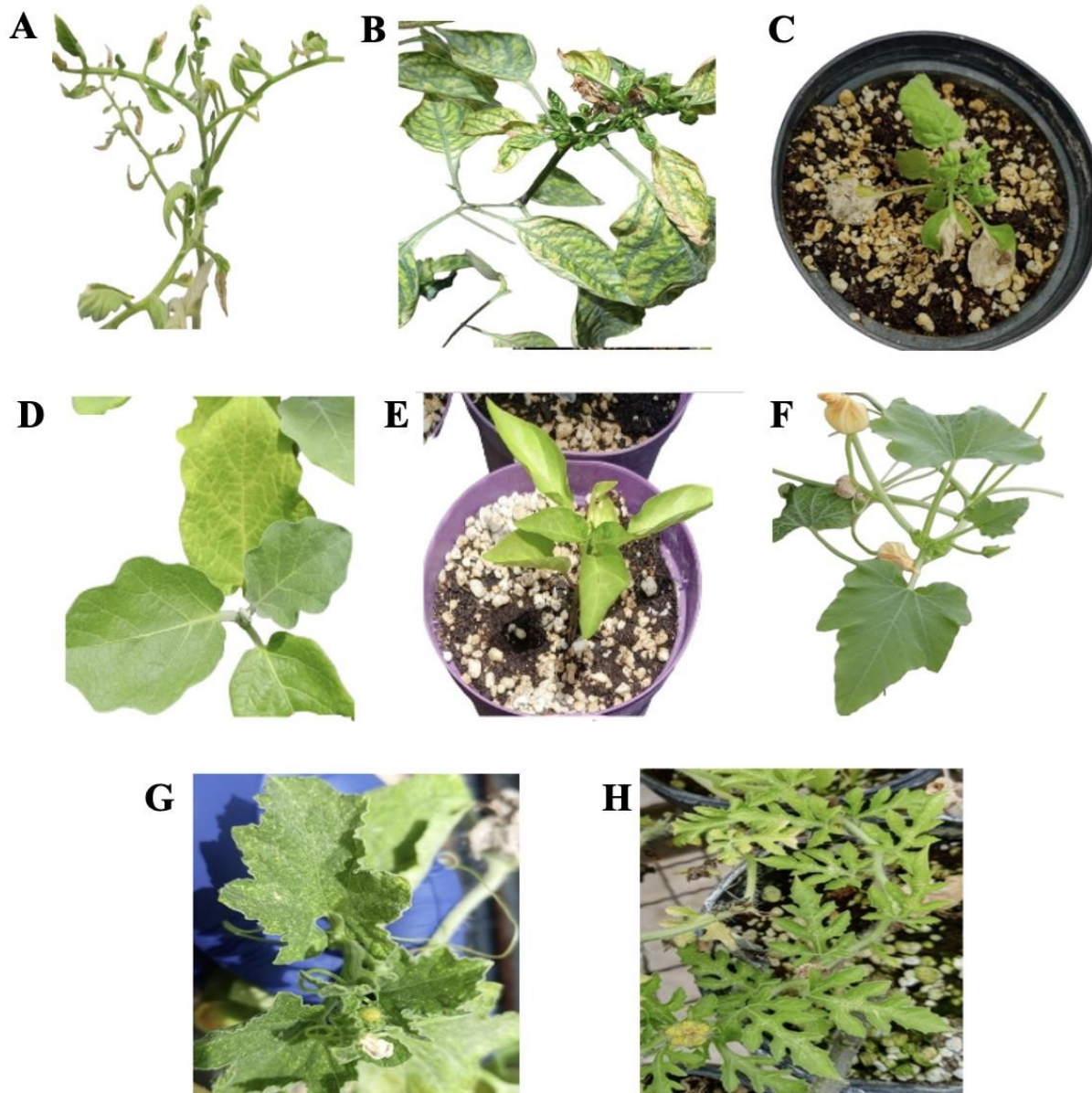


Figure 1. Plant species inoculated with CpCDV-JO infectious clone. A: tomato (Balady), B: pepper (Peperone Corno di Toro Rosso), C: *N. benthamiana*, D: eggplant (Melanzana BIANCA A UOVO), E: pepper (Peperone yolo wonder), F: zucchini (Black Beauty), G: melon (Melon piel de sapo) and H: watermelon (Sugar Baby).

3.2. Development of Tomato leaf curl New Delhi virus infectious clone and pathogenicity test

Before the ToLCNDV-JO clone was used to inoculate test plants, pBINPLUS-5.4-ToLCNDV-DNA-A and pBINPLUS-5.4-ToLCNDV-DNA-B plasmids were subjected to PCR using ToLCNDV-F2/ToLCNDV-R1 and ND-B-Clal1419R/ND-B-BamHI921F specific primer pairs, respectively (Table 1). Figure (2) shows the specificity of the recombinant plasmids. Plants belonging to *Solanaceae* and *Cucurbitaceae* families were inoculated with ToLCNDV-JO. All *N. benthamiana*, zucchini and melon plants developed disease symptoms, and only one tomato

and watermelon plant was symptomatic (Fig. 3). Symptoms of chlorotic mosaic and curling developed on tomato plant cv. Balady, while severe leaf curling (Fig. 3, A), leaf yellowing and leaf malformation were observed on zucchini cv Black Beauty plants (Fig. 3, E). Melon plants showed chlorotic lesions, downward leaf curling and yellowing and *N. benthamiana* plants developed leaf curling, yellowing and mottling symptoms (Fig. 3. I). On the other hand, all eggplant and pepper plants remained symptomless throughout the period of the experiment (Fig. 3 H and I).

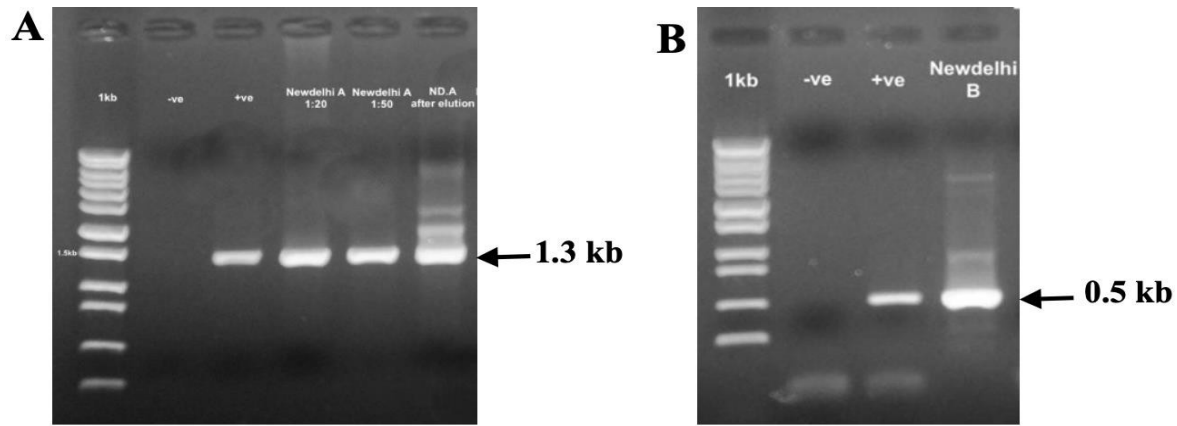


Figure 2. Confirmation of pCambia2300-5.4-ToLCNDV-DNA-A and pCambia2300-5.4-ToLCNDV-DNA-B identities by PCR. A: pCambia2300-5.4-ToLCNDV-DNA-A amplified using primer pair ToLCNDVF1/ToLCNDV R2; B: pCambia2300-5.4-ToLCNDV-DNA-B amplified using ND-B-ClaI1419R/ND-B-BamHI921F primer pair. 1kb: 1 kb DNA ladder; -ve: negative control (PCR mix components without template); +ve: positive control (DNA-A or DNA-B of ToLCNDV-JO).

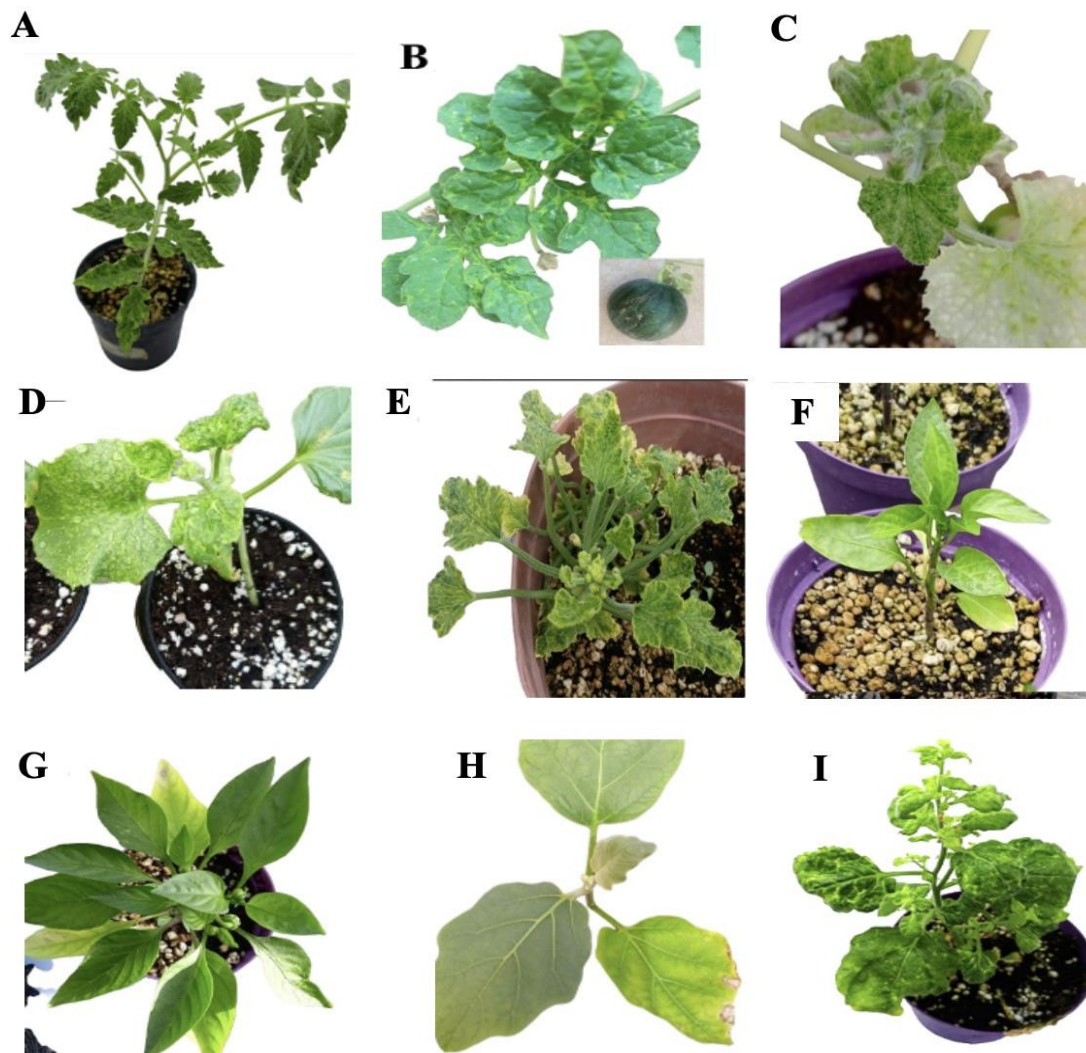


Figure 3. Plant species inoculated with ToLCNDV-JO infectious clone. A: tomato (Balady); B: watermelon (Sugar baby); C: squash cv. JAZYF1; D: melon cv. piel de sapo; E: zucchini cv. Black Beauty; F: pepper (Peperone Corno di Toro Rosso); G: pepper cv. peperone yolo wonder; H: eggplant cv. Melanzana BIANCA; I: *N. benthamiana*.

It is important to note that when asymptomatic plants were tested for ToLCNDV-JO infection by PCR, many plants were found positive for the virus (Table 2). For example, one tomato plant showed disease symptoms while 13 other asymptomatic plants out of 20 plants were found positive for the virus by PCR. Similarly, one

watermelon plant showed chlorotic mosaic and curling, while 6 plants were asymptomatic but were positive for the virus as confirmed by PCR. PCR results demonstrated that the infection rate of ToLCNDV-JO was 77.5%.

4. Discussion

The primary goal of this study was to develop infectious clones of two plant viruses belonging to the *Geminiviridae* family and to investigate their pathogenicity in host plants. This goal is accomplished by inserting multimers of virus genomes into binary vectors, which are subsequently introduced into an *Agrobacterium* and used to infect host plants (Saad *et al.*, 2021).

The first infectious clone developed in this study was CpCDV-JO. Previous studies examined the pathogenicity of CpCDV as a control on *N. benthamiana* plants in the presence of betasatellite of CLCuMV (CLCuMβ). The results and symptoms development were in accordance with results obtained in this study; however, the pathogenicity of the CpCDV-JO infectious clone has been investigated without the co-infection with beta-satellites. In a previous study, Khalid *et al.* (2017) tested the pathogenicity of a CpCDV infectious clone on tomato plants. Infected plants showed yellow lesions and curling of newly developed leaves, and plants were severely stunted. Similar disease symptoms were also observed on tomato plants inoculated with CpCDV-JO two months post inoculation. Possible explanations of symptoms delay include low number of CpCDV-JO particles (titer) introduced into tomato cells and the number of tomato cells that were successfully infiltrated with the bacterial suspension. Furthermore, environmental conditions such as temperature, light and humidity prevailed in our greenhouses may also played a role in symptoms expression (Pares *et al.*, 1996).

CpCDV-JO was able to induce upward leaf curling and yellowing symptoms on pepper plants cv. Peperone Corno di Toro Rosso. Similar results were reported by Akhtar *et al.* (2011). It is worth mentioning that the infection rate of CpCDV-JO was low (7 symptomatic plants out of 20 infected plants). This may be due to low viral load (Kumar *et al.*, 2022). It is important to note that although all CpCDV-JO infected pepper, watermelon and zucchini plants did not develop disease symptoms; however, the virus could be detected in these plants by PCR. These results suggest that asymptomatic plants may possess high level of resistance against CpCDV-JO infection. From epidemiological point of view, asymptomatic host plants infected with plant viruses pose high risk especially in the presence of insect vectors.

CpCDV was reported to infect cucurbit plants including watermelon and squash. In Egypt, squash plants with severe leaf curling, yellowing, stunting and reduced fruit size were observed in different fields in Giza governorate. PCR results showed that plants were mixed infected with CpCDV and *Squash leaf curl virus* (SLCV). However, authors did not show whether the observed disease symptoms were due to SLCV or CpCDV infections (Fahmy *et al.*, 2015). In another study, Zaagueri *et al.* (2017) reported that CpCDV is the causal agent of hard fruit syndrome of watermelon cv. Crimson in Tunisia. On the light of this study, it can be speculated that watermelon plants inoculated with CpCDV-JO may show disease symptoms on fruits. This may be true since 8 out of 9 plants were PCR positive for CpCDV-JO infection. In addition, further attempts must be done to inoculate other watermelon cultivars to investigate the susceptibility of these cultivars to CpCDV-JO infection.

ToLCNDV-JO was recently detected on cucurbits, tomato and pepper crops in Jordan (unpublished data). In this study, an infectious clone of the virus has successfully been developed. The infectivity assay test showed high (100%) infection rate of ToLCNDV-JO clone. Data presented in this study demonstrated that ToLCNDV-JO clone could infect melon, watermelon, tomato, squash, zucchini and *N. benthamiana* plants. A large body of literature showed similar results obtained by different research groups (Cifuentes *et al.*, 2017; Mei *et al.*, 2023; Vo *et al.*, 2023). It is worth noting that only one ToLCNDV-JO infected tomato plant cv. Balady out of 20 plants showed mild vein-yellowing symptoms. In agreement with these data, Yamamoto *et al.* (2021) reported that most tomato plants inoculated with ToLCNDV-[ES-Alm-Cuc-16] were symptomless and only few plants developed slight vein yellowing symptoms. In the present study, latent infections with ToLCNDV-JO were observed in pepper and eggplants. No disease symptoms could be detected on these plants; however, PCR results showed that most of eggplants were positive for ToLCNDV-JO infection. It is important to note that only 2 out of 7 pepper plants were found infected with ToLCNDV-JO. A possible explanation may be that the used pepper cultivars were not susceptible to the virus. Therefore, more trials must be conducted to inoculate other pepper cultivars, especially those commonly grown in Jordan. Against our results, Mnari-Hattab and co-authors reported that pepper plants were severely infected with ToLCNDV (Mnari-Hattab *et al.*, 2022). Data presented in this study also showed that eggplant is a symptomless host of the ToLCNDV-JO. However, eggplants infected an Italian isolate of the virus developed severe disease symptoms (Parrella *et al.*, 2018). More studies are necessary to evaluate the response of other host plants, commonly grown in Jordan, to CpCDV-JO and ToLCNDV-JO infections.

5. Conclusions

This is the first study in Jordan that demonstrates the development of infectious clones to CpCDV and ToLCNDV. These clones should be available to other scientists in Jordanian universities who are doing research in the field of plant virology. Furthermore, the outcomes of this study can be useful to seed companies who are interested in developing germplasm resistant to plant viruses.

Acknowledgment

This study was supported by grant no. SIS70019GR0087 funded by the government of U.S.A.

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Optimization and Assessment of Gallic Acid Proniosomal Gel for Transdermal Application

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Received: September 19, 2025; Revised: December 12, 2025; Accepted: January 8, 2026

Abstract

The current study investigates the development and assessment of a proniosomal gel incorporating gallic acid to treat *Staphylococcus aureus* infection. Gallic acid exhibits strong antimicrobial activity; its proniosomes were prepared to enhance its stability and transdermal delivery. Different formulations were developed by the coacervation phase separation approach in combination with a response surface design for optimization. Span 60 and soy lecithin served as the independent variables, with drug release and entrapment efficiency chosen as the dependent responses. The best formulation obtained was further analysed, incorporated into a Carbopol 940 gel base, and evaluated. The entrapment efficiency and drug release of different batches of proniosomes ranged between 76 ± 0.81 to $92.69 \pm 0.55\%$ and 44.06 ± 0.53 to $88.34 \pm 0.41\%$, respectively. The results demonstrated that the optimized batch of proniosomal gel exhibited a uniform vesicle size of 299.7nm with a polydispersity index of 0.232 and a zeta potential of -49.3 mV. pH and drug content of gel were 6.2 ± 0.23 and $99.51 \pm 0.65\%$, respectively. The zone of inhibition of proniosomal gel, marketed gel, and gallic acid was 33 ± 3.87 , 30 ± 2.89 , and 27 ± 3.19 mm, respectively; these results confirmed the superior antimicrobial effect of the formulation. To conclude, gallic acid-loaded proniosomal gel was found to be a promising formulation for the effective treatment of the infection caused by *Staphylococcus aureus*, with its superior drug entrapment and skin absorption properties, which enable the formulation to deliver the drug to the site of infection efficiently.

Keywords – Proniosomes, gallic acid, transdermal, coacervation phase separation method

1. Introduction

Gallic acid is a naturally occurring phenolic compound and is widely distributed among different families of higher plants. It is present throughout different parts of the plant, such as bark, wood, leaves, fruits, seeds, and roots, often occurring either in free form or as derivatives. It exists both in its free form and as components of more intricate structures, including ester derivatives and polymers (Daglia *et al.*, 2014). The therapeutic potential of gallic acid is quite comprehensive. It exhibits diverse biological traits such as antioxidant, anti-inflammatory, anticancer, antiviral, and anti-allergic. Furthermore, research has demonstrated its efficacy in addressing cardiovascular and metabolic disorders. Various investigations have highlighted the antibacterial properties of gallic acid. However, the precise mechanisms underlying its antimicrobial effects remain unclear in several cases. The antimicrobial mechanisms of polyphenols may involve various factors, which act independently or in synergy. These factors include alterations in the function of the cytoplasmic membrane, interference with intracellular processes, and induction of programmed cell death (Hadidi *et al.*, 2024). Despite numerous therapeutic benefits, applications of gallic acid

are restricted owing to fast metabolism, poor absorption, suboptimal distribution, low bioavailability, and quick elimination from the body (Bhuia *et al.*, 2023).

To date, gallic acid has been studied in various forms, including gels, microparticles, emulsions, microemulsions, and micelle formulations, to explore its therapeutic benefits. Stability aspects are a major concern about emulsion formulation (Dhandhi *et al.*, 2025). Vesicular drug delivery systems, such as liposomes, solid lipid nanoparticles, metal nanoparticles, and polymeric nanoparticles, were investigated by various researchers to overcome some drawbacks of gallic acid (Shukla *et al.*, 2022; Monteiro e Silva *et al.*, 2017; Consoli *et al.*, 2016; Patil *et al.*, 2021). Liposomes of gallic acid suffer from the drawbacks of poor encapsulation efficiency, burst release, and oxidative degradation (Enaru *et al.*, 2021). Nanoparticles in general undergo aggregation and are rapidly cleared, costly, and exhibit inconsistent drug release (Holghoomi *et al.*, 2024). The advancement of vesicular drug delivery systems has seen significant interest in the development of niosomes. Niosomes are advantageous owing to low cost, good stability, ease of formulation, and scaling-up (Ge *et al.*, 2019). These are preferred vesicles for transdermal drug delivery due to the modulation of drug transfer through the skin. The alterations in stratum corneum structures are among the

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primary factors contributing to increased permeability of drugs. The treatment with niosomes appears to significantly modify the intercellular lipid barrier within the stratum corneum, resulting in a more relaxed and permeable configuration (Barry, 2001). Niosomes, while beneficial, are not without their limitations. The formulations and compositions of niosomes can be physically unstable, leading to potential aggregation if the established preparation protocols are not strictly followed. Additionally, the process of preparing niosomes can be quite time-consuming, and there are infrequent instances of insufficient drug loading. Furthermore, variations in surface charges on vesicles can occur, which increases the risk of fusion, as vesicles with opposing charges are more likely to attract one another (Bashkeran *et al.*, 2023). These drawbacks can be overcome by proniosomes. These are liquid crystalline-compact niosomal hybrids, which are widely preferred for transdermal drug delivery (Tareen *et al.*, 2020). Upon skin application, these get hydrated with water from skin under occlusion and convert into niosomes (Keyvani-Ghamsari *et al.*, 2023). Some phospholipids can disrupt and fluidize the lipid bilayer of the stratum corneum, thereby improving diffusion across the skin layers. Proniosomes also provide practical benefits in terms of handling, storage, distribution, and dosing. Unlike conventional aqueous niosomal dispersions, they minimize common stability issues like aggregation, vesicle fusion, and drug leak. They also have the potential to improve the skin barrier's recovery rate (Ammar *et al.*, 2011).

Taking into account the above-mentioned factors, the aim of present study is to enhance penetration, stability, and modulate release of gallic acid by loading it into the proniosomal vesicular system. The formulation is further loaded into gel base for transdermal application to counteract microbial infection.

2. Materials and methods

2.1. Materials

Gallic acid, cholesterol, and soy lecithin were obtained from Research-Lab Fine Chem Industries, Mumbai, India. Additional ingredients, including Span 60, ethanol, Carbopol 940, triethanolamine, sodium benzoate, potassium dihydrogen orthophosphate, sodium hydrogen phosphate, and sodium hydroxide, were sourced from Thermosil Fine Chemical Industries Co., Ltd., India. All chemicals and reagents utilized in the formulation process were of analytical grade and highest purity.

2.2. Experimental design

A response surface optimal custom design was employed using Design Expert® software (Version 11, Stat-Ease, Minneapolis, USA) to generate different formulation batches (Table 1). Soya lecithin (X1) and Span 60 (X2) were chosen as independent variables; however, percent entrapment efficiency (Y1) and drug release (Y2) were taken as response factors. Statistical evaluation was done through analysis of variance (ANOVA) within software, and regression models were constructed to interpret the experimental domain. A significance level of $p \leq 0.05$ was considered for all analyses (Soliman *et al.*, 2016).

Table 1. Preparation of proniosomes by response surface optimal custom design

Independent variables	Symbols	Levels		
		Low	Medium	High
Amount of soya lecithin (mg)	X1	10	15	20
Amount of Span 60 (mg)	X2	160	180	200
Dependent variables				
Y1	Entrapment efficiency (%)			
Y2	Drug release (%)			

2.3. Preparation of gallic acid-loaded proniosomes

The preparation of gallic acid-loaded proniosomes was carried out using the coacervation phase separation method (Mehta *et al.*, 2016). Accurately measured quantities of gallic acid, soya lecithin, cholesterol, and Span 60 were placed into a 5 mL glass vial, subsequently by the addition of 0.3 mL of absolute ethanol. The mixture was blended with a glass rod, and the vial was immediately capped to avoid solvent loss through evaporation. The mixture was then subjected to heating in a water bath (Thermostate, India) at a temperature range of 60 to 65°C for approximately five min, ensuring that the surfactant blend was fully dissolved. After a clear solution was obtained, the aqueous phase (0.1 mL) was added at 60°C, and then the system was reheated. After achieving a transparent mixture, the vial was removed from the water bath and allowed to cool down at room temperature (Mohsen *et al.*, 2023). Different formulations are shown in Table 2.

Table 2. The composition of gallic acid proniosomes formulations

Batch no.	Drug (mg)	Cholesterol (mg)	Soya lecithin (mg)	Span 60 (mg)
P1	5	15	20	180
P2	5	15	15	200
P3	5	15	20	160
P4	5	15	10	180
P5	5	15	10	200
P6	5	15	15	160
P7	5	15	15	180
P8	5	15	20	200
P9	5	15	10	160

2.4. Characterization of proniosomes

2.4.1. Entrapment efficiency

0.2 g of the proniosomal gel was added to 10 mL of phosphate buffer (pH 7.4). The mixture was sonicated for 3 minutes to achieve uniform dispersion and subsequently centrifuged at 8000 rpm for 90 minutes at 4 °C (C24 Plus, Remi, India). The supernatant obtained was collected and analysed spectrophotometrically at 259 nm, using phosphate buffer (pH 7.4) as the reference blank. Entrapment efficiency was calculated in triplicate by equation (1) (Kandekar *et al.*, 2025).

$$EE\% = \frac{\text{Total amount of drug} - \text{Untrapped drug}}{\text{Total amount of drug}} \times 100 \quad (1)$$

2.4.2. Drug release

The in-vitro release profile of gallic acid from the proniosomal gel was evaluated using a Franz diffusion cell

(DBK Instruments, India), featuring a receptor compartment with a 25 mL volume and a diffusion area of 1 cm². A dialysis membrane (150 LA 401, HiMedia Laboratories, India) was positioned between the donor and receptor chambers to act as a permeation barrier. A sample containing 2 mg of gallic acid was introduced into the donor compartment, while the receptor side was filled with phosphate buffer at pH 7.4. The system was maintained at a temperature of 32 ± 0.5 °C and stirred continuously using a magnetic stirrer. At scheduled time points ranging from 1 to 8 hours, 1 mL samples were extracted from the receptor compartment. To preserve sink conditions, each withdrawn sample was immediately replaced with an equal volume of preheated phosphate buffer (pH 7.4). The samples were then analysed using UV spectrophotometry at 259 nm, with phosphate buffer (pH 7.4) serving as the blank.

2.4.3. Optimization of gallic acid proniosomes

Optimization was carried out to identify the levels of independent variables (X1 and X2) that would achieve the highest entrapment efficiency and sustain drug release over an 8h. The overlay plot was produced by Design Expert software, and the optimized formulation was selected. Then, it was evaluated and loaded into a gel base.

2.4.4. Characterization of optimized formulation

2.4.4.1. Particle size, polydispersity index (PDI), and zeta potential

0.2g of an optimized batch of proniosomal gel was added to 20 mL of phosphate buffer at pH 7.4, and then it was sonicated in a water bath for 10min. Following sonication, a 1 mL sample was extracted for analysis. Using dynamic light scattering (DLS) with a laser diffraction analyzer (SZ-100, Horiba, Osaka, Japan), vesicle size, polydispersity index, and zeta potential were assessed (Abu El-Enin *et al.*, 2019).

2.4.4.2. Scanning electron microscopy (SEM)

Vesicle morphology was studied by a scanning electron microscope operated at 20 kV. Precisely, 0.2g of proniosomes was dispersed in 10mL of pH 7.4 phosphate buffer. It was affixed to double-sided carbon tape and then positioned on an aluminium stub, where it underwent drying in a vacuum, following which the scanning process was conducted (Kandekar *et al.*, 2025).

2.4.4.3. Differential scanning calorimetry (DSC)

The thermal analysis of gallic acid, soya lecithin, Span 60, and the optimized formulation of proniosomes was performed using a differential scanning calorimeter (Mettler Toledo, SW STAR, United States). The scan was carried out at a heating rate of 10°C/min under a nitrogen atmosphere over a temperature range of 30°C-300°C (Kandekar *et al.*, 2021).

2.5. Preparation of proniosomal gel

Prepared proniosomes are incorporated into the gel base of Carbopol 940. Precisely, 1g of Carbopol 940 was soaked overnight. Optimized formulation of proniosomes was incorporated in a swollen base with constant stirring at a slow speed till a homogeneous mass was formed. Triethanolamine (0.1% v/w) was incorporated in the gel to adjust pH, while glycerine and sodium benzoate (each

0.1%) were added as humectant and preservative, respectively (Cheng *et al.*, 2024).

2.5.1. Determination of pH, viscosity, and drug content

The pH of the optimized proniosomal gel was measured using a pH meter in triplicate. The viscosity was assessed by Brookfield's viscometer (Brookfield Engineering Labs, USA) at 30, 50, and 100 rpm by using spindle number 64. To evaluate drug content, 1g of the prepared gel was mixed with 25mL of ethanol to produce the stock solution. The stock solution was then sonicated in a water bath for 10min, filtered, and analysed spectrophotometrically at 259nm. The content was estimated by equation (2).

$$\text{Drug Content (\%)} = (\text{Actual amount of gallic acid} / \text{Theoretical amount of gallic acid}) \times 100 \quad (2)$$

2.5.2. Texture analysis

The gel's texture was evaluated using a CT3-100 Texture Analyser (Brookfield Engineering Labs, USA) in triplicate. The formulation was placed into the female probe, and the test was conducted in compression mode. A conical male probe (35 mm diameter, 45° angle) was injected to the sample at a speed of 0.50 mm/s and trigger load of 2 g until reaching a depth of 10 mm. All measurements were performed in triplicate at 25 °C. (Dhakane *et al.*, 2023).

2.6. In-vitro diffusion study of gel

The *in-vitro* investigation of gallic acid-loaded carbopol gel, proniosomal gel, and marketed gel was carried out by the Franz diffusion cell apparatus by adopting the same procedure as mentioned under the *in-vitro* diffusion study of proniosomes and gallic acid (Pandit *et al.*, 2023).

2.7. Ex-vivo diffusion study of gel

A Franz diffusion cell with goat skin as the diffusion membrane was used for Ex-vivo diffusion studies of the drug-loaded Carbopol gel, proniosomes, proniosomal gel, and a marketed formulation. Fresh goat skin was collected from a local slaughterhouse. During transport, the skin was preserved in Ringer solution. The hair from the skin was shaved off, and care was taken to avoid transverse cuts. The skin was then attached between compartments, with the stratum corneum facing the donor chamber, and the diffusion study was carried out following the same process described for the *in-vitro* release study. Additionally, fresh goat skin was used with a placebo gel in the donor compartment and phosphate buffer (pH 7.4) in the receptor compartment to serve as a blank control (Shah *et al.*, 2021).

2.7.1. Ex-vivo skin permeation

Ex-vivo permeation of gallic acid through the skin membrane was determined by calculating steady-state flux ($J_{\max}$). The drug flux was calculated by the following equation (3).

$$J_{\max} = \frac{dQ}{dt} / A \quad (3)$$

Where dQ/dt is the skin permeation rate that indicates the cumulative amount of permeated gallic acid Vs time, and A is the effective diffusion area of the skin membrane (Dhakane; Pandit, 2023).

2.8. Antimicrobial activity against *Staphylococcus aureus*

The antimicrobial activity against *S. aureus* was evaluated using the agar well diffusion method against *Staphylococcus aureus* (*S. aureus*). Nutrient agar was prepared by dissolving in distilled water and sterilized in an autoclave (Space Lab Instruments, India) at 121 °C and 15 psi. The *S. aureus* culture was then spread uniformly over the surface of the agar plate. Sterilized borers were used to create wells in the agar plates, into which various samples were added separately. The plates were then transferred to an incubator and incubated at 37°C for 24 h. (Selvaraj *et al.*, 2022). The zone of inhibition (mm) was recorded in triplicate.

3. Result and discussion

3.1. Preparation of gallic acid-loaded proniosomes

Initial trials were conducted to select the level of Span 60 in the range of 5 to 25 mg and soy lecithin in the range of 150 to 250 mg based on literature survey. The proniosomes were not formed below 10 mg of soy lecithin. Similarly, for Span 60 proniosomes were not generated below 160 mg and above 200 mg. Hence, Span 60 and soy lecithin was selected in the range of 160 to 200 mg and 10 to 20 mg, respectively. Span 60 is a sorbitan sterate belonging to the category of non-ionic surfactants. It is a key structural component of proniosomes. The enhancement of the entrapment efficiency of the drug is mainly attributed to the presence of Span 60 (Barani *et al.*, 2018). Cholesterol is included in proniosomes at an optimal level to enhance formulation stability through its interaction with non-ionic surfactants. Cholesterol alone is not capable of forming the bilayer structure; however, it can integrate with the bilayer membrane, serving as a crucial regulator of both the membrane's structure and its flexibility (Ge;Wei;He;Yuan, 2019). Soya lecithin has the ability to form micelles in aqueous solution, significantly improving the drug entrapment characteristics. It not only facilitates the permeation of drugs but also plays a crucial role as a stabilizing agent (Govindarajan *et al.*, 2022).

3.2. Effect of soya lecithin and Span 60 on entrapment efficiency

Entrapment efficiency of vesicles depends on their stability, which is largely determined by the bilayer-forming components. The study focused on maintaining consistent levels of drug and cholesterol while systematically altering concentrations of soya lecithin and Span 60 to investigate their impact on entrapment efficiency. The different formulations showed entrapment efficiency between 76 ± 0.81 and $92.69 \pm 0.55\%$ (Figure 1a). Such high efficiency not only enhances the potential therapeutic efficacy of the formulation but also suggests its robustness as a carrier for targeted and sustained drug delivery applications. The results revealed that the higher entrapment was associated with a higher amount of Span 60. The increased entrapment can be attributed to the hydrophobic properties, solid characteristics, and higher phase transition temperature of Span 60. The results were found in agreement with Wen *et al.* (2014), where researchers reported that Span 60 and 80 showed better entrapment than Brij 52 (Wen *et al.*, 2014). The effect of soya lecithin on entrapment efficiency was found to vary,

but overall it contributed to an enhancement in entrapment efficiency. Soya lecithin, along with cholesterol, provides rigidity to the vesicle and prevents the leakage of the drug (Mehta *et al.*, 2016). These results were supported by the polynomial equation (4), contour (Figure 1b), and 3D graphs (Figure 1c).

$$\text{Entrapment efficiency (Y1)} = 91.24 + 4.95A + 2.14B - 3.14AB - 3.43A^2 - 0.9894B^2 \quad (4)$$

The positive sign for Span 60 and soya lecithin denotes the enhanced entrapment efficiency within the vesicles. The ANOVA test was used to analyse the effect of Span 60 and soy lecithin on entrapment efficiency. The test was found significant and confirmed by p-value of 0.0001 and F-value was 175.10. Adjusted and predicted R² values were 0.9831 and 0.9580, respectively. The difference between adjusted and predicted values was less than 0.2, indicating precise data. The lack of fit value is 4.99.b

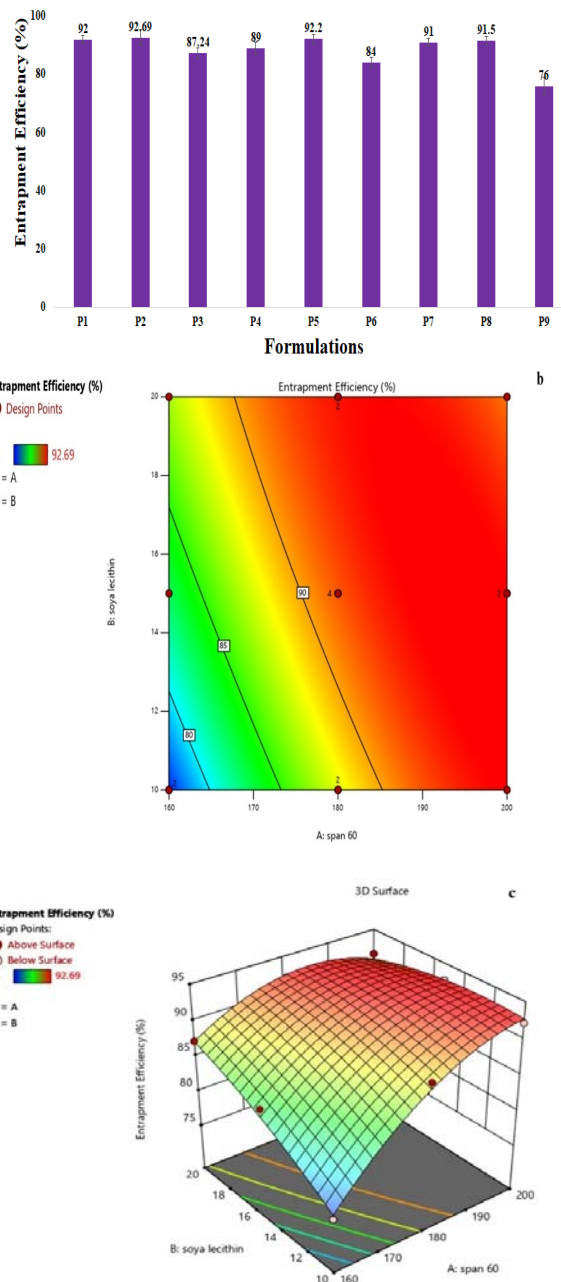


Figure 1. a) Entrapment efficiency, b) Contour plot, c) 3D graph

As evident in Figures 1b and c, the entrapment efficiency of proniosomes was directly proportional to the amounts of soya lecithin and Span 60. The change in colour from blue to red was indicative of enhanced entrapment.

3.3. Effect of soya lecithin and Span 60 on drug release

In-vitro release experiments revealed that Span 60 and soya lecithin had a notable impact on the drug release behaviour. The formulations exhibited drug release percentages ranging from $44.06 \pm 0.53\%$ to $88.34 \pm 0.41\%$. A higher concentration of soya lecithin was associated with enhanced drug release. Within vesicular delivery systems, phosphatidylcholine lecithin's primary constituent serves as an effective permeation enhancer, attributed to its elevated phase transition temperature.

Additionally, it acts to inhibit the leakage of the encapsulated drug (Wen;Farid;Kassem, 2014). The polynomial equation (5) generated during the ANOVA analysis is also supportive of the positive impact of Span 60 and the adverse effect of soya lecithin on drug release.

$$\text{Drug release (\%)} = 66.71 - 16.01A + 4.43B \quad (5)$$

The effect of Span 60 and soy lecithin was also analysed by ANOVA, where a p-value of 0.0001 indicates the test significance. F-value, adjusted and predicted R^2 values were 199.36, 0.9636 and 0.9448, respectively. The difference between the adjusted R^2 and the model predicted R^2 values was below 0.2, which further confirmed the adequacy of model. The lack of fit value is 7.22.

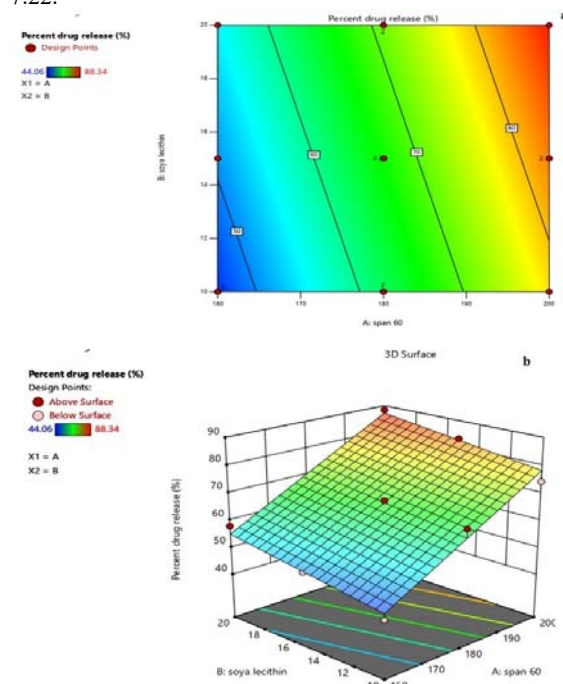


Figure 2. a) Contour plot, b) 3D graph of drug release from different formulations

Figures 2a and b were suggestive of drug release retardation with enhanced amounts of soya lecithin as indicated by blue colour. The opposite effect was observed

for Span 60, where the colour transitioned from blue to red.

3.4. Optimized formulation selection

Based on the results of drug release and entrapment efficiency, the optimized formulation was selected. According to the overlay plot (Figure 3), the predicted optimized batch demonstrates the entrapment efficiency and drug release of 91.82% and 79.79%, respectively. Practically, the value of entrapment efficiency and drug release was $92.51 \pm 0.62\%$ and $79.72 \pm 0.54\%$, respectively.

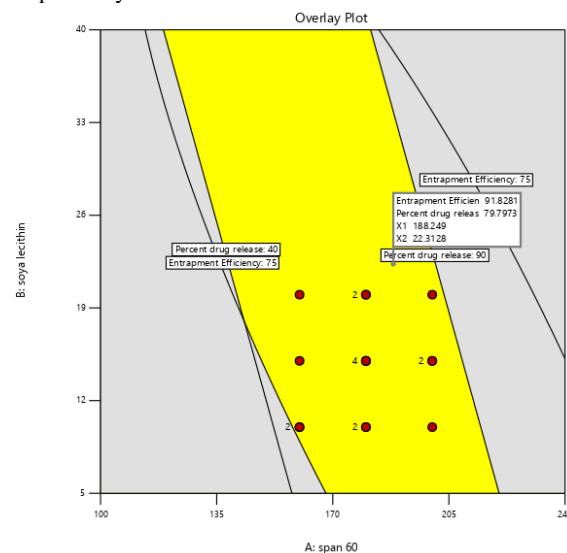


Figure 3 Overlay plot of entrapment efficiency and drug release

3.5. Characterization of optimized formulation

3.5.1. Vesicle size, PDI, and zeta potential

The vesicle size (Figure 2a) of proniosomes was 299.7 nm. This characteristic is especially beneficial for the transdermal administration of drugs. The polydispersity index was found to be 0.232. A poly-dispersity index (Figure 4a) of less than 0.3 is generally considered acceptable for drug delivery formulations. This index indicates the uniformity or homogeneity of particle sizes within the formulation. A lower polydispersity index suggests minimal variation in particle sizes, which is crucial for consistent drug delivery performance. The optimized formulation exhibited a zeta potential (Figure 4b) of -49.3 mV. This high negative zeta potential suggests strong repulsive forces between particles, which are beneficial for maintaining stability by preventing aggregation and flocculation. The presence of a high negative charge is often due to negatively charged excipients like soya lecithin, contributing to the formulation's stability (Danaei *et al.*, 2018).

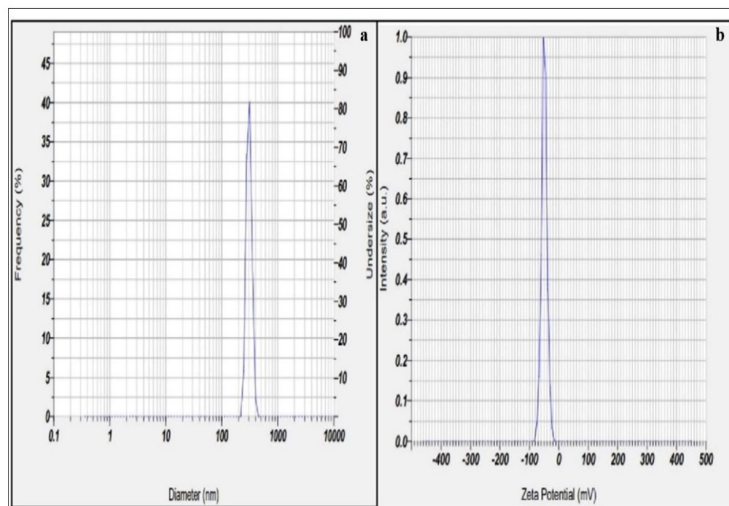


Figure 4. a) Particle size of optimized formulation, b) Zeta potential of optimized formulation

3.6. Scanning electron microscopy

Figure 4c presents the scanning electron microscopy (SEM) photomicrograph of the proniosomal formulation. According to the findings, the vesicles displayed a spherical shape, smooth texture, and were free from aggregation (Pinzaru *et al.*, 2021).

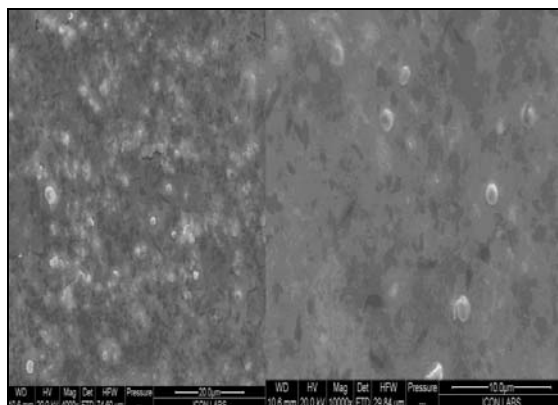


Figure 4c .SEM image of Proniosomes

3.7. Differential scanning calorimetry (DSC)

Thermal analysis (Figure 4d) studies were conducted for the drug, Span 60, soy lecithin, and proniosomes. The thermogram of gallic acid, Span 60, and soy lecithin exhibited an endothermic peak at 264.64°C, 57.42°C, and 262. 60°C, respectively. In contrast, the broad endothermic peak of the formulation was displayed at 107.72°C. This broad peak suggests potential interactions between the drug, Span 60, and soya lecithin within the proniosomal structure. The disappearance of the endothermic peak of gallic acid in the formulation indicates that the drug has been encapsulated successfully within vesicles formed by Span 60 and soya lecithin (Shah;Nair;Shah;Jacob;Bharadia;Haroun, 2021).

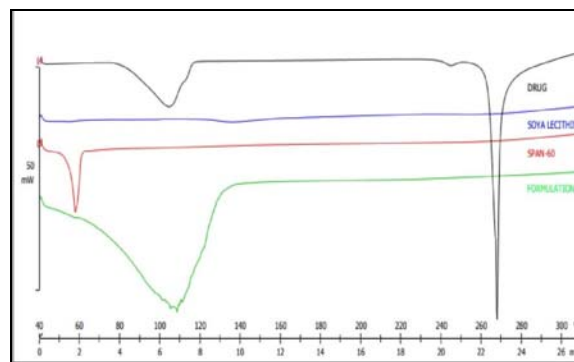


Figure 4d. DSC thermogram of gallic acid, soya lecithin, Span60 and proniosomal gel

The Composition and summary of various characteristics of the optimized formulation are listed in Table 3

Table 3 Composition and summary of key characteristics of optimized formulation

Soya lecithin (mg)	Span 60 (mg)	Vesicle size (nm)	Entrapment efficiency (%)	PDI	Zeta potential (mV)
22.31	188.24	299.7	92.51 ± 0.62	0.232	-49.3

3.8. Preparation of proniosomal gel

Carbopol 940 was employed as the gelling agent to convert the optimized proniosomal formulation into a gel-based system. Carbopol® 940 is a white, crosslinked polyacrylic acid polymer that acts as an efficient rheology modifier, providing high viscosity and forming clear gels.

3.9. Evaluation of proniosomal gel

3.9.1. Determination of pH, viscosity, and drug content

The gallic acid-loaded proniosomal gel had a pH of 6.2 ± 0.23 , which is matching to the pH of the skin. This

result was indicative of the appropriateness of the gel for topical application. The viscosity of proniosomal gel at speeds of 30 rpm, 50 rpm, and 100 rpm was 7900 ± 155 , 6389 ± 204 , and 3900 ± 139 cP. The viscosity of the marketed gel at speeds of 30 rpm, 50 rpm, and 100 rpm was 6440 ± 162 , 4820 ± 224 , and 2825 ± 192 cP, respectively. The formulation gel is more viscous than the marketed gel. As viscosity is directly proportional to adhesiveness, the results were suggestive of a prolonged contact time of the proniosomal gel with skin. The drug content of the optimized formulation was $99.51 \pm 0.65\%$, indicative of good entrapment of gallic acid in proniosomes.

The firmness of the proniosomal gel and the marketed gel (Figure 5a and 5b) was 750 g and 675 g, respectively. The tackiness of the proniosomal gel and the marketed gel was 200 g and 380 g, respectively (Figure 5a and 5b). The Work of adhesion, which is the ratio of area under the curve of tackiness and area under the curve of firmness of the proniosomal formulation and marketed gel, was 0.266 g/sec and 0.56 g/sec, respectively (Dhakane; Pandit, 2023). These findings may be due to the higher viscosity of the proniosomal gel compared to the marketed formulation.

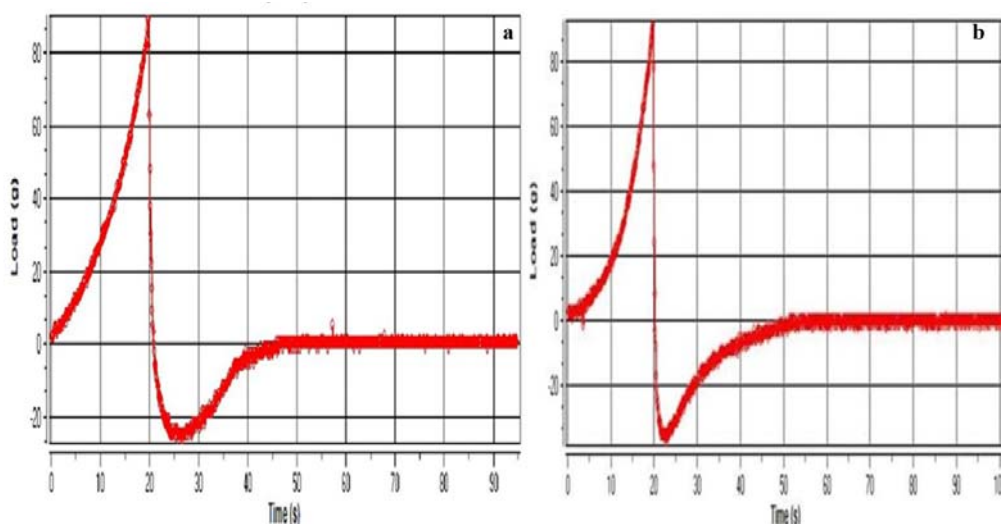


Figure 5. Texture analysis of a) Proniosomal gel, b) Marketed gel

3.9.2. In-vitro drug diffusion study

The diffusion of gallic acid from proniosomes and proniosomal gel was 86.55 ± 2.54 and $83.18 \pm 2.23\%$, respectively, whereas gallic acid without entrapment in vesicles exhibited $58.42 \pm 1.87\%$ drug diffusion within 8 h. The drug diffusion from gallic acid-loaded gel was 50.07 ± 2.87 (Figure 6a). A biphasic drug release pattern helps in the avoidance of saturation of the drug while diffusing through the skin. Initially, fast release leads to achieving transdermal delivery with a high concentration of the drug. Results are as per the given literature (Lather *et al.*, 2016). The release of proniosomes and proniosomal gel follows first-order diffusion kinetics; gallic acid and plain gel follow Korsmeyer-Peppas diffusion kinetics.

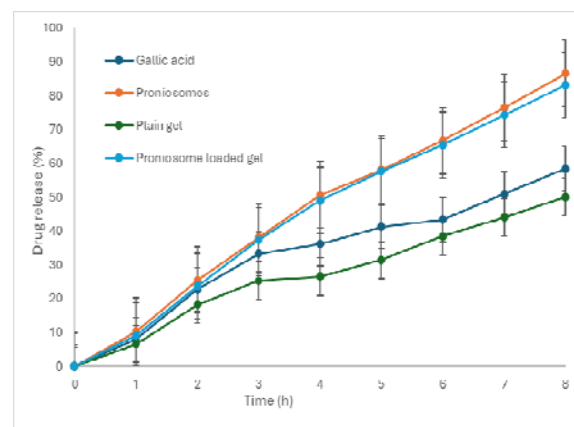


Figure 6a. In vitro drug diffusion study

3.9.3. Ex-vivo drug diffusion study

The ex-vivo drug diffusion (Figure 6b) for gallic acid-loaded proniosomes, proniosomal gel, marketed gel, and gallic acid-loaded gel were 82.5 ± 2.46 , 79.72 ± 2.89 , 75.99 ± 3.12 , and $47.47 \pm 2.43\%$, respectively. These results showed an increase in the drug diffusion of gallic acid by using the proniosomal vesicular system (Aldawsari *et al.*, 2023). The release of proniosomes and proniosomal gel follows first-order diffusion kinetics; gallic acid and plain gel follow Korsmeyer-Peppas diffusion kinetics.

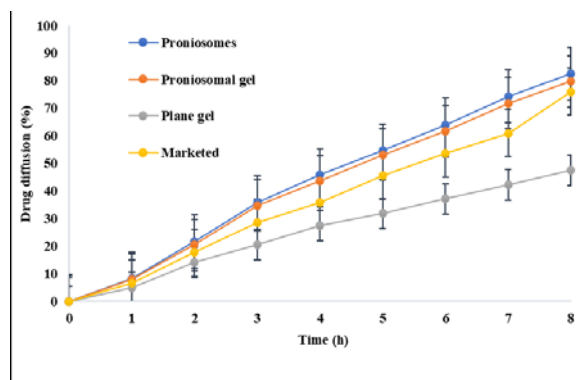


Figure 6b. Ex vivo drug diffusion study

3.9.4. Skin permeation study

The steady-state flux equation was used to determine the drug flux of the plain gel, marketed gel, proniosomes, and proniosomal gel. As per the results, the order of transdermal flux was proniosomes ($0.08610 \mu\text{g}/\text{cm}^2/\text{min}$) > proniosomal gel ($0.08237 \mu\text{g}/\text{cm}^2/\text{min}$) > marketed gel ($0.06032 \mu\text{g}/\text{cm}^2/\text{min}$) > plain gel ($0.03792 \mu\text{g}/\text{cm}^2/\text{min}$). Flux refers to the amount of drug permeated per unit time. In the case of the proniosome formulation, the drug was solubilized in the matrix of soya lecithin, and hence the amount of drug permeated was found more. In the gel formulation, the release of proniosomes was hindered due to entrapment in the gel matrix; hence, the flux was slightly low. In the marketed formulation, attempts were not made to enhance the solubility, and therefore the flux is lower. As gallic acid is insoluble in nature and entrapped in the gel base for plain gel, the flux is further lowered. These parameters illustrated that proniosomes containing surfactant Span 60 and soya lecithin improved the diffusion of gallic acid across the membrane (Dhakane; Pandit, 2023).

3.9.5. Antimicrobial activity against *Staphylococcus aureus*

The zone of inhibition (Figure 7) of proniosomal gel, marketed gel, and gallic acid was 33 ± 3.87 , 30 ± 2.89 , and 27 ± 3.19 mm. The value of the antibacterial study of gallic acid was consistent with the reported literature (Yu *et al.*, 2022). This study showed (Figure 9) that the proniosomal gel has a larger zone of inhibition than the pure drug and the marketed gel incorporated into the well.

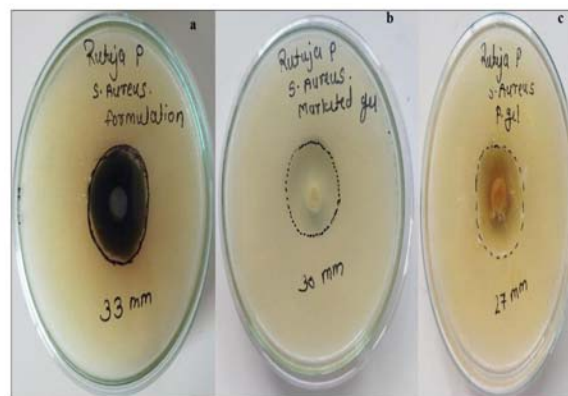


Figure 7 Zone of inhibition of a) Proniosomal gel, b) Marketed gel, c) Plain gel

The comparative summary of proniosomes, proniosomal gel, plain gel, and marketed gel is elaborated in Table 4.

Table 4: Comparative summary of proniosomes, proniosomal gel, plain gel, and marketed gel

Formulation	In-vitro diffusion (%)	Ex-vivo drug diffusion (%)	Flux ($\mu\text{g}/\text{cm}^2/\text{min}$)	Zone of inhibition (mm)
Proniosomes	86.55 ± 2.54	82.5 ± 2.46	0.08610	-
Proniosomal gel	83.18 ± 2.23	79.72 ± 2.89	0.08237	33 ± 3.87
Marketed gel	58.42 ± 1.87	75.99 ± 3.12	0.06032	30 ± 2.89
Plain gel	50.07 ± 2.87	47.47 ± 2.43	0.03792	27 ± 3.19

4. Conclusion

The findings of this study confirm the successful development of a gallic acid-loaded proniosomal gel. The optimized formulation exhibited high entrapment efficiency. SEM analysis revealed well-defined vesicles without signs of aggregation. The release of the drug in the formulation was extended. Antimicrobial activity test suggests that proniosomes hold strong potential as transdermal delivery systems for gallic acid to treat *S. aureus* infection. Further, preclinical studies must be carried out to evaluate the effectiveness of the formulation *in-vivo*.

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Purification and Characterization of α -methylene- γ -butyrolactone (Tulipalin A) from *Tulipa gesneriana* Callus Extracts

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Received: October 1, 2025; Revised: November 28, 2025; Accepted: January 15, 2026

Abstract

In this study, α -methylene- γ -butyrolactone (Tulipalin A) from *Tulipa gesneriana* L. callus extracts was purified and characterized via high-performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR spectroscopy). Red callus generated in tissue culture was extracted with methanol, providing a crude extract with numerous components. HPLC analysis of the crude sample revealed three different peaks at 3.8, 4.4, and 6.0 minutes, indicating the presence of several metabolites. Following purification, a single strong peak was observed at 3.8 minutes, which was identical to that of the authentic Tulipalin A standard. The isolated compound's identification and purity were further validated by the co-injection test, which revealed only one co-eluted peak. FTIR examination of the purified fraction revealed γ -lactone absorption bands, such as a strong carbonyl band at approximately 1760 cm^{-1} , a conjugated C=C bond near 1660 cm^{-1} , and terminal vinyl group signals at $910\text{--}990\text{ cm}^{-1}$. These findings provide quantitative and qualitative evidence of the compound's identity and purity. Overall, this study shows that methanolic extraction and chromatographic purification are effective methods for isolating Tulipalin A from *Tulipa gesneriana* callus, and it emphasizes the importance of combining HPLC and FTIR for the structural characterization of bioactive metabolites.

Keywords: *Tulipa gesneriana*, red callus, Tulipalin A, HPLC, FTIR, purification, tissue culture.

1. Introduction

Tulip (*Tulipa gesneriana* L.), often known as the garden tulip, serves as a perennial ornamental plant in the family *Liliaceae* that is extensively cultivated for its beautiful spring blossoms (Kavaiya *et al.*, 2025). Tulip tissue, in addition to its aesthetic appeal, has several phytochemicals with traditional therapeutic effects such as antipyretic, anticancer, and laxative (Krzyńska, Gąsecka and Magdziak, 2020). Tulipalin A (α -methylene- γ -butyrolactone) represents a biosynthetic product formed from the precursor 6-Tuliposide A. Tulipalin A is kept as an inactive glucoside in the vacuoles of healthy tulip plant cells, away from the enzyme accountable for its conversion (Nomura, 2017).

Tuliposide A-converting enzyme (TCEA) was described as the first known lactone-forming carboxylesterase, which converts Tuliposide A to the active compound Tulipalin A by enzymatic transesterification without hydrolysis, and thus the conversion is enzyme-mediated and not spontaneous (Nomura *et al.*, 2015). When tissues are injured or exposed to a pathogenic attack, this enzyme is released from the plastids and reaches the substrate (Nomura *et al.*, 2015; Nomura *et al.*, 2017). Spatial separation of substrates and enzymes represents a subtle defense mechanism; Tulipalin A is produced in high quantities only upon cell rupture, protecting the plant from autotoxicity under normal conditions (Nomura, Ogita and Kato, 2012).

Tulipalin A is the main component responsible for the allergenic and bioactive effects of tulips (Nomura *et al.*, 2023; Phan *et al.*, 2025). It is the main cause of a phenomenon known as “tulip fingers,” a type of occupational contact dermatitis that affects tulip growers and florists (Hassan *et al.*, 2018). These compounds have a protective role within the plant, acting as natural antibiotics with fungicidal effects that protect the bulbs from pathogenic infections (McCluskey, Bourgeois and Harbison, 2014). A proteomic study demonstrated that Tulipalin A exerts immunostimulatory effects, activating stress response proteins and altering purine metabolism in human immune cell models (Zwicker *et al.*, 2016).

Plant tissue culture is a technique that involves growing plant tissue segments (explants) in the laboratory on artificial media under aseptic conditions (Hasnain *et al.*, 2022). It provides a sustainable source for active chemicals. Callus cultures and cell suspensions are commonly utilized to create secondary metabolites, ensuring consistent synthesis regardless of environmental conditions (Chandran *et al.*, 2020; Jdayea & Neamah, 2024; Abdelazeez *et al.*, 2025).

Tulipalin A and its precursors are isolated and identified from *T. gesneriana* using modern chromatographic and spectroscopic techniques. Tuliposides and Tulipalins are mostly separated from complicated plant extracts using high-performance liquid chromatography (HPLC) for analytical purposes (Nomura, Ogita and Kato, 2020). Fourier transform infrared (FTIR) spectroscopy is a crucial tool for identifying plant

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compounds. It quickly confirms the unique functional groups of Tulipalin A, including the α -methylene- γ -butyrolactone ring, which makes it an effective technique for confirming the metabolite's identity (Lee *et al.*, 2021). Although the biochemical pathway of Tulipalin A has been thoroughly described, the controlled production of Tulipalin A through *in-vitro* callus culture in *Tulipa gesneriana* has not been sufficiently investigated. This is because most studies have concentrated on extracting the compound from natural plant organs, which are subject to a wide variation depending on the environmental and seasonal conditions (Nomura, Ogita and Kato, 2012). Van Rossum, Alberda and van der Plas (1998) reported that tissue-cultured tulip explants can accumulate Tuliposide A and Tulipalin A up to ~1300% above their initial levels, supporting the superior capacity of *in-vitro* cultured tissues such as callus for enhanced Tulipalin production. The purpose of this study was to use tissue culture techniques in tulip (*T. gesneriana*) to yield homogeneous and sustainable plant material, then to extract and characterize some important secondary metabolites, especially Tulipalin A and its precursors Tuliposides, utilizing advanced analytical techniques such as high-performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR).

2. Materials and Methods

This work was conducted in the tissue culture facilities of the University of Baghdad's College of Science for Women.

2.1. Laboratory Sterilization

Forceps, scalpels, and Petri dishes were sterilized in an autoclave at 121°C with 1.04 kg/cm² for 30 minutes. The instruments were then soaked in 96% ethanol to quickly sterilize and remove any impurities. They were then allowed to dry in a laminar airflow cabinet. To guarantee sterility, the cabinet was UV-sterilized for 30 minutes and then sprayed with 70% ethanol (Kareem, 2021).

2.2. Media Preparation

Standard Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was prepared by dissolving 4.9 g/L MS powder (Himedia, India), 30 g/L sucrose, and 8 g/L agar (Biolife, India) in sterile distilled water. The pH was adjusted to 5.8 using HCl or NaOH. After sterilization, the medium was permitted to cool before being used.

2.3. Callus induction

T. gesneriana leaves were cut into 0.2 cm pieces under sterile conditions and cultivated on MS medium supplemented with plant growth regulators as follows: 2,4-Dichlorophenoxyacetic acid (2,4-D; Himedia, India) (1 mg/L) + Thidiazuron (TDZ; Himedia, India) (1 mg/L). The media were stored in a growth chamber at 25 ± 2°C, with a 16-hour light/8-hour dark cycle (Tahtamouni *et al.*, 2020).

2.4. 2.4. Extraction of the Active Compounds

Five grams of the collected callus (wet weight) were taken and mixed with 50 mL of 70% methanol (Alpha-Chemika, India). The samples were placed in a reflux apparatus under standard conditions at 60–80°C for 6 hours. After the extraction process was complete, the

samples were allowed to cool and then filtered using sterile filter paper. The crude extract was collected. The resulting extracts were stored at 4°C until chemical tests were performed (Alwasha, Shlahib and Hamad, 2018).

2.5. Chromatographic and Spectroscopic Methods

The analysis was carried out at the Scientific Research Authority, Environmental and Water Research Center, to detect Tulipalin A (α -methylene- γ -butyrolactone) using HPLC (SYKAM, Germany). A C18-ODS column (25 cm in length, 4.6 mm internal diameter) was used. The mobile phase consisted of distilled water and acetonitrile at a ratio of 20:80 (v/v), with a flow rate of 1.0 mL/min. Detection of the active compound was performed using a UV detector at 208 nm. Identification was confirmed by co-analysis with Tulipalin A standard (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-239165) (Harada *et al.*, 2002).

FTIR spectroscopy was also employed to confirm the functional groups of Tulipalin A. A known quantity of the sample intended for FTIR analysis was taken and mixed with potassium bromide (KBr) powder at a ratio of ten times the sample weight, to ensure proper homogenization and to minimize strong absorption effects. The mixture was extensively crushed in a porcelain mortar to produce a homogenous blend, which was then pressed into a specific die to generate a transparent pellet appropriate for spectroscopy (Das *et al.*, 2024). Prior to this operation, the sample was dried at room temperature to remove residual moisture, which could cause unwanted absorption peaks in the spectrum and interfere with the analysis of functional group bands. This procedure is commonly used to prepare KBr pellets for spectroscopic examination. Other studies have confirmed how absorbed moisture in KBr pellets can interfere with hydroxyl peaks, reducing the quality of the data (Dai *et al.*, 2023).

3. Results and Discussion

T. gesneriana leaf explants cultivated on MS medium supplemented with 2,4-D (1 mg/L) + TDZ (1 mg/L) demonstrated a unique reaction in callus formation, resulting in the production of a friable callus with a delicate texture. The callus formed as a result of this treatment has a distinct red coloration.

Active compounds were extracted utilizing methanol by using the conventional method. The extract obtained from the red-colored callus showed a red coloration, reflecting the chemical composition of the callus used.

To establish the identity and purity of the active ingredient recovered from the red callus, HPLC analysis was carried out as follows:

100 μ L of Tulipalin A standard (α -MGBL) was injected through the HPLC system to measure the retention time along with chromatographic properties. The resulting chromatogram showed a sharp and distinct peak at a retention time of 3.8 min, with a recorded peak area and height consistent with the standard specifications of the compound (Figure 1).

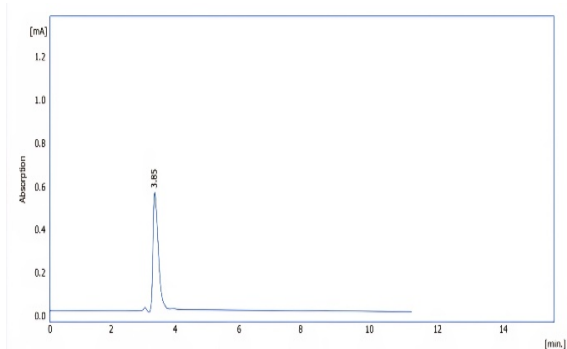


Figure 1: HPLC chromatogram of the Tulipalin A standard (α -MGBL) showing a single pure peak at a retention time of 3.8 min.

Additionally, 100 μ L of the crude sample was injected under the same chromatographic conditions. The chromatogram displayed several major peaks at different retention times, including a prominent peak at 3.8 min, in addition to other peaks that appeared at retention times beyond 4 minutes (Figure 2).

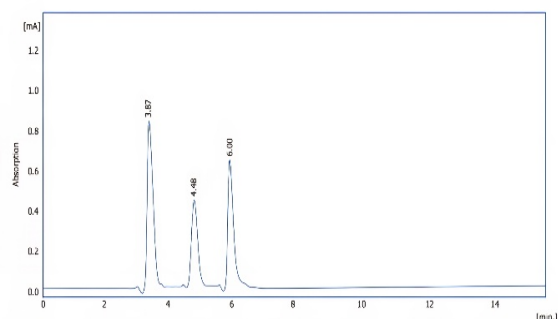


Figure 2: HPLC chromatogram of the crude extract showing three major peaks, one of which at 3.8 min.

To obtain the active compound in a purified form, multiple injections were performed and fractions were collected using a fraction collector. When the collected fraction was reinjected into the HPLC system, the resulting chromatogram showed a single peak at 3.8 min, with no additional peaks detected in the profile (Figure 3).

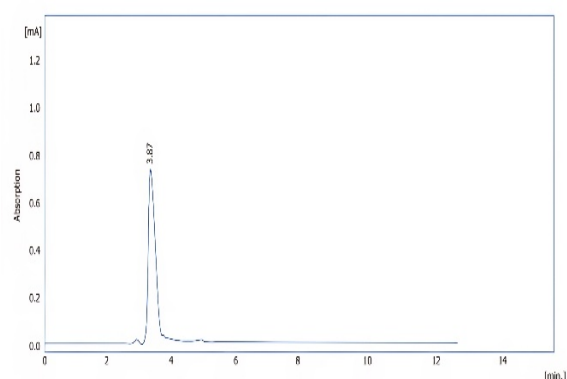


Figure 3: HPLC chromatogram of the isolated compound showing a single peak at 3.8 min.

An additional test was conducted by mixing 50 μ L of the isolated compound with 50 μ L of the Tulipalin A standard (α -MGBL) and injecting the mixture into the system. The resulting chromatogram revealed a single peak at 3.8 min, with nearly double the peak area compared with the individual injections of either the standard or the isolated compound (Figure 4).

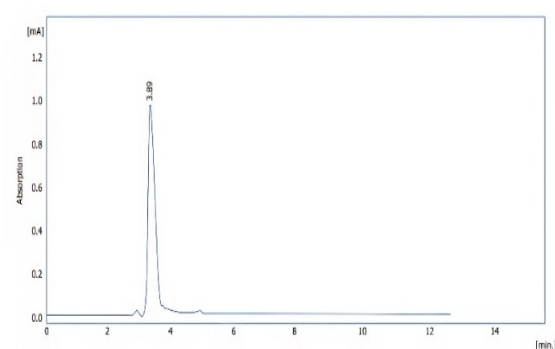


Figure 4: HPLC chromatogram of co-injection (isolated compound + standard) showing a single peak at 3.8 min with doubled area.

The findings obtained from HPLC were reinforced by FTIR analysis to verify the functional groups and structural identity of the compound (Figure 5), which illustrates a comparative representation of the FTIR spectra of the Tulipalin A standard (a) and the isolated compound (b), showing the distribution of characteristic absorption bands across the spectral range (4000–500 cm^{-1}). The FTIR spectrum of the Tulipalin A standard (α -MGBL), shown in Figure (5a), revealed several characteristic absorption bands distributed across the range of 4000–500 cm^{-1} . The major peaks were observed at 3506, 2938, 1880, 1632, 1439, 1270, 1116, 1024, and 767 cm^{-1} . The FTIR spectrum of the isolated compound Figure (5b) demonstrated a pattern of absorption bands within the same spectral range (4000–500 cm^{-1}). The main absorption bands appeared at 3512, 2936, 1883, 1630, 1432, 1272, 1118, 1022, 823, and 764 cm^{-1} .

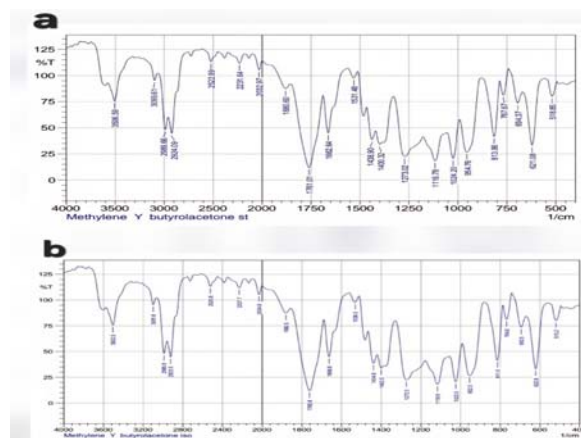


Figure 5: FTIR spectra of Tulipalin (α -methylene- γ -butyrolactone) standard and the isolated compound, showing the major absorption bands in the range of 4000–500 cm^{-1} . (a) FTIR spectrum of Tulipalin standard. (b) FTIR spectrum of the isolated compound.

The results of this study indicated that the formation of red callus in tulip tissue culture was associated with an increased accumulation of bioactive secondary metabolites. This interpretation agrees with the findings of (Lai *et al.*, 2022), who discovered that red callus produced by *Vitis davidii* contained excessive levels of anthocyanins and that the amount of red pigmentation raised significantly under blue and violet light, with important pigments such as cyanidin-3-glucoside as well as peonidin-3-glucoside accumulating beneath these conditions. Similarly, Yao *et al.* (2023) reported that

culture factors-including sugar source, light quality, and temperature-significantly enhanced anthocyanin and phenolic accumulation in red pear callus. Sucrose supplementation, paired with red-blue light and relatively low temperature ($\sim 15^{\circ}\text{C}$), resulted in increased production of anthocyanins and secondary metabolites, as well as activation of biosynthetic genes. Compared with our findings, the red coloration of tulip callus serves as a clear indicator of secondary metabolic activation, supporting the concept that pigmentation is linked to the accumulation of other defense-related metabolites such as Tulipalin.

In terms of extracting and purifying the active ingredient, methanolic extraction from the red callus allowed for the isolation of Tulipalin A, which was compatible with the findings of (Christensen and Kristiansen, 1999). They observed that Tuliposide A is the major precursor to Tulipalin and that both chemicals are plentiful in tulip tissues, accounting for more than 1% of the fresh weight. Likewise, Nomura and colleagues (Nomura, Ogita and Kato, 2020) demonstrated that Tuliposide A quickly transforms into Tulipalin after cell injury or during extraction, and chromatographic examination of tulip extracts revealed the existence of several Tuliposides (A, B, D, and F). In a more recent study (Lee *et al.*, 2021) isolated three new Tuliposides (H–J) alongside known compounds from a methanolic extract of *Amana edulis* bulbs (a close relative of tulip in the Liliaceae family), using spectroscopic analyses to confirm their identities. Therefore, the findings on the isolation of pure Tulipalin from the red callus agree with these studies and further highlight the efficiency of methanolic extraction in releasing the active compound from tulip tissues.

3.1. Retention Time Matching with the Standard

The chromatogram of the crude tulip extract displayed three distinct peaks at retention times of 3.8, 4.4, and 6.0 minutes. The presence of multiple peaks in the crude extract indicates the coexistence of several compounds. Indeed, previous studies have reported that α -methylene- γ -butyrolactone (commonly known as Tulipalin A) typically appears as one of the major peaks within complex plant extracts. Following the isolation and purification steps, a single sharp peak was obtained at a retention time of 3.8 minutes, which matched exactly with that of the authentic standard (reference compound). Such correspondence of retention time between the isolated compound and the standard, under identical HPLC conditions, provides strong evidence of the compound's identity. In chromatographic analysis, this retention-time matching is considered a decisive criterion for confirming that the unknown and the standard are the same compound. Accordingly, the agreement of the isolated sample's peak with that of the Tulipalin A standard strongly supports that the purified compound is indeed α -methylene- γ -butyrolactone.

3.2. Purity of the Isolated Compounds and Removal of Impurities

The appearance of a single chromatographic peak for the isolated compound directly indicates its high purity. In HPLC analysis, a pure sample yields a single sharp peak, whereas the presence of impurities is manifested by additional peaks, shoulders, or peak tailing adjacent to the main peak. In this case, the two peaks initially observed at

4.4 and 6.0 minutes in the crude extract completely disappeared after purification, confirming the removal of these unwanted components. The HPLC profile of the purified sample showed one dominant peak occupying nearly the entire signal area, suggesting that the target compound now represents the overwhelming majority of the sample. Quantitatively, the peak area of the target compound (at 3.8 minutes) increased from being only a fraction of the total chromatographic area in the crude extract to accounting for nearly 100% of the total area in the purified sample. This clearly demonstrated that the isolation procedure concentrated the desired compound while eliminating most other constituents. Similarly, in a comparable study on the purification of a natural metabolite, the chromatographic analysis of the purified fraction displayed a single retention-time peak identical to that of the standard, with a purity approaching 99%, thereby confirming the efficiency of purification in eliminating impurities and achieving a high degree of compound purity.

To provide additional confirmation of the compound's identity, a co-injection test was performed by introducing a mixture of the isolated compound and the standard into the HPLC system. If the isolated compound had been different from the standard, two distinct peaks or a change in peak shape would have been expected. Instead, the analysis yielded a single, sharp peak at 3.8 minutes with no overlap or splitting. This behavior demonstrates that the isolated compound and the reference standard co-elute under identical chromatographic conditions, indicating a perfect match of identity. Therefore, this test provided unequivocal evidence that the compound isolated from the tulip extract was pure α -methylene- γ -butyrolactone, as its retention peak was identical to that of the standard with no detectable trace of other components.

FTIR analysis of the standard and the isolated sample from tulip extract showed a clear match in the positions of the absorption bands, confirming that the compound isolated after purification is α -methylene- γ -butyrolactone (Tulipalin A) with high purity, as no additional peaks indicative of impurities were observed. The main bands detected included a strong absorption band at $\sim 1760\text{ cm}^{-1}$ corresponding to the carbonyl group of the γ -lactone ring, and a band at $\sim 1660\text{ cm}^{-1}$ indicative of an α , β -unsaturated double bond conjugated with the carbonyl. Additional features comprised the region at $2850\text{--}3000\text{ cm}^{-1}$ due to stretching vibrations of both saturated and unsaturated C–H bonds, a band at $\sim 1450\text{ cm}^{-1}$ associated with methylene scissoring vibrations, characteristic absorptions between $1100\text{--}1200\text{ cm}^{-1}$ assigned to C–O stretching in the lactone ester, and distinct bands at 910 and 990 cm^{-1} corresponding to out-of-plane vibrations of the terminal vinyl group ($=\text{CH}_2$), which serve as a fingerprint for the compound. These values are fully consistent with previous reports describing α -methylene- γ -lactones, where recent studies (Smith, 2016; Graur *et al.*, 2022) highlighted that the combination of a strong carbonyl band near 1760 cm^{-1} , a conjugated double bond around 1660 cm^{-1} , and vinyl group signals at $910\text{--}990\text{ cm}^{-1}$ provides conclusive evidence of this structural unit. Such spectral concordance strongly supports the earlier HPLC findings, which revealed a single peak at a retention time identical to the standard, thereby providing combined quantitative and qualitative evidence that the isolated compound is pure

Tulipalin A. This confirms the success of the purification process and validates the reliability of the sample for subsequent biological and analytical applications.

4. Conclusion

This study demonstrated that red callus of *Tulipa gesneriana* is an in vitro source of secondary metabolites. Methanolic extraction enabled the successful isolation of Tulipalin A (α -methylene- γ -butyrolactone). HPLC analysis confirmed the identity and purity of the compound, as the isolated fraction yielded a single peak at 3.8 minutes, identical to the standard, with co-injection further validating the match. FTIR spectra revealed characteristic absorption bands consistent with Tulipalin A, including the γ -lactone carbonyl and terminal vinyl group. Together, these findings provide strong quantitative and qualitative evidence of the compound's identity and purity, highlighting the efficiency of red callus culture for producing valuable bioactive metabolites.

Acknowledgments

The authors would like to thank the Department of Biology/ College of Sciences for Women/University of Baghdad for providing the scientific laboratories to complete this work.

Conflict of interest

There are no conflicts of interest in this paper.

Funding

Self-funding.

Author contribution

- Israa Adnan Salman: Conceptualization, study design, supervision, Data analysis, figure preparation, manuscript writing.
- Sumaya F. Hamad: Methodology, experiments, manuscript editing, sample collection.

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Development of an Image-Based Identification System for Freshwater Diatoms Using Computer Vision Techniques

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Received: October 17, 2025; Revised: December 21, 2025; Accepted: January 16, 2026

Abstract

Diatoms are widely used indicators of environmental condition because of their rapid and sensitive responses to changes in water quality. However, routine identification and enumeration using morpho-taxonomic and molecular approaches remain labor-intensive and time-consuming. This study evaluates the performance of a YOLO-based object detection framework for automated detection, localization, and genus-level classification of freshwater diatoms from microscopic images. Using annotated image datasets, model performance was assessed at the cell level, with detection and classification accuracy quantified using mean average precision (mAP). YOLOv4 achieved a mAP of 56.64% for diatom detection and localization. In multi-class classification experiments involving 16 genera, YOLOv4 outperformed YOLOv7, yielding a higher mAP of 19.21%. Performance analysis revealed that class imbalance and limited cell counts negatively affected detection accuracy in several genera. Restricting the dataset to eight genera with higher cell representation improved performance, with YOLOv4 trained on raw images reaching a mAP of 41.98%. In terms of efficiency, the automated system processed and quantified diatom cells from approximately 100 images within minutes, compared with several days required for manual analysis of a smaller image set. These results demonstrate that YOLO-based models can support rapid, cell-level diatom analysis, with performance strongly influenced by dataset composition, and highlight their potential as complementary tools for ecological monitoring workflows.

Keywords: Digital image processing, Freshwater diatoms, Philippines, YOLO (You Only Look Once)

1. Introduction

Diatoms, unicellular phototrophs with approximately 100,000 extant species worldwide, play an important role in biomass production and have various applications in industrial, cell biological, and molecular fields (Bozarth *et al.*, 2009; Wang and Seibert, 2017). With a reproduction cycle of around two weeks, diatoms have one of the shortest generation times among all species. Due to their rapid responsiveness and high sensitivity to various factors including changes in nutrient concentration, organic pollution, and aquatic productivity, diatoms help researchers monitor early warning signs of increasing pollution and track the success of habitat restoration efforts within ecosystems (Rimet, 2012; Masouras *et al.*, 2021; Arguelles, 2022). Their robustness and potential as water quality indicators have even prompted the European Water Framework Directive to advocate for and require the use of bioindicators, including benthic diatoms, for assessing stream ecosystems (Masouras *et al.*, 2021). The crucial role of diatoms as bioindicators underscores the need for effective methods to identify and categorize the diverse species present in aquatic environments.

In the Philippines, numerous studies have documented the diversity, ecological significance, and bioindicator value of diatoms across marine, freshwater, and extreme environments. Investigations have reported diverse assemblages from the West Philippine Basin (Shen *et al.*, 2017), toxin-producing *Pseudo-nitzschia* species in San Pedro Bay (Yap-Dejeto *et al.*, 2013), epiphytic communities associated with seagrasses (Yap-Dejeto and Baqueros, 2015), thermophilic taxa from hot springs (Martinez-Goss *et al.*, 2019), and pollution-tolerant species in urban river systems such as the Marikina River (Martinez-Goss *et al.*, 2023). Additional studies have highlighted diatom epiphytes in Laguna de Bay and other freshwater systems (Arguelles *et al.*, 2019). While these works underscore the ecological relevance of diatoms for environmental assessment, they also highlight the labor-intensive and time-consuming nature of traditional morpho-taxonomic identification, which requires extensive expertise and manual microscopy (Arguelles and Monsalud, 2017; Arguelles *et al.*, 2018; Arguelles, 2021; Arguelles, 2023).

To address these limitations, automated image-based approaches using digital image processing and machine learning have gained increasing attention. Convolutional neural networks (CNNs) and object detection frameworks,

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including YOLO-based architectures, have been successfully applied to diatom, phytoplankton, and microalgal detection and classification in international studies, demonstrating varying levels of accuracy depending on dataset size, taxonomic complexity, and image quality (Dimitrovski *et al.*, 2012; Wang *et al.*, 2021; Liu *et al.*, 2022; Syed *et al.*, 2024). These studies have established YOLO and related CNN-based methods as effective tools for microscopic organism recognition, particularly for high-throughput detection and counting tasks. However, reported performance metrics such as mean average precision, precision, and recall vary widely across studies, reflecting differences in training data composition, class balance, and annotation strategies (Kloster *et al.*, 2014; Pedraza *et al.*, 2017; Libreros *et al.*, 2019; Siddik and Bostan, 2019; Arguelles, 2020; Geronimo *et al.*, 2023).

In this context, the present study evaluates the applicability of YOLO-based object detection models (YOLOv4 and YOLOv7) for automated detection, counting, and genus-level classification of freshwater diatoms using locally generated microscopic image datasets. Rather than introducing YOLO as a novel methodology, this work focuses on assessing model performance under conditions relevant to Philippine laboratories, including dataset size, class imbalance, and taxonomic complexity. Specifically, the study examines detection and classification performance across 16 diatom genera and explores how reducing class complexity influences mean average precision at the cell level. By comparing model performance and processing efficiency with conventional manual approaches, this study aims to clarify the strengths and limitations of YOLO-based systems as complementary tools for routine diatom-based environmental monitoring.

2. Materials and Methods

2.1. Sample Collection, Preservation, and Slide Preparation

Benthic diatom samples (10–15 mL) were collected from freshwater sampling sites using sterile Tarson tubes (Arguelles, 2019). Samples were stored in insulated coolers during transport and processed within 24 h of collection. For microscopic examination, 3 mL subsamples were preserved in 4% formaldehyde to maintain cellular integrity. Preserved samples were gently homogenized prior to slide preparation to ensure even distribution of diatom cells. Temporary wet mounts were prepared for light microscopy, following standard diatom examination protocols, without chemical digestion to preserve natural assemblage structure.

2.2. Image acquisition

Microscopic images were captured using an Olympus CX31 binocular light microscope equipped with an Infinity X digital camera at 1000× magnification (oil immersion). Images contained heterogeneous diatom assemblages exhibiting variation in size, morphology, and abundance across taxa. A total of 237 images were collected across three independent sampling batches to reflect realistic variability in environmental samples.

The first batch (108 images) was used exclusively for model training. The second and third batches, comprising

84 and 45 images respectively (129 images total), were reserved for model testing. Genus-level representation varied across batches, with the first batch dominated by *Aulacoseira*, *Encyonema*, *Cymbella*, and *Fragilaria*, while *Achnantheidium* was more prevalent in the latter batches. Several genera (e.g., *Cocconeis*, *Cyclotella*, *Rhoicosphenia*, *Surirella*, *Achnanthes*, *Planothidium*) were sparsely represented in both training and testing datasets.

2.3. Dataset Annotation and Composition

All images were manually annotated using Labellmg software. Annotation was performed by one trained diatom taxonomist with extensive experience in freshwater diatom identification. Each visible diatom cell was enclosed in a bounding box and labeled at the genus level. Annotation was performed at the cell level, rather than per image, and exported in YOLO-compatible format.

Overlapping or clustered cells were annotated as separate objects where cell boundaries were visually distinguishable. Ambiguous cells (those with unclear morphological features due to orientation, overlap, or partial visibility) were excluded from annotation to minimize labeling uncertainty. Given that a single expert performed annotation, formal inter-rater validation was not conducted; however, annotations were reviewed iteratively to ensure internal consistency.

Table 1 summarizes the number and proportion of annotated diatom cells per genus in the training and testing datasets. Class imbalance was evident, with several genera represented by fewer than 10 annotated cells. This imbalance was retained to reflect real-world environmental sample composition rather than artificially balancing the dataset.

Table 1. Number of cells found per diatom genus in the training and testing datasets.

Class	Training (no. of cells)	Training (%)	Testing (no. of cells)	Testing (%)
<i>Aulacoseira</i>	198	20.62%	21	2.88%
<i>Fragilaria</i>	189	19.68%	35	4.80%
<i>Encyonema</i>	115	11.97%	149	20.46%
<i>Cymbella</i>	201	20.93%	82	11.26%
<i>Cocconeis</i>	5	0.52%	16	2.19%
<i>Synedra</i>	79	8.22%	43	5.90%
<i>Gomphonema</i>	13	1.35%	49	6.73%
<i>Achnanthes</i>	7	0.72%	0	0.00%
<i>Nitzschia</i>	45	4.68%	87	11.95%
<i>Planothidium</i>	1	0.10%	1	0.13%
<i>Sellaphora</i>	15	1.56%	0	0.00%
<i>Surirella</i>	6	0.62%	0	0.00%
<i>Navicula</i>	73	7.60%	33	4.53%
<i>Cyclotella</i>	10	1.04%	9	1.23%
<i>Rhoicosphenia</i>	1	0.10%	0	0.00%
<i>Achnantheidium</i>	2	0.20%	203	27.88%

2.4. Image Pre-processing

Image pre-processing was applied as a controlled experimental factor rather than a default step. Two pre-processing techniques were evaluated: bilateral filtering and contrast-limited adaptive histogram equalization (CLAHE). Bilateral filtering was used to reduce background noise and small debris while preserving cell

edges, which is particularly important for microscopy images derived from preserved environmental samples. CLAHE was applied to enhance local contrast and improve edge definition under variable illumination conditions.

Pre-processing was applied consistently to both training and testing datasets within the designated experimental setups. Raw (unprocessed) images were also retained to evaluate whether pre-processing significantly influenced detection and classification performance.

2.5. Experimental setup

The experimental workflow was structured in two sequential stages to systematically evaluate the feasibility and performance of YOLO-based object detection for freshwater diatom analysis (Table 2). In the first stage, YOLOv4 was applied exclusively to raw microscopic images to assess its capability to detect and localize individual diatom cells under minimally processed conditions. This preliminary stage served to confirm whether the YOLO object detection framework could reliably identify microscopic diatom structures within complex environmental images containing debris and heterogeneous cell assemblages.

In the second stage, classification performance was evaluated using four experimental setups that combined two YOLO architectures (YOLOv4 and YOLOv7) with two image conditions (raw and pre-processed). This design enabled direct comparison of (i) model architecture effects and (ii) the influence of image pre-processing on genus-level classification accuracy. All experimental setups used the same training and testing datasets to ensure comparability of results.

Table 2. List of experimental setups for the classification of 16 diatom genera.

Setup no.	Pre-processing	Classifier
1	Bilateral Filtering and Contrast-Limited Adaptive Histogram Equalization (CLAHE)	YOLOv4
2	None	YOLOv4
3	Bilateral Filtering and Contrast-Limited Adaptive Histogram Equalization (CLAHE)	YOLOv7
4	None	YOLOv7

For the initial detection experiment, a binary classification task was performed to distinguish diatom cells from non-diatom objects in microscopic images. A total of 237 expert-annotated images were used to train a YOLOv4 model using raw images without additional pre-processing. Model evaluation was conducted using an independent test set consisting of 27 images. Performance was assessed using an Intersection over Union (IoU) threshold of 0.50, following standard object detection evaluation protocols.

2.6. Model Training Configuration

A total of four object detection models were trained: YOLOv4 using raw images, YOLOv4 using pre-processed images, YOLOv7 using raw images, and YOLOv7 using pre-processed images. Model training was conducted on a workstation running Windows 11, equipped with an Intel

Core i7-8700 processor and an NVIDIA GeForce GTX 1070 graphics processing unit.

For all models, the learning rate was fixed at 0.001, and the maximum number of training iterations was set to 32,000 to ensure sufficient convergence. Input image dimensions and other hyperparameters followed the default settings recommended by the official YOLOv4 and YOLOv7 implementations. No additional data augmentation techniques, such as rotation or flipping, were applied beyond the defined pre-processing steps, in order to isolate the effects of image enhancement on model performance.

Training time differed markedly between architectures. YOLOv4 models required approximately 12 h to complete training, whereas YOLOv7 models completed training in approximately 30 min, reflecting architectural optimizations that reduce computational complexity.

2.7. Cell Detection, Classification, and Counting

Model inference was performed exclusively on the independent testing dataset. During inference, each trained model generated bounding boxes and corresponding genus-level predictions for individual diatom cells present in each image. Detection outputs were filtered based on confidence thresholds defined by the YOLO framework to reduce false-positive predictions.

Detected diatom cells were counted on a cell-by-cell basis, and the number of cells per genus was recorded for each image. These cell counts formed the basis for both quantitative comparison across experimental setups and subsequent performance evaluation. This approach allowed assessment of the models' ability to simultaneously detect, classify, and enumerate diatom cells in complex microscopic images.

2.8. Performance Evaluation

Model performance was evaluated at the cell level, rather than at the image level, to accurately reflect the object detection nature of the task. Mean average precision (mAP) was used as the primary performance metric and was computed by comparing predicted bounding boxes and genus labels against expert-annotated data. A prediction was considered correct when both the localization and classification criteria were satisfied.

Diatom curated annotations generated by the taxonomist served as the reference standard for evaluation. Performance metrics therefore reflect the system's ability to correctly detect, localize, and classify individual diatom cells across multiple genera, providing a robust measure of applicability for automated diatom-based environmental monitoring.

3. Results and Discussion

The study investigated the use of YOLO architecture for detecting, identifying, and counting local freshwater diatoms in the Philippines. The system successfully recognized and categorized 16 genera of freshwater diatoms exhibiting variations in color, size, and shape but with room for improvement. This technological advancement holds potential significance for diatom taxonomists, demonstrating the feasibility of a computational recognition approach for automated identification of selected freshwater diatoms.

The YOLOv4 model achieved a mean average precision (mAP) of 56.64% at an IoU threshold of 0.50 for the binary diatom detection task. The model obtained a precision of 71%, recall of 48%, and an F1-score of 57%. The relatively high precision indicates that the model was effective at correctly identifying diatom cells when detections were made. However, the lower recall suggests that a substantial proportion of expert-labeled diatom cells were not detected, which negatively influenced the overall mAP value. This result highlights the challenge of detecting small and morphologically diverse diatom cells in complex microscopic images using raw data alone.

The system automated the detection, classification, and counting of diatom cells. The second experimental setup specifically assessed its performance in classifying and enumerating cells from 16 freshwater diatom genera. Out of the 237 images prepared for the second setup, a total of 129 images were reserved for the testing phase and were used to evaluate the performance of the system. The remaining 108 images were already used to train both the YOLOv4 and the YOLOv7 models in both the raw and pre-processed datasets.

Table 3. Mean average precision (mAP), precision, and recall of each experimental setup.

Classifier	mAP	Precision	Recall
YOLOv4 on pre-processed dataset	18.32	40%	25%
YOLOv4 on raw dataset	19.21	41%	25%
YOLOv7 on pre-processed dataset	13.3%	54.2%	28.3%
YOLOv7 on raw dataset	2.88%	2.63%	3.31%

The diatom detection results were compared with the prepared labels of the test dataset to evaluate the accuracy of the system in detecting and classifying diatoms in an image. An IoU threshold of 0.50 was set for the confidence scores generated by the trained YOLO models. This means that the detections made by the models need to match at least 50% of the prepared labels in the test dataset. It was observed that YOLOv4, trained on a pre-processed image dataset, successfully detected diatoms with a mean average precision of 18.32%. Additionally, it demonstrated a precision of 40%, recall of 25%, and an F1-score of 31%. Conversely, YOLOv4, trained on a raw image dataset, achieved a mean average precision of 19.21%. Furthermore, it generated a precision of 41%, recall of 25%, and an F1-score of 31%. The YOLOv7 trained on a pre-processed dataset, exhibited a mean average precision of 13.3%, precision of 54.2%, and recall of 28.3%. Meanwhile, the YOLOv7 model trained on the raw dataset yielded a mean average precision of 2.88%, precision of 2.63%, and recall of 3.31%.

Interestingly, YOLOv7, despite being a more recent architecture, underperformed compared to YOLOv4 on this dataset. Several factors may have contributed to this outcome. First, YOLOv7 is sensitive to small datasets and class imbalance, and the present dataset contained several underrepresented genera (≤ 15 cells per genus), which likely hampered the model's ability to learn reliable features. Second, training instability due to the complexity of the YOLOv7 architecture may have further reduced performance, particularly for minority classes. Third, the morphological similarity among certain diatom genera (e.g., *Cymbella* vs. *Encyonema*) adds to the challenge of

generalizing from limited samples. These observations suggest that YOLOv7 may require larger and more balanced datasets to outperform YOLOv4 in diatom classification tasks.

Table 3 shows the mean average precision, precision, and recall scores for each model. The YOLOv4 model, without additional preprocessing, achieved the highest mean average precision score of 19.21% among all models. This indicates that the YOLOv4 model, trained on the raw dataset, can detect more cells and therefore count more cells compared to other setups. However, it still fails to detect some existing cells in the image, as indicated by its low recall score. On the other hand, the YOLOv7 model, trained on processed images, detects fewer cells, as shown by its 13.3% mAP, but excels in distinguishing among cells, as demonstrated by its 54.2% precision score.

To contextualize the performance of the proposed system, it is important to compare it with prior international work on diatom detection and CNN-based image analysis. Previous studies applying deep learning to diatom classification have reported mAP values ranging from 60% to 85% for well-curated datasets with larger sample sizes per class (Valova *et al.*, 2020). Segmentation-based approaches have similarly achieved high accuracy in detecting diatoms but often require extensive manual annotation and are sensitive to overlapping cells. Compared to these studies, the present system shows moderate performance (maximum mAP of 19.21% for YOLOv4 on raw images), which reflects the challenges of working with naturally distributed, morphologically diverse diatoms under limited and imbalanced dataset conditions. This comparison highlights the contribution of the current study in demonstrating the feasibility of automated recognition for local Philippine freshwater diatoms and underscores areas for improvement, particularly dataset balancing and increasing cell representation per genus.

Looking closer at the performance of the YOLOv7 model on the raw dataset through the confusion matrix, *Aulacoseira* produced the highest accuracy of 70%. This is followed by *Fragilaria*, *Encyonema*, and *Cymbella* at 5%, 1%, and 10%, respectively. This shows how the model can only detect and classify these particular genera while failing on others. On the other hand, the YOLOv7 model on the preprocessed dataset achieved better results than the raw dataset. *Aulacoseira* still boasts the highest accuracy of 82%, followed by *Fragilaria* and *Encyonema* at 7%, *Synedra* at 3%, and *Cymbella* at 28%. The PR-curve, F1-curve, P-curve, and R-curve from both YOLOv7 models also showed better scores for *Aulacoseira*, *Cymbella*, and *Encyonema*, both of which represented at least 20% of the total number of cells for training.

The YOLOv4 models were also able to get significantly higher accuracy precision scores for classes *Aulacoseira*, *Encyonema*, and *Cymbella*. In YOLOv4 trained on the raw image dataset, the model achieved 75.90% accuracy precision score for *Aulacoseira*, 46.40% accuracy precision score for *Encyonema*, and 40.18% accuracy precision score for *Cymbella*. Notably, *Cyclotella* also showed a high accuracy precision score of 40.74%. This can be attributed to the unique circular shape of this particular genus compared to the rest of the classes. In the YOLOv4 model trained on the preprocessed image dataset, the model achieved accuracy precision scores of 67.70%

for *Aulacoseira*, 52.06% for *Encyonema*, and 41.08% for *Cymbella*. Similarly, *Cyclotella* also exhibited a high accuracy precision score of 49.85%. The numbers also indicate a slight increase in the number of false positives in the YOLOv4 model for the preprocessed dataset, affecting its overall mean accuracy precision compared to the raw dataset. This demonstrates how the YOLOv4 model trained on the raw dataset outperforms the one trained on the preprocessed dataset.

Based on the listed results, diatoms under the genera *Aulacoseira*, *Encyonema*, *Cymbella*, *Synedra*, *Navicula*, and *Cyclotella* have generally higher average precision on all four models trained for this study. This is directly correlated with the number of labelled cells under these genera which were used to train the YOLO models. The remaining genera, especially the *Achnanthes*, *Planothidium*, *Sellaphora*, *Surirella*, and *Rhoicosphenia* demonstrate very low precision scores. This discrepancy may be attributed to the lower number of cells from these genera in the dataset, which is consistently less than or equal to 15. Given that CNNs (Convolutional Neural Network) rely on analyzing and learning patterns to make accurate predictions, the underrepresentation of certain classes significantly impacted the system's performance. Figure 1 illustrates the substantial discrepancy between the actual cell labels and the predicted labels by the YOLOv7 model for the raw dataset. It is noticeable that genera with larger samples in the training dataset, such as *Aulacoseira*, *Encyonema*, and *Cymbella*, are detected more frequently than genera with fewer samples, like *Achnanthidium*, even though the latter is more prevalent in the test dataset. *Achnanthidium*, for example, has only about two (2) cell samples in the training dataset. This pattern was consistently observed across the other YOLO setups which further emphasizes the significance of a well-balanced and diverse training dataset for achieving optimal performance across various diatom genera. However, it is crucial to note that well-represented classes were still accurately detected and identified, as seen in the example of *Aulacoseira* cells within the blue bounding boxes. This underscores the system's potential to reduce the burden of classification and counting on diatom experts.

It is also noticeable that the performance of YOLOv4 and YOLOv7 differed when subjected to an additional pre-processing step. YOLOv4 achieved a mean average precision of 18.32% when trained on pre-processed images, whereas the model trained on raw images yielded a higher mean average precision of 19.21%. For YOLOv7, the model trained on raw images demonstrated a mean average precision of 2.88%, while the model trained on processed images exhibited a substantially higher mean average precision of 13.3%. This underscores the impact of pre-processing techniques on the performance of both YOLO models and emphasizes the importance of considering such factors in optimizing their effectiveness.

The impact of pre-processing on model performance differed between the two architectures. For YOLOv7, pre-processing significantly improved mAP (from 2.88% to 13.3%), suggesting that image enhancement-such as background normalization, contrast adjustment, or noise reduction-helped the deeper network better extract features from small and morphologically diverse cells. Conversely, YOLOv4 exhibited a slight decrease in mAP after pre-processing (from 19.21% to 18.32%), which may be due to

the model's simpler architecture already being well-suited to raw image patterns, and pre-processing potentially altering subtle textures or colors that the network relied on for detection. These results underscore the importance of tailoring pre-processing steps to the architecture being used and highlight that pre-processing is not universally beneficial.

Building on the insights gained from the analysis of the 16-class experimental setup, which evaluated the performance of YOLOv4 and YOLOv7, assessed the impact of image pre-processing, and highlighted the significance of the number of cells required to represent a diatom genus during training, a subsequent experiment focused on the top eight diatom genera. The genera *Aulacoseira*, *Fragilaria*, *Encyonema*, *Cymbella*, *Synedra*, *Nitzschia*, *Navicula*, and *Achnanthidium* were selected due to their high cell abundance (Table 4 and 5). The images used for both training and testing were also divided to achieve a close to 80-20 division of the cell numbers per genus for training and testing, respectively. In this additional experiment, the highest-performing model from previous analyses, YOLOv4 trained on a raw image dataset, was employed. The key distinction in this experiment was the exclusion of the other eight classes characterized by a lower cell count.

Table 4. Number of cells found per classification in the eight-class experimental setup.

Class	Training (no of cells)	Testing (no of cells)
<i>Aulacoseira</i>	179	40
<i>Fragilaria</i>	186	38
<i>Encyonema</i>	215	49
<i>Cymbella</i>	198	85
<i>Synedra</i>	81	41
<i>Nitzschia</i>	111	21
<i>Navicula</i>	88	18
<i>Achnanthidium</i>	196	9

Table 5. Average precision (AP), true positive (TP), and false positive (FP) of each class in the eight-class experimental setup using the YOLOv4 model trained on raw image dataset.

Class	AP	TP	FP
<i>Aulacoseira</i>	71.61%	33	29
<i>Fragilaria</i>	22.89%	11	17
<i>Encyonema</i>	49.15%	33	46
<i>Cymbella</i>	59.08%	51	26
<i>Synedra</i>	26.54%	11	7
<i>Nitzschia</i>	41.70%	11	12
<i>Navicula</i>	51.52%	9	4
<i>Achnanthidium</i>	13.33%	3	12



Figure 1. Comparison between the actual labels (top) and the predicted labels (bottom) of the YOLOv7 for the raw dataset.

The system demonstrated significant improvement, as indicated by its mean average precision score of 41.98%, a substantial increase compared to the 19.21% mAP achieved in the 16-class experiment. Additionally, the system achieved a precision of 51%, recall of 54%, and an F1-score of 53%. This underscores the importance of a large number of diatom cells per genus needed to effectively train a YOLO model, enabling not only the detection but also the classification of diatoms in a digital image.

Figure 2 displays the PR-curve for the one-class, 16-class, and eight-class experimental setups across all classes. PR-curves were used to gauge the model's performance in the positive class, which is the focus of this study – i.e., to detect and classify diatoms in a digital image. In the eight-class PR curve, precision values range from 0.25 to 0.63, with the highest precision at 0.63. This indicates that when the model predicts a positive class, it is correct approximately 63% of the time. The recall values range from 0.44 to 0.65, with the highest recall being 0.65. This implies that the model can correctly identify 65% of the actual positive instances. Comparing this with the PR curves for the one-class experiment and 16-class experiment shows the added complexity of the problem when introducing additional or reducing the number of diatom genera for classification.

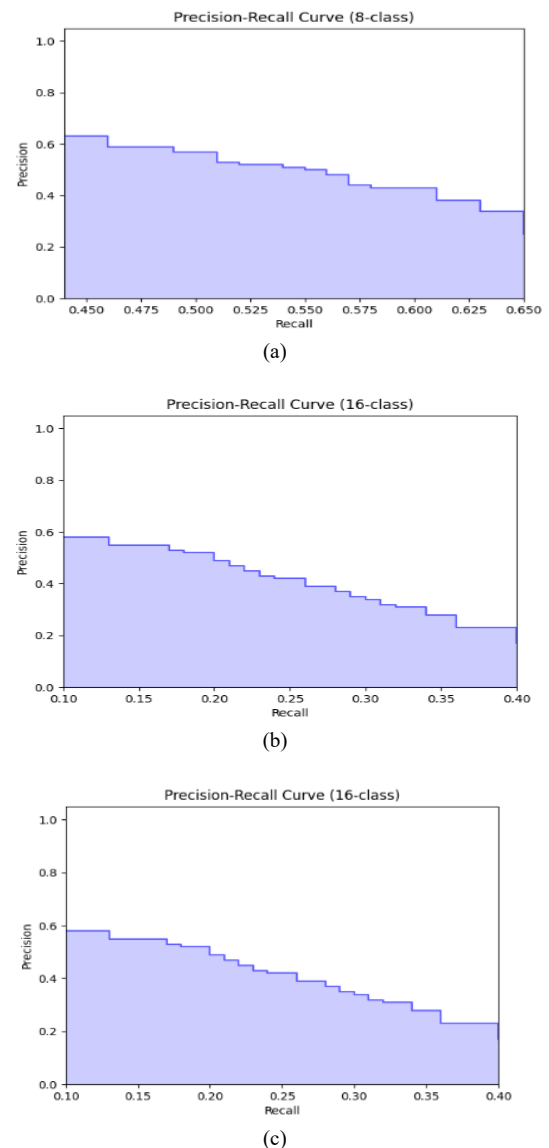


Figure 2. PR-curves for all classes of the (a) one-class, (b) 16-class, and (c) eight-class experimental setup.

Closer inspection of these scores also revealed the impact of imbalanced datasets on the average precision scores of each genus. Imbalanced datasets tend to exhibit a bias toward the majority classes. In extreme cases, the model may completely ignore the minority classes (Valova *et al.*, 2020). In this study, it can be observed that a higher number of false positives is present in most of the majority classes, especially *Aulacoseira*, *Encyonema*, and *Cymbella*. This indicates that a significant portion of the false detections made by the model falls under these classifications.

The decrease in the mAP score of the system can be attributed to another factor: the misidentification of certain genera due to similarities in their morphology. For instance, both *Cymbella* and *Encyonema* exhibit a boat-like or crescent shape in the images used for this study. The primary distinction lies in the appearance of their central lines, with *Encyonema* having a straight line and *Cymbella* featuring a curved one. In some instances, the system erroneously identified *Cymbella* as *Encyonema*, highlighting the challenge posed by their close resemblance. Another diatom genus, *Synedra*, and

Fragilaria, also faced a similar issue due to their close resemblance to each other. While *Fragilaria* typically has a rectangular shape compared to the pointed ends of *Synedra*, both exhibit elongated structures that may confuse the model.

Another factor contributing to the decrease in the mAP score of the system is the varying orientation of cells in the images, for example the *Cymbella* cell in a side view, unlike the typical front view seen in most images. This difference in orientation can lead to confusion in the pattern learned by the YOLOv4 model, ultimately impacting its performance on test images. *Fragilaria* faced a similar issue due to the variability of its samples across the datasets. In numerous instances, *Fragilaria* cells were found in groups, often overlapping with each other. However, there were also instances where a single cell appeared without any group, and instances where their sizes differed. This variability could potentially affect the system's performance in identifying cells under this genus. To enhance the model's ability to recognize the various configurations present in each image, more samples for training would be needed.

The automated approach offers a significant time advantage compared to traditional methods. The system requires only seconds to minutes for identifying and counting diatoms in 100 images, whereas the conventional method typically necessitates at least two days for 50 images. It is crucial to acknowledge that during the identification process, subtle distinctions in the cellular characteristics, such as shape, diameter, and size, of diatom species can be time-consuming and susceptible to human bias without the assistance of computer-aided automation procedures. To address this challenge, future studies employing this system could explore alternative pre-processing techniques that further emphasize the features of these cells. Improving the visibility of cellular edges and enhancing color in the images has the potential to enhance the classification outcomes of the system. It is also recommended to integrate CNNs with complementary techniques tailored specifically for diatom morphology analysis. Moreover, exposing the system to a diverse range of images taken under various conditions, including different lighting conditions, microscope magnifications, and capturing devices, can enhance the model's adaptability in identifying and recognizing diatoms in digital images.

The proposed automated approach demonstrates that reliable diatom identification can be achieved without removing the organic cover by leveraging visual features extracted from light microscopy images, such as overall cell shape, size, and geometric outlines, which remain discernible even when fine frustule details are partially obscured. Under these conditions, the system is most effective at the genus level, as major morphometric characteristics are sufficient for robust classification, whereas species-level identification remains constrained by subtle intra-genus morphological differences, cell orientation, and overlapping individuals inherent to light microscopy. Consequently, the method is positioned as a rapid, high-throughput screening tool that substantially reduces manual workload for diatomists, while acknowledging that species-level resolution would require higher-resolution imaging or complementary validation approaches. The use of 4% formalin as a preservative,

although not ideal for preserving delicate frustule features over long periods, was justified by its practicality and effectiveness in maintaining overall cell integrity for timely image acquisition; moreover, the reliance on general morphological patterns rather than ultrastructural details mitigates the impact of minor preservative-induced distortions. In comparison with earlier and contemporary automated identification frameworks, the present methodology advances the field by applying modern YOLO-based deep learning to enable simultaneous detection, classification, and counting of multiple freshwater diatom genera within complex images, achieving substantial gains in processing speed and scalability. While traditional and SEM-based methods remain indispensable for high-precision species-level taxonomy, the proposed approach provides a practical and efficient complement for large-scale, genus-level monitoring and ecological assessments.

The study aimed to mirror the natural process undertaken by diatom experts during microscopic slide inspections. Consequently, the distribution of each genus in the images could not be controlled, reflecting real-world scenarios. To mitigate this limitation, future efforts should consider introducing new datasets to the model where other classes are more prominently featured. This approach would enable a more comprehensive training experience, allowing the models to adapt to a wider range of scenarios and potentially leading to substantial improvements in their mean average precision scores. Fine-tuning the models with diverse datasets could enhance their performance and make them more robust in handling variations in diatom distribution in digital images.

The inclusion of additional diatom genera into the model will enhance the identification system for freshwater diatom species and contribute to its overall robustness. Integrating the developed identification system into a mobile application would offer a more convenient, flexible, and faster means of recognizing and identifying freshwater diatoms. It is recommended that future research concentrate on establishing a database management system for all images of Philippine freshwater diatom species. This proposed system should be accessible via the Internet to encourage ongoing indexing and tagging of new images. Furthermore, online databases can facilitate swift access and retrieval of historical data.

4. Conclusion

The study applied YOLO architecture to detect, identify, and count freshwater diatoms in the Philippines, achieving significant progress in computational recognition. Using YOLOv4, the initial detection phase reached a mean average precision (mAP) of 56.64%. In the classification phase, YOLOv4 without pre-processing performed best in detecting 16 genera, with a mAP of 19.21%, precision of 41%, recall of 25%, and an F1-score of 31%. Focusing on the eight most abundant genera in a third setup improved the mAP to 41.98%. Challenges such as dataset imbalance, morphological similarities, and cell orientation affected precision, but the system drastically outpaced manual analysis, processing 100 images in minutes. While currently limited to 16 genera, the study demonstrates machine vision's potential to automate and enhance diatom identification. Future work includes

diversifying the dataset, refining pre-processing, integrating CNNs, expanding genera coverage, developing a mobile app, and building an online Philippine diatom database to advance taxonomy and ecological monitoring.

Conflict Of Interests

The authors have no conflict of interests to declare.

Acknowledgment

The authors acknowledge the Institute of Computer Science and the National Institute of Molecular Biology and Biotechnology (BIOTECH-UPLB) for the technical and financial support provided for the study. Lastly, the authors are grateful for the constructive comments and suggestions of the journal editors and reviewers.

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Optimizing Lipid and Astaxanthin Accumulation in *Chromochloris zofingiensis* Through Nitrogen Limitation and Phosphorus Enrichment

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Received: September 20, 2025; Revised: January 6, 2026; Accepted: January 17, 2026

Abstract

Chromochloris zofingiensis is a promising microalga capable of accumulating both lipids and astaxanthin, two high-value bioproducts relevant to biofuel, aquaculture, and nutraceutical industries. This study aimed to investigate how nitrogen limitation combined with varying phosphorus enrichment affects the growth, pigment composition, lipid content, and astaxanthin accumulation of *C. zofingiensis* under photoautotrophic conditions. A batch culture experiment was conducted with four treatments: standard nitrogen and phosphorus as the control (N & P), nitrogen limitation with normal phosphorus levels (N_{lim} & P), nitrogen limitation with moderate phosphorus enrichment (N_{lim} & P₊), and nitrogen limitation with high phosphorus enrichment (N_{lim} & P₊₊). The major hypothesis is that an elevated phosphorus supply helps alleviate the negative impacts of nitrogen deprivation on growth, while simultaneously promoting the biosynthesis of lipids and astaxanthin. Results showed that nitrogen and phosphorus sufficiency led to the highest growth rate and chlorophyll content, while nitrogen deprivation severely limited photosynthetic pigment and protein synthesis. However, high phosphorus supplementation (N_{lim} & P₊₊) under nitrogen-limited conditions mitigated biomass suppression, leading to biomass yields statistically comparable to the control. This treatment also induced the highest lipid content (34.11% DW) and lipid productivity (33.16 mg L⁻¹ d⁻¹) and significantly elevated astaxanthin concentration (1.81 mg L⁻¹). Moderate phosphorus enrichment (N_{lim} & P₊) maximized per-cell astaxanthin content, highlighting a trade-off between cell-specific pigment accumulation and volumetric productivity. These findings demonstrate that nutrient modulation can redirect metabolic fluxes, enabling dual enhancement of lipids and carotenoids in *C. zofingiensis*. The synergistic effects of nitrogen limitation and phosphorus enrichment offer a practical nutrient engineering strategy for optimizing microalgal bioprocesses aimed at producing biofuels and high-value compounds.

Keywords: astaxanthin production, *Chromochloris zofingiensis*, excessive phosphorus, lipid biosynthesis, nitrogen limitation

1. Introduction

Microalgae are photosynthetic organisms that are recognized as a renewable and sustainable feedstock for biofuel production, animal feed, and high value biochemicals due to their high photosynthetic efficiency, rapid growth, and ability to accumulate a diverse range of compounds, including lipids and pigments (Babu *et al.*, 2022; Ezhumalai *et al.*, 2024). *Chromochloris zofingiensis* is particularly noteworthy among microalgae strains due to its unique capability to co-accumulate substantial amounts of lipids and astaxanthin, a high value ketocarotenoid with potent antioxidant activity (Mao *et al.*, 2018). This species has demonstrated superior adaptability under diverse cultivation regimes, including photoautotrophic, heterotrophic, and mixotrophic conditions, making it a promising candidate for integrated biomass valorization (Wood *et al.*, 2024; Zhang *et al.*, 2019). However, despite

its potential, large-scale application remains constrained by low biomass and product yields under standard cultivation conditions (Wood *et al.*, 2024). Enhancing the biosynthetic capacity of *C. zofingiensis* through nutrient modulation strategies thus remains a critical area of investigation for advancing sustainable microalgal bioprocessing.

A commonly adopted approach for improving microalgal lipid and astaxanthin productivity involves the induction of stress conditions, particularly nitrogen deprivation (Mao *et al.*, 2018; Zhu *et al.*, 2015), which commonly arises in large-scale batch or semi-batch cultivation as nutrients are rapidly exhausted at high cell densities and is also deliberately imposed to redirect cellular metabolism toward high-value metabolite accumulation (Yadav *et al.*, 2025). Nitrogen starvation has been shown to redirect cellular metabolism toward synthesizing storage lipids and secondary carotenoids, at

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the cost of inhibited cell division and diminished biomass accumulation (Maltsev *et al.*, 2023). This metabolic trade-off poses a significant limitation, as the reduction in biomass can offset the gains in product concentration, thereby constraining overall productivity. In *C. zofingiensis*, nitrogen limitation has been reported to induce lipid content and concurrent astaxanthin accumulation, yet biomass yields decline sharply under such conditions (Mao *et al.*, 2018; Zhu *et al.*, 2015).

Research has increasingly focused on phosphorus repletion as a combinatorial nutrient strategy to mitigate the adverse effects of nitrogen limitation on microalgal growth and lipid production. As an essential macronutrient, phosphorus plays a critical role in energy metabolism, nucleic acid synthesis, and membrane stability (Bossa *et al.*, 2024), and its supplementation under nitrogen-deficient conditions has been shown to enhance lipid accumulation without severely compromising biomass productivity (Chu *et al.*, 2014; Zhu *et al.*, 2015). This effect is largely attributed to luxury phosphorus uptake, leading to intracellular polyphosphate accumulation that functions as an energy reserve to sustain anabolic processes during nitrogen starvation (Zhu *et al.*, 2015). Empirical evidence supports this mechanism, as phosphorus repletion under nitrogen deprivation significantly increased lipid productivity and upregulated oxidative phosphorylation-related genes in *Chlorella vulgaris* (Chu *et al.*, 2019).

Beyond lipid enhancement, recent attention has also focused on the relationship between phosphorus availability and carotenoid synthesis. Astaxanthin production in *C. zofingiensis* has been linked to oxidative stress responses and energy-demanding biosynthetic pathways (Zhang *et al.*, 2019). Given that polyphosphate reserves can act as high-energy molecules akin to ATP (Chu *et al.*, 2013), it is plausible that phosphorus repletion may provide the metabolic energy required for sustained astaxanthin synthesis under nitrogen stress.

Although the individual impacts of nitrogen limitation and phosphorus enrichment on lipid and other biomass components have been investigated in other microalgal species (Chu *et al.*, 2019; Fu *et al.*, 2017), their combined effects in *C. zofingiensis* remain largely unexplored. Given the species' unique ability to simultaneously produce lipids and astaxanthin, along with its potential for high-value biomass generation, there is a clear need to evaluate the interactive roles of these two macronutrients systematically. Moreover, previous work has largely focused on heterotrophic or mixotrophic systems (Fu *et al.*, 2017; Shen *et al.*, 2016), leaving a critical knowledge gap regarding photoautotrophic cultivation, which is more sustainable and scalable for outdoor applications.

This study addresses the aforementioned research gap by examining the effects of phosphorus repletion on lipid and astaxanthin production in *C. zofingiensis* under nitrogen-limited, photoautotrophic conditions. The major hypothesis is that a high supply of phosphorus helps reduce the negative impacts of nitrogen deprivation on growth while at the same time promoting the biosynthesis of lipids and astaxanthin. To confirm the hypothesis, we examined different phosphorus concentrations in nitrogen-deficient BG-11 media and then monitored growth, biomass, photosynthetic pigments, protein content, lipid production, and astaxanthin level. The novelty of this

research lies in its focus on the dual enhancement of lipid and astaxanthin productivity in *C. zofingiensis* under strictly photoautotrophic regimes, which has not been systematically studied to date. The outcomes of this work are expected to offer new insights into nutrient engineering strategies for microalgal bioprocess optimization, particularly for strains capable of producing multiple high-value bioproducts.

2. Materials and Methods

2.1. Strain and cultivation conditions

The microalga *C. zofingiensis* UTEX 32 was provided from University of Texas Culture Collection of Algae. This strain was selected for this study due to its recognized potential for concurrent lipid and astaxanthin production. Under static photoautotrophic conditions, stock cultures were maintained in 250 mL Erlenmeyer flasks containing 150 mL of standard BG-11 medium (Stanier *et al.*, 1971). Illumination was provided continuously using cool white, fluorescent light at an intensity of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 25 °C.

The experimental design employed a batch culture system with four treatment groups: (1) sufficient nitrogen and phosphorus (N & P; 240 mg L⁻¹ NO₃-N and 5.56 mg L⁻¹ phosphorus), (2) nitrogen-deficient and sufficient phosphorus (N_{lim} & P; 8 mg L⁻¹ NO₃-N and 5.56 mg L⁻¹ phosphorus), (3) nitrogen-deficient and moderate phosphorus repletion (N_{lim} & P₊; 8 mg L⁻¹ NO₃-N and 27.8 mg L⁻¹ phosphorus), and (4) nitrogen-limitation and high phosphorus repletion (N_{lim} & P₊₊; 8 mg L⁻¹ NO₃-N and 55.6 mg L⁻¹ phosphorus). The concentrations for sufficient nitrogen and phosphorus were based on the standard BG-11 formula, while the nitrogen-deficient condition was set at 3.3% of the standard BG-11 nitrate concentration. Phosphorus levels were varied up to 1,000% of the original concentration (Table 1).

Pre-cultivation was performed for 4 days to establish exponential growth. Algal cells were harvested by centrifugation at 1,800g for 15 min, washed twice with sterile distilled water to remove residual nutrients, and resuspended to an initial OD₆₈₀ of 0.123 for all treatments. Each culture was maintained in 500 mL flasks with 250 mL working volume, under constant aeration at a flow rate of 0.4 L/min with sterilized air and illumination at 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under continuous light period. Cultures were incubated at 25 °C for 5 days.

Table 1. Initial nitrogen and phosphorus concentrations in the modified BG-11 medium

	N & P	N _{lim} & P	N _{lim} & P ₊	N _{lim} & P ₊₊
Nitrogen (NO ₃ -N) (mg L ⁻¹)	240 (100%)	8 (3.3%)	8 (3.3%)	8 (3.3%)
Phosphorus (PO ₄ ³⁻ -P) (mg L ⁻¹)	5.56 (100%)	5.56 (100%)	27.8 (500%)	55.6 (1000%)

*Values inside parenthesis show the ratio of the changed nitrogen/phosphorous concentrations to those in the original BG-11 medium.

2.2. Growth and biomass determination

Growth was monitored daily by measuring optical density at 680 nm (OD₆₈₀) using a GENESYS 20 UV-Vis spectrophotometer (Thermo Scientific, USA). Specific growth rate (μ) was calculated according to standard logarithmic formulas:

$$\mu \text{ (d}^{-1}\text{)} = \frac{[\ln(N_t) - \ln(N_0)]}{\Delta t} \quad (1)$$

N_t and N_0 are final and initial OD₆₈₀ values, respectively, and Δt is the time interval in days.

For biomass estimation, 25 mL of culture was filtered through 1.2 μm GF/C filters (Whatman, USA), rinsed with deionized water, and dried at 105 °C for 2 hours until constant weight was achieved. Filters were cooled in a desiccator and weighed to determine dry biomass in g L⁻¹ (Fakhri *et al.*, 2021).

2.3. Chlorophyll analysis

Chlorophyll *a* and *b* (Chl *a+b*) of *C. zofingiensis* were extracted using 90% methanol, with samples collected at the end of the experimental period (Fakhri *et al.*, 2024). Two mL of algal culture was centrifuged at 1,800g for 15 min, washed with distilled water, and resuspended in 2 mL of 90% methanol. The suspension was sonicated for 10 min and incubated in a water bath at 70 °C for 10 min. After centrifugation, absorbance readings were taken at 652 and 665 nm. Pigment concentrations were calculated using the following equations (Ritchie, 2006):

$$\text{Chl } a \text{ (}\mu\text{g mL}^{-1}\text{)} = 16.5169 \times A_{665} - 8.0962 \times A_{652} \quad (2)$$

$$\text{Chl } b \text{ (}\mu\text{g mL}^{-1}\text{)} = 27.4405 \times A_{652} - 12.1688 \times A_{665} \quad (3)$$

2.4. Lipid determination

Lipid content was determined gravimetrically based on extraction with a chloroform-methanol (2:1 v/v) mixture following the Bligh and Dyer (1959) method. After 5 days of cultivation, microalgae were harvested and oven-dried at 65 °C for 30 h. Approximately 30 mg of dry biomass (M1) was extracted overnight, and lipids were separated, dried, and weighed. Lipid content was expressed as % of dry weight (% DW), while lipid productivity was determined as mg L⁻¹ d⁻¹:

$$\text{Lipid content (\% DW)} = \frac{M3-M2}{M1} \times 100\% \quad (4)$$

M1 refers to the total dry biomass, M2 represents the weight of the empty vial, while M3 corresponds to the weight of the vial containing the extracted lipid.

$$\text{Lipid productivity (mg L}^{-1}\text{ d}^{-1}\text{)} = \frac{W2-W1}{\Delta t} \times \text{lipid content} \quad (5)$$

W1 is designated as the biomass measured at the initiation of the cultivation process, whereas W2 denotes the biomass obtained at the termination of cultivation.

2.5. Astaxanthin analysis

Astaxanthin content was analyzed using a spectrophotometric method adapted from Boussiba and Vonshak (1991). 10 mL of algal culture was harvested by centrifugation at 1,800g for 15 min. The resulting cell pellet was washed with 5 mL of distilled water to remove residual culture medium. To remove chlorophyll, the pellet was resuspended in 5 mL of 5% (w/v) potassium hydroxide (KOH) prepared in 30% (v/v) methanol. The mixture was vortexed for 30 sec, then incubated in a water bath at 75 °C for 10 min. After incubation, the sample was centrifuged again at 1,800g for 15 min, and the supernatant

was discarded. If the supernatant still exhibited a green color, indicating the presence of chlorophyll, the dechlorophyllation process was repeated until the supernatant became colorless. The dechlorophyllated pellet was then treated with 25 μL of acetic acid and 5 mL of DMSO to facilitate astaxanthin extraction. The sample was vortexed thoroughly and subjected to sonication using an ultrasonic cleaner for 15 min. After sonication, the mixture was incubated in a water bath at 75 °C for another 10 min to enhance pigment release. The final extract was centrifuged, and the supernatant containing astaxanthin was collected. The absorbance of the extract was measured at a wavelength of 530 nm using a spectrophotometer. The astaxanthin concentration, expressed in mg L⁻¹, and cellular astaxanthin content, expressed in mg g⁻¹, were calculated using the following equation (Li *et al.*, 2012).

$$\text{Astaxanthin concentration (mg L}^{-1}\text{)} = \frac{A_{530} - 0.0107}{0.1556} \quad (6)$$

$$\text{Cellular astaxanthin content (mg g}^{-1}\text{)} = \frac{\text{Astaxanthin concentration}}{\text{Biomass concentration}} \quad (7)$$

2.6. Statistical analysis

All experiments were conducted in triplicate. Results are presented as means $\pm$ standard deviation (SD). Statistical significance among treatments was assessed using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test ($p < 0.05$) using SPSS 25.0 software.

3. Results and Discussion

3.1. Growth and biomass of *C. zofingiensis*

The growth profile (OD₆₈₀) of *C. zofingiensis* under the four culture conditions (N & P, N_{lim} & P, N_{lim} & P₊, and N_{lim} & P₊₊) in the photoautotrophic system is shown in Figure 1A. On day 1, a slight increase in OD₆₈₀ was observed in all groups, with no noticeable divergence among treatments, suggesting comparable initial growth responses. By day 2, OD₆₈₀ values continued to rise gradually across all conditions, though the N & P group began to show a marginally higher increase compared to nitrogen-limited treatments. From day 3 onward, distinct growth patterns became more apparent. The N & P culture showed a steeper rise in OD₆₈₀ compared to nitrogen-limited conditions, indicating a more robust exponential growth phase supported by sufficient nitrogen availability. On day 5, *C. zofingiensis* under the N & P condition reached its highest OD₆₈₀ (1.14). In contrast, all N-deficient treatments attained much lower OD₆₈₀ values (0.870 to 0.932), suggesting that *C. zofingiensis* growth was clearly constrained by nitrogen limitation. In a similar study, Fakhri *et al.* (2025) found that the growth of *C. pyrenoidosa* was severely inhibited under nitrogen deficiency and P repletion. In addition, further increasing concentration of phosphorus in the media could not promote the growth of microalgae.

Biomass concentration, however, showed a different response (Figure 1B). The N & P condition produced the highest biomass at 0.53 $\pm$ 0.07 g L⁻¹, while N_{lim} & P and N_{lim} & P₊ produced lower values of approximately 0.38 $\pm$ 0.05 and 0.40 $\pm$ 0.06 g L⁻¹, respectively. Interestingly, N_{lim} & P₊₊ yielded 0.49 $\pm$ 0.03 g L⁻¹ of biomass, which was statistically not different from the N & P group ($p > 0.05$).

This outcome contradicts the assumption that all N-deficient treatments uniformly suppress biomass and suggests that phosphorus enrichment at sufficiently high levels (as in N_{lim} & P_{++}) can partially offset the adverse effects of nitrogen deprivation. In accordance with our study, Fu *et al.* (2017) reported that phosphorus supplementation up to 45 mg L⁻¹ under nitrogen-limited conditions can alleviate growth inhibition and support biomass accumulation of *Chlorella regularis*.

These observations suggest a synergistic, though not compensatory, effect of phosphorus under nitrogen-limited conditions. Nitrogen is essential for synthesizing cellular macromolecules such as proteins, nucleic acids, and chlorophyll; thus, its deficiency is expected to constrain biomass production regardless of other nutrient availability (Nagappan & Kumar, 2021). The enhanced biomass in the N_{lim} & P_{++} treatment may result from improved energy metabolism or maintenance of homeostasis enabled by

luxury phosphorus uptake and polyphosphate accumulation, as described by Zhu *et al.* (2015) and Chu *et al.* (2019). In such scenarios, excess phosphorus could support ATP generation and modulate enzymatic functions, enabling modest growth despite persistent nitrogen stress.

Nonetheless, the biomass recovery in N_{lim} & P_{++} was incomplete and did not reach the level of nitrogen-sufficient control, reinforcing that phosphorus cannot fully substitute for nitrogen. The data, therefore, supports a nuanced conclusion: while nitrogen remains the critical driver of algal growth, high phosphorus availability can modestly mitigate the impact of nitrogen starvation, thereby enhancing biomass accumulation in *C. zofingiensis* under suboptimal nitrogen conditions.

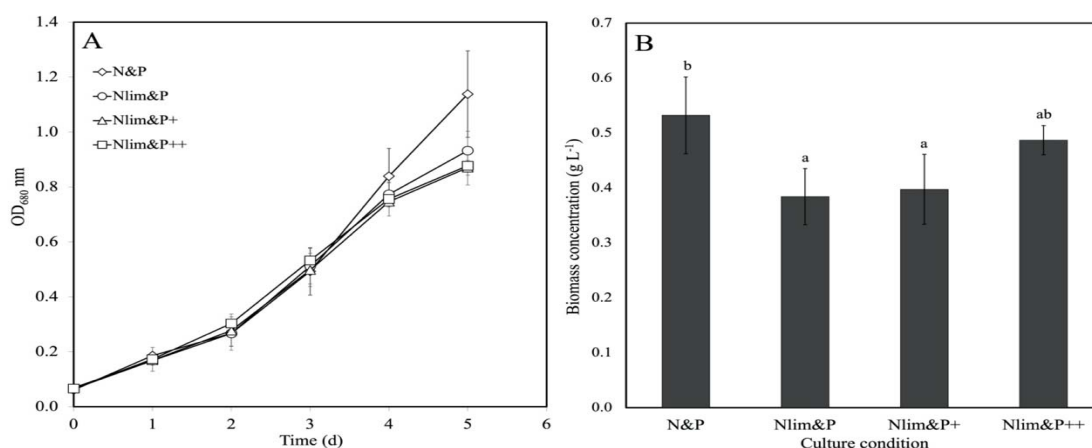


Figure 1. Growth of the *Chromochloris zofingiensis* cells: A) changes in optical density at 680 nm (OD₆₈₀); and B) biomass concentration of *C. zofingiensis* grown in different culture conditions. Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). Same letters show that there was no difference between treatments (p > 0.05)

3.2. Chlorophyll and protein content of *C. zofingiensis*

Nitrogen limitation profoundly affected photosynthetic pigments and protein content (Figure 2). Chl *a+b* concentrations were highest in the N & P control, reflecting robust photosynthetic capacity under nutrient-replete conditions. By contrast, N-deficient cultures exhibited dramatically lower chlorophyll: for example, the N_{lim} & P treatment contained only 5.77 µg mL⁻¹ Chl *a+b* at day 5, versus 18.96 µg mL⁻¹ in N & P. The drop is more than threefold and was statistically significant (Figure 2A). Supplementing excess P in the N-deficient media did not increase chlorophyll levels of *C. zofingiensis*. This phenomenon was also observed in our previous investigation of *C. pyrenoidosa*. Such reductions are expected because nitrogen is a major constituent of chlorophyll and associated proteins (Fakhri *et al.*, 2025). Limited nitrogen causes the destruction of nitrogenous compounds such as chlorophyll and the degeneration of chloroplasts, resulting in a loss in photosynthetic performance (Liu *et al.*, 2022).

Protein content exhibited a similar nitrogen-dependent pattern (Figure 2B). Protein, which is rich in nitrogen, was highest in the N & P treatment and lowest in N_{lim} & P_{++} . N-limited cultures rely on internal protein reserves and accumulate less new protein, so protein per cell or biomass fell sharply. Additional phosphorus did not reverse this effect. This result is consistent with some studies reporting that nitrogen scarcity constrains protein synthesis in microalgae (Liu *et al.*, 2022; Zarrinmehr *et al.*, 2020). Importantly, phosphorus is essential for nucleic acids and energy metabolism, but it plays a less direct role in protein production. Thus, in our data, the P-repleted (N_{lim} & P_{++} , N_{lim} & P_{++}) cultures did not improve protein relative to N_{lim} & P. Our findings align with the study in *Tetraselmis subcordiformis* Yao *et al.* (2018). Despite the abundance of phosphorus, there is no chlorophyll or protein regeneration, implying that energy or substrate availability from polyphosphates is inadequate to resume biosynthetic processes without nitrogen.

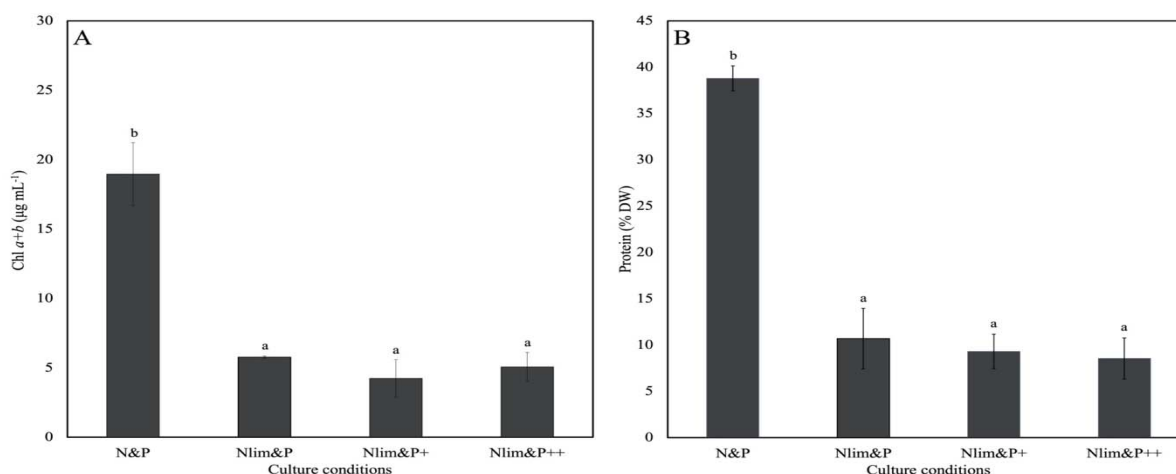


Figure 2. Chl *a+b* of *Chromochloris zofingiensis* cultivated in different culture conditions (A). Protein content of *C. zofingiensis* cultivated in different culture conditions (B). Error bars demonstrate the standard deviations from the triplicate experiments ($n = 3$). At a 95% confidence level, different letters show that there was a remarkable difference between treatments

3.3. High phosphorus triggered enhanced lipid accumulation

Figure 3 illustrates the effects of different nitrogen and phosphorus regimes on lipid accumulation and lipid productivity in *C. zofingiensis*. As shown in Figure 3A, lipid content varied significantly across treatments ($p < 0.05$). The control culture (N & P) exhibited the lowest lipid content at approximately $18.11 \pm 0.19\%$ DW. Under nitrogen-limiting conditions with normal phosphorus supplementation (N_{lim} & P), lipid content markedly increased to about 30.56% DW, representing a significant improvement compared with the control ($p < 0.05$). This finding aligns with well-documented responses in microalgae where nitrogen deprivation triggers reallocation of carbon flux toward storage lipids, particularly triacylglycerols (TAGs) (Huang *et al.*, 2019; Maltsev *et al.*, 2023). Nitrogen stress upregulates the malic enzyme, generating NADPH that favors fatty acid synthesis. Under limited nitrogen conditions, these enzymes would drive carbon into lipids (Ma *et al.*, 2018).

A similar lipid content was observed in N_{lim} & P_+ cultures, indicating that moderate phosphorus enrichment did not further enhance lipid accumulation. However, the highest P treatment (N_{lim} & P_{++}) yielded the greatest lipid levels of all (34.11% DW), which corresponds to an 88% increase over the N & P control and an 11.64% increase over N_{lim} & P (Figure 3). This progressive enhancement is consistent with the notion that luxury phosphorus uptake can be channeled into storage products under nutrient stress (Chu *et al.*, 2013; Fu *et al.*, 2017). For example, Chu *et al.* (2013) reported that *C. vulgaris* with ample P under N starvation achieved the highest lipid accumulation of any condition tested. In their study, phosphorus taken up by the cells was stored as polyphosphate and accompanied by increased lipid yield. Similarly, our *C. zofingiensis* cultures likely use extra phosphorus to fuel energetic and reductive processes for fatty acid synthesis. Phosphorus repletion may have enhanced this effect by providing additional ATP and phosphate donors; indeed, microalgae can rapidly assimilate surplus P when starved of N (Chu *et al.*, 2019; Chu *et al.*, 2013). The excess polyphosphate and associated metabolic energy can indirectly promote fatty

acid assembly. We also note that simply increasing P in N-replete cultures does not usually raise lipids, but in the context of N deficiency, it seems to prime the cells for even greater TAG storage. Thus, our data demonstrates a synergistic effect: nitrogen limitation sets the stage for lipid accumulation, and phosphorus repletion further enhances it.

Lipid productivity of *C. zofingiensis* exhibited a parallel pattern. The control treatment yielded approximately $19 \text{ mg L}^{-1} \text{ d}^{-1}$. Nitrogen-limited treatments with normal (N_{lim} & P) and moderate phosphorus (N_{lim} & P_+) achieved similar values ($23\text{--}25 \text{ mg L}^{-1} \text{ d}^{-1}$), with no significant differences ($p > 0.05$). Conversely, N_{lim} & P_{++} strongly improved lipid productivity to $33.16 \text{ mg L}^{-1} \text{ d}^{-1}$, a statistically significant enhancement relative to all other groups ($p < 0.05$). This outcome highlights the critical role of phosphorus in sustaining cellular metabolism under nitrogen stress. Whereas nitrogen limitation tends to suppress cell division and biomass accumulation, high phosphorus availability counterbalances this limitation, enabling the cells to maintain higher growth rates while still channeling carbon into lipid synthesis. The net effect is a synergistic increase in biomass and lipid content, culminating in maximal lipid productivity.

From a bioprocessing perspective, these results are highly relevant. For industrial biofuel production, lipid productivity is a more critical metric than lipid content (% DW), since it integrates the fraction of lipids and the biomass accumulation rate (Senousy & Ellatif, 2020). Although nitrogen limitation reduced growth relative to the nutrient-replete control (N & P), the N_{lim} & P_{++} condition achieved the highest lipid productivity among all treatments, exceeding not only other nitrogen-limited conditions but also the control culture. Specifically, lipid productivity under N_{lim} & P_{++} reached $33.16 \text{ mg L}^{-1} \text{ d}^{-1}$, representing a 41.7% increase compared with N_{lim} & P and a substantial improvement over N & P. This outcome indicates that high phosphorus availability can effectively compensate for nitrogen-induced growth suppression by sustaining sufficient biomass formation while simultaneously enhancing lipid biosynthesis.

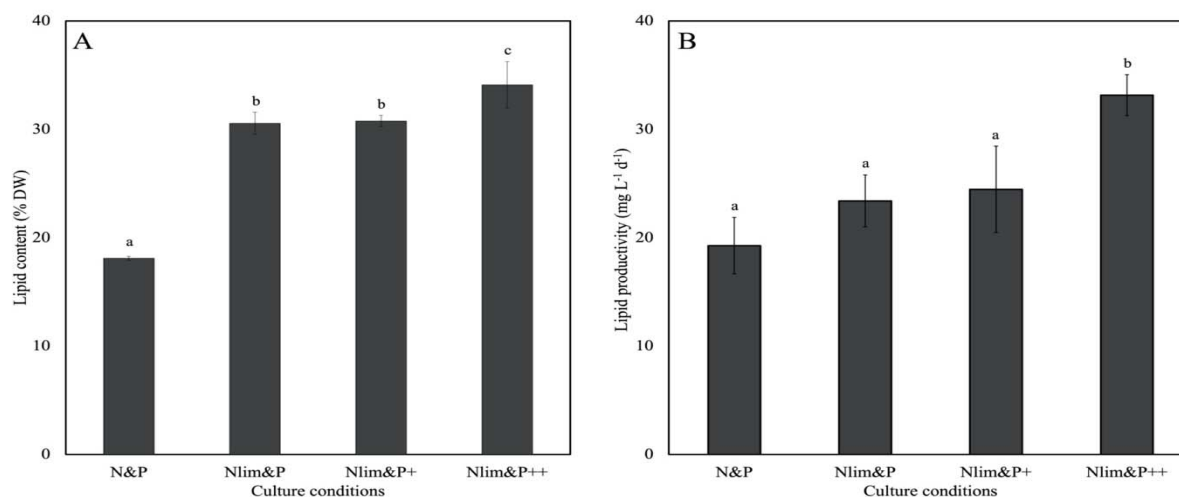


Figure 3. (A) Lipid content of *Chromochloris zofingiensis* under different phosphorus and nitrogen supply; (B) Lipid productivity of *C. zofingiensis* under different phosphorus and nitrogen supply. Error bars demonstrate the standard deviations from the triplicate experiments ($n = 3$). At a 95% confidence level, different letters show that there was a remarkable difference between treatments.

3.4. Astaxanthin production was triggered by nitrogen deficiency and phosphorus supply

Figure 4 illustrates the effects of nitrogen limitation and varying phosphorus supply on astaxanthin accumulation in *C. zofingiensis*. Astaxanthin concentration in the control (N & P) was the lowest, averaging 0.45 mg L^{-1} (Figure 4A). Under $N_{\text{lim}} \& P$ condition, astaxanthin concentration increased significantly to approximately 1.29 mg L^{-1} ($p < 0.05$). In a similar study, Li *et al.* (2022) demonstrated that *Haematococcus pluvialis* exposed to nitrogen starvation exhibited a marked increase in astaxanthin accumulation. A further increase was observed under moderate and high phosphorus supplementation ($N_{\text{lim}} \& P_+$ and $N_{\text{lim}} \& P_{++}$), with concentrations about 1.81 mg L^{-1} in the latter. The cellular astaxanthin content followed a similar trend (Figure 4B). The control culture accumulated 1.0 mg g^{-1} , whereas $N_{\text{lim}} \& P$ increased the content to 3.3 mg g^{-1} . The maximum value of 4.3 mg g^{-1} was obtained under $N_{\text{lim}} \& P_+$, with a slight decline under $N_{\text{lim}} \& P_{++}$ (3.7 mg g^{-1}), although still significantly higher than the control.

Our study confirms that nitrogen limitation is a strong inducer of astaxanthin biosynthesis in *C. zofingiensis*. Nitrogen deprivation reduces chlorophyll synthesis and photosynthetic proteins, creating oxidative stress that stimulates carotenoid accumulation as a protective mechanism (Shi *et al.*, 2020). Certain proteins undergo biodegradation, and the resulting carbon skeletons are partially redirected into the biosynthetic pathway of astaxanthin (Juneja *et al.*, 2013; Ren *et al.*, 2021). The 3–4-fold increase in cellular astaxanthin under nitrogen limitation highlights the metabolic shift from growth-oriented processes toward secondary metabolite synthesis.

Phosphorus availability further modulated this response. Moderate phosphorus supplementation ($N_{\text{lim}} \& P_+$) yielded the highest per-cell astaxanthin content, suggesting phosphorus supports metabolic activity required for carotenoid biosynthesis. Phosphorus plays essential roles in ATP production, nucleic acid metabolism, and redox balance, which may enhance precursor availability for the carotenoid pathway. However, excessive phosphorus ($N_{\text{lim}} \& P_{++}$) slightly reduced per-cell astaxanthin, although volumetric concentration continued to rise, likely due to sustained

biomass production. This indicates a trade-off: higher phosphorus supports biomass growth and volumetric pigment yield, while moderate supplementation optimizes per-cell astaxanthin accumulation.

The pigmentation switches in *C. zofingiensis* are directly related to astaxanthin production, as evidenced by the significant increase in astaxanthin content under phosphorus-enriched conditions (Figure 4C), which quantitatively demonstrates the stimulatory effect of phosphorus supply on astaxanthin accumulation. When provided with nutrient-adequate (N & P) culture medium, the cells showed a rich green pigmentation indicative of elevated chlorophyll production and low induction of carotenoid pigments. However, the nitrogen-deficient treatments showed a vivid yellow–orange pigmentation indicative of chlorophyll breakdown and the production of secondary carotenoid pigments such as astaxanthin. This pigmentation change reflects the visible manifestation of cells undergoing the process of reprogramming the rate at which the cell produces photosynthetic pigments and channeling the flow of carbon for the production of astaxanthin. Microscopic observations revealed changes in cellular appearance accompanying the yellow–orange pigmentation. Together with the quantitative astaxanthin measurements, these phenotypic changes are consistent with enhanced secondary carotenoid accumulation under nitrogen-limited conditions.

3.5. Biotechnological Implications and Future Directions

The capacity of *C. zofingiensis* to co-accumulate astaxanthin and lipids under nitrogen stress combined with phosphorus optimization distinguishes this species from most commercially exploited microalgae, which typically specialize in single target metabolite. For example, *Haematococcus pluvialis*, the dominant industrial source of natural astaxanthin, can accumulate exceptionally high astaxanthin contents but exhibits slow growth, high sensitivity to stress, and negligible lipid productivity, leading to elevated production costs (Shah *et al.*, 2016). Conversely, lipid-oriented strains such as *Nannochloropsis* spp. and *Chlorella vulgaris* are widely applied for biofuel and aquafeed production due to their robust growth and high triacylglycerol yields, yet they lack the capacity to

synthesize high-value ketocarotenoids like astaxanthin (Cecchin *et al.*, 2022). In this context, *C. zofingiensis* offers a unique “double-duty” platform capable of producing both astaxanthin and lipid-rich biomass, which confers a substantial biotechnological advantage within integrated biorefinery frameworks, where astaxanthin recovery can offset the costs associated with biofuel or feedstock production.

Future research should focus on metabolic engineering to improve astaxanthin biosynthetic pathways, particularly control of central enzymes β -carotene ketolase and

hydroxylase. It may also be possible to upgrade the cultivation strategy by utilizing two-stage processes, such as photobioreactor design or nutritional stress mixing. The integration of process engineering and the implementation of the toolbase of omics will be the most critical factors in achieving the organism's metabolic potential. Lastly, the future of *C. zofingiensis* as a foundation organism for sustainable biotechnology lies very strongly at the intersection of renewable energy, the aquaculture industry, and human health.

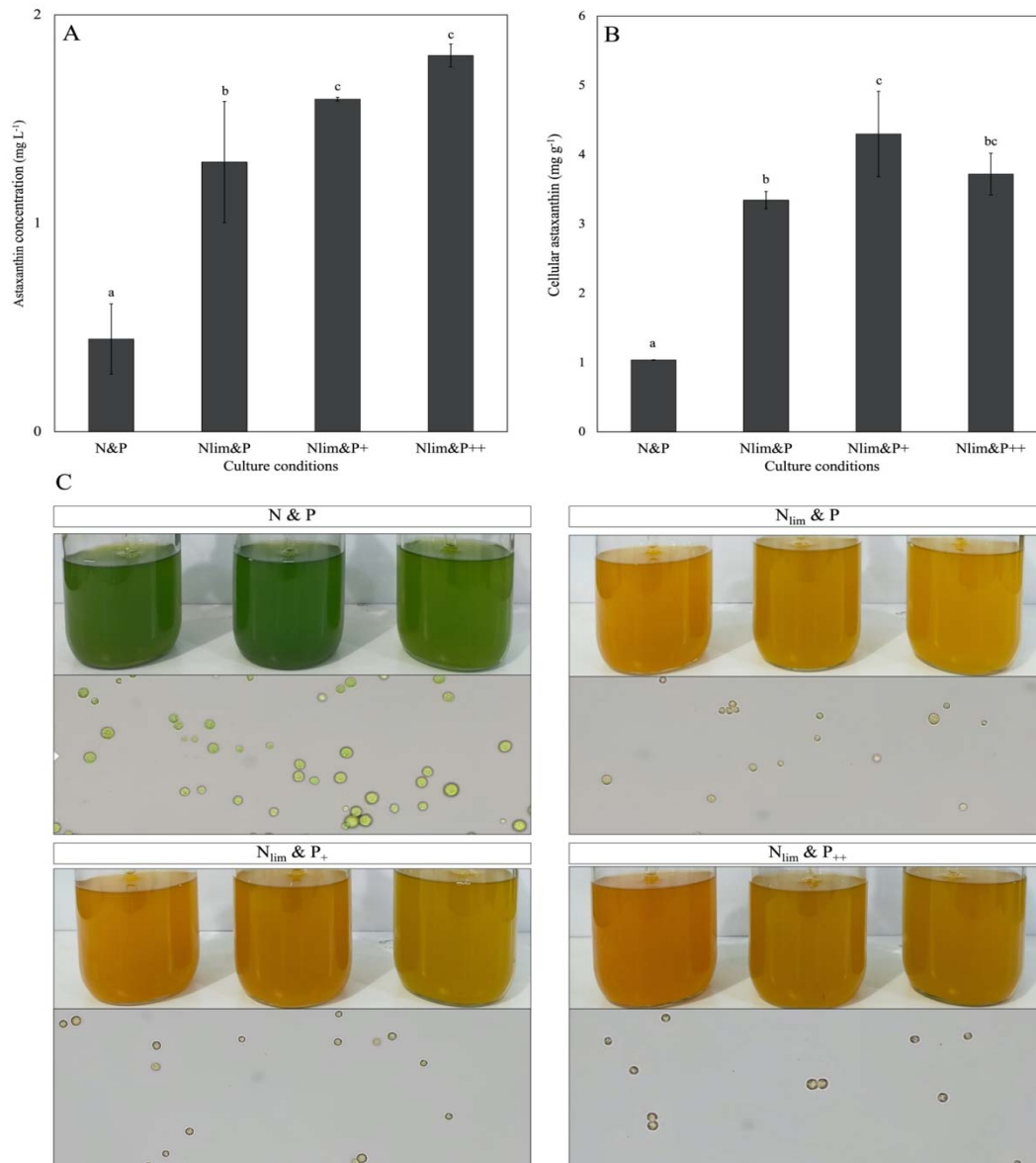


Figure 4. (A). Astaxanthin concentration (mg/L) of *Chromochloris zofingiensis* under different phosphorus and nitrogen supply; (B). Cellular astaxanthin (mg g⁻¹) of *C. zofingiensis* under different phosphorus and nitrogen supply; (C). Changes in coloration of *C. zofingiensis* cultures at day 5. Error bars demonstrate the standard deviations from the triplicate experiments (n = 3). At a 95% confidence level, different letters show that there was a remarkable difference between treatments.

4. Conclusions

This study demonstrated that the strategic manipulation of nitrogen and phosphorus availability effectively enhances the dual production of lipids and astaxanthin in *Chromochloris zofingiensis* under photoautotrophic conditions. Nitrogen limitation alone suppressed growth

and photosynthetic pigment biosynthesis but triggered metabolic reprogramming toward storage lipid and carotenoid accumulation. When combined with phosphorus enrichment, particularly at high levels (N_{lim} & P₊₊), this stress condition significantly increased lipid productivity and astaxanthin accumulation, while partially restoring biomass levels. Notably, the highest astaxanthin concentration and lipid productivity were achieved in the

Nlim & P⁺⁺ treatment, suggesting phosphorus can provide the metabolic energy and cofactors necessary to support biosynthesis under nitrogen-deficient conditions. These results confirm that phosphorus repletion under nitrogen stress synergistically improves both volumetric and per-cell yields of target bioproducts. This dual-stimulus strategy provides a promising route for optimizing high-value compound production without severely compromising growth—a common bottleneck in microalgal bioprocesses. The findings contribute to the growing knowledge on nutrient stress responses in microalgae and offer practical insights for scalable applications in biofuel, aquaculture, and pharmaceutical sectors. Future studies should explore long-term cultivation strategies, integrate multi-stage cultivation systems, and apply transcriptomic or metabolomic tools to unravel further the regulatory pathways involved. Overall, this research establishes *C. zofingiensis* as a robust microalgal platform for sustainable biotechnology, bridging the fields of renewable energy and natural antioxidant production.

Acknowledgements

This work was supported by Faculty of Fisheries and Marine Science, Universitas Brawijaya [grant number 121, 2024] (granted to Muhammad Fakhri).

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Identification of taurocyamine compound against histidinol-phosphate aminotransferase of *Mycobacterium tuberculosis* through bioinformatics and molecular docking approaches

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Received: October 17, 2025; Revised: January 17, 2026; Accepted: January 26, 2026

Abstract

Mycobacterium tuberculosis contaminates all types of environments, and it is a biohazard of tuberculosis disease. This concern has provided a strong motivation to search for a natural compound to inhibit the identified target enzyme against that pathogen, which is contaminated in the environment before spreading to other living things and the ecology. The procedure of finding and developing inhibitors begins with target identification. Therefore, the more accurate the identification, the more opportunity for the success of inhibition. Many academic databases and bioinformatics tools have been applied to complete this study via subtractive proteomics, biological networks, and molecular docking approaches. Many databases including Kyoto Encyclopedia of Genes and Genomes (KEGG), the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), the Database of Essential Genes (DEG), the virulence factor database (VFDB), The Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), and The National Center for Biotechnology Information (NCBI) were used. Eventually, according to the subtractive proteomics results, a total of 30 cytoplasmic proteins were obtained. Finally, histidinol-phosphate aminotransferase was identified as a potential target via protein-protein interaction network analysis. Then molecular docking of 12 compounds obtained from the KEGG database was performed against this enzyme target. Only a taurocyamine compound was proposed successfully as the potential candidate based on its binding energy (-6.25 kcal/mol) compared to an original inhibitor (-7.10 kcal/mol). Moreover, the binding patterns, including hydrogen bonds and hydrophobic interactions between the taurocyamine and targeted enzyme were similar to those of the original inhibitor. The results reveal that the taurocyamine compound might be useful as an antibacterial agent in environmental remediation. However, experimental validation needs to be performed. Moreover, the added benefit from this study is that the *in silico* platform could be applied to identify the potent enzyme target and its inhibitor quickly, cheaply, and credibly.

Keywords: Biological network, Bioinhibitor, Comparative proteomics, Subtractive proteomics, Target identification

1. Introduction

The causative agent of tuberculosis disease, also called TB (tuberculosis), in humans is *Mycobacterium tuberculosis*. This bacterium is a large, non-motile, rod-shaped, aerobic, non-spore-forming, acid-fast bacterium, and has a slow generation period (Singh, 2014; Zhang *et al.*, 2022a). Unfortunately, TB has most likely reverted to being the most common infectious agent-related cause of death worldwide (World Health Organization, 2024). *M. tuberculosis* transmission occurs both with and without patient contact, causing the environment to become a source of *M. tuberculosis* infection (Velayati *et al.*, 2015). Moreover, *M. tuberculosis* contaminates many types of environments, including natural environments (e.g., air, soil, and rivers) and others (e.g., wastewater, raw sewage, sewage effluent, sanatorium sewage, inlet and outlet settling tanks, sewage from cattle farms, sewage sediment, dam sediment, and household dust) (Zhang *et al.*, 2022a). Moreover, this pathogen remains virulent in contaminated

environments, especially in soil (Velayati *et al.*, 2015). Therefore, it is necessary to inhibit *M. tuberculosis* to reduce the likelihood of epidemics and transmission to humans.

The greater the accuracy and speed of target identifying, the greater the rate of inhibitor discovery for pathogens contaminating the environment. Generally, in the past, traditional target identification has relied on experimental study, which is costly, time-consuming, and inaccurate (Zhang *et al.*, 2022b). To date, *in silico* strategies have gained prominence and the acceptance of researchers' attention because they enable the identification of possible targets (Melak and Gakkhar, 2015; Chowdhury *et al.*, 2021; Ashraf *et al.*, 2022; Khan *et al.*, 2022; Zhang *et al.*, 2022b; Duguma and Dinka, 2024). Additionally, computational techniques have facilitated the identification of effective targets for such a pathogen (Ashraf *et al.*, 2022).

Among the diversity of *in silico* strategies, comparative analysis is applied to find the unique enzymes of the pathogen by comparing the metabolic pathways of the

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pathogens with the host (Zhang *et al.*, 2022b). Subtractive proteomics analysis is a notable approach to detect novel targets in the pathogen (Chowdhury *et al.*, 2021). It is a powerful concept via sequence subtraction between the host and pathogen proteomes (Ashraf *et al.*, 2022). Moreover, the protein-protein interaction network is also used to identify a hub-bottleneck node, which can be the potential target (Nithaya *et al.*, 2023). The biological network utilized for target identification operates under the assumption that the impact of particular nodes within the network might disseminate along its connections (Zhang *et al.*, 2022b). Generally, the interesting potential targets are based on the following characteristics: 1) an essential protein, 2) a non-homologous protein in the human host, 3) existing in unique metabolic pathways, 4) a cytoplasmic virulent enzyme, and 5) a hub-bottleneck node (Ashraf *et al.*, 2022; Khan *et al.*, 2022; Zhang *et al.*, 2022b; Nithaya *et al.*, 2023). If this enzyme loses function due to inhibition, the pathogen will die. Meanwhile, the two-dimensional (2D) similarity search combined with molecular docking performs well in compound screening (Szilágyi *et al.*, 2021). The 2D similarity search assumes that similar molecules are more likely to have similar biological properties than dissimilar ones (Szilágyi *et al.*, 2021). It is suitable for virtual screening due to its simplicity, ease of interpretation, and reasonably high recovery rate (Szilágyi *et al.*, 2021). The molecular docking is commonly used to calculate the binding affinity between a ligand and receptor in 3D space. Identifying the “best-fit” orientation of a ligand and a specific protein by reducing the system’s free energy helps to predict the molecule’s affinity and efficacy (Abuhamdah *et al.*, 2020).

The objectives in this study are 1) to identify and prioritize the potential target of *M. tuberculosis* H37Rv by a comparative, subtractive proteomics approach and biological network analysis, and 2) to propose a lead compound that could inhibit the potential target by molecular docking.

2. Material and Methods

2.1. Hardware and overall computational methodology

A laptop with an Intel (R) Core™ i7-10750H CPU at 2.60 GHz, 16 GB of RAM, and a 64-bit operating system was used in all steps of this study. Many databases and programs were used in this study (Fig. 1).

2.2. Comparative proteomics analysis

The metabolic pathways of *M. tuberculosis* H37Rv, which is the most studied strain (Melak and Gakkhar, 2015), and *Homo sapiens* were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). The organism codes, including “mtu” and “has”, were used for searching the metabolic pathways of *M. tuberculosis* and *Homo sapiens* (human), respectively. Those metabolic pathways were compared using the Venny 2.1 online tool (Oliveros, 2007). Subsequently, all proteins of each unique metabolic pathway were collected. The repeated proteins were discarded using the Cluster Database at High Identity with Tolerance (CD-HIT) program with cd-hit function at a 60% threshold (Li and Godzik, 2006). Finally, the non-redundant amino acid sequences in the unique metabolic pathways were obtained as dataset-1.

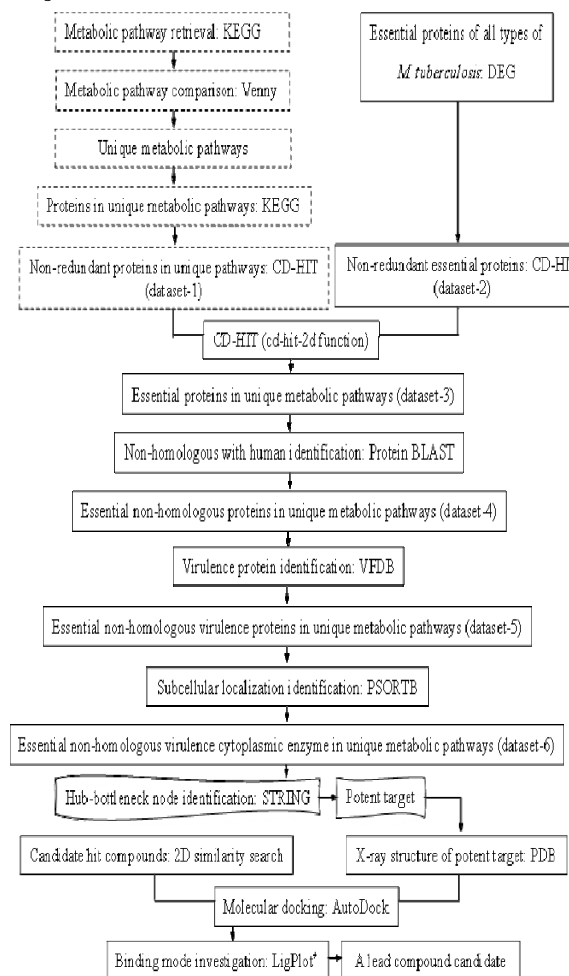


Figure 1. Methodology of the computational experiment. Dash, solid, and sketched boxes represent the comparative, subtractive proteomics, and biological analysis, and molecular docking, respectively.

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2.3. Subtractive proteomics analysis

First, the essential genes of *M. tuberculosis* were downloaded from the Database of Essential Genes (DEG) (Luo *et al.*, 2021). A total of 614, 771, and 687 essential genes in *M. tuberculosis* H37Rv, H37Rv II, and H37Rv III, respectively, were downloaded (Sasseti *et al.*, 2003; Griffin *et al.*, 2011; Zhang *et al.*, 2012). The repeated sequences were then removed by the cd-hit function at a 60% threshold (Li and Godzik, 2006). After that, the non-redundant essential proteins were kept for the next step as dataset-2. Second, the cd-hit-2D function was utilized to compare dataset-1 and dataset-2 at a 100% threshold (Li and Godzik, 2006). This step was executed twice. The essential proteins in the unique metabolic pathways were then obtained as dataset-3. Third, it was subjected to the Basic Local Alignment Search Tool (BLAST) available on the NCBI database using BLASTp with the expectation threshold 10^{-5} against *Homo sapiens* (human) Protein BLAST (Altschul *et al.*, 1990). The amino acid sequences of the essential non-homologous proteins in the unique metabolic pathways were then obtained as dataset-4. Fourth, for identifying the proteins having the highest virulence factor, the shortlisted proteins of dataset-4 were subjected to BLASTp against the virulence factor database

(VFDB) (Liu *et al.*, 2019) by setting the E-value cut-off to 10^{-5} . The essential non-homologous virulent proteins were obtained as dataset-5. Fifth, PSORTb version 3.0.3 was employed to identify the subcellular location. Gram-positive with an outer membrane was chosen, and the return results are cytoplasm, cytoplasmic membrane, periplasm, outer membrane, extracellular space, and unknown (Yu *et al.*, 2010). These areas are critical because proteins are delivered to specific places once released (Ashraf *et al.*, 2022). Finally, the essential non-homologous virulence cytoplasmic enzymes in unique metabolic pathways were obtained as dataset-6.

2.4. Biological network analysis

The protein-protein interaction (PPI) was generated through the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 12.0 (Szklarczyk *et al.*, 2015). Twenty-one cytoplasmic proteins with scores higher than 9.97 of dataset-6 were used to generate the PPI with the *M. tuberculosis*.

2.5. 2D similarity search and molecular docking

The query ligand, 2-(N-morpholino) ethanesulfonic acid (MES), was structurally searched via the SuperNatural_3 (Gallo *et al.*, 2023), the Natural Product Activity and Species Source (NPASS) (Zhao *et al.*, 2023), and the SIMilar COMpound (SIMCOMP) (Hattori *et al.*, 2010). For SIMCOMP, the query ligand was subjected to a target database, including KNApSACk (Afendi *et al.*, 2012) and compounds. Each database used a structure search with the default parameters. The x-ray structure of the enzyme (PDB_ID: 4R8D) was used as a receptor (Nasir *et al.*, 2016), and was visualized by Discovery Studio Visualizer (Biovia, 2024). AutoDock 4.2.6 and AutoDockTools 1.5.7 (Morris *et al.*, 2009) was used for molecular docking. The optimal parameters were screened prior to redocking (data not shown). Redocking was performed so as to calculate the root mean square deviation (RMSD) value. The lower the RMSD value, the smaller the error deviation in the docking (Frimayanti *et al.*, 2021). If this value is less than 2 Å, it means that the method is credible (Shamsian *et al.*, 2024). The grid box size was 40 Å x 40 Å x 40 Å. The grid center was X: 12.762, Y: -4.456, and Z: -15.695. The number of individuals in the population, the maximum number of energy evaluations, and the hybrid Genetic Algorithm with Local Search (GA-LS) runs were set to 300, 2500000, and 50, respectively (Mishra *et al.*, 2023). The other parameters were set to default values. For candidate docking, all compounds having structural similarity with MES were docked in the receptor as well as the redocking technique. For post-docking analysis, enzyme-inhibitor interactions were analyzed by Ligplot⁺ tool (Laskowski and Swindells, 2011).

3. Results and Discussion

3.1. Proteins in unique metabolic pathways

The comparative analysis has now helped in generating a set of proteins existing in the unique metabolic pathways of *M. tuberculosis*. A total of 137 and 360 metabolic pathways were found in *M. tuberculosis* and humans, respectively. Consequently, 51 unique metabolic pathways of *M. tuberculosis* were identified, as shown in Fig. 2 and

Table 1. Because they are absent in humans (Khan *et al.*, 2022), they are important for *M. tuberculosis* in survival and growth. Most of them are involved in the compound biosynthesis and degradation, such as lysine and peptidoglycan biosynthesis, and also benzoate and flavonoid degradation. A total of 1113 proteins of unique pathways were obtained. The 540 redundant proteins were removed. A total of 573 proteins in unique metabolic pathways were identified. They participate in different cellular functions of the pathogen (Duguma and Dinka, 2024).

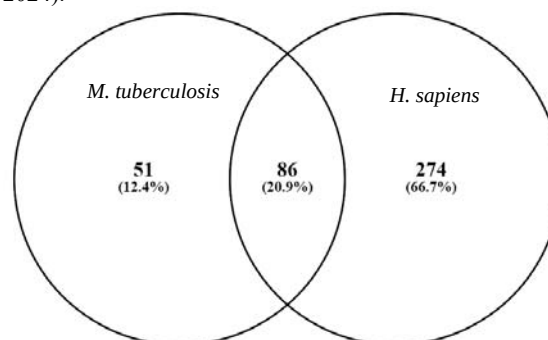


Figure 2. Metabolic pathways between pathogen and human using Venny diagram.

3.2. Subtractive proteomics analysis

Subtractive proteomics analysis has helped in identifying the set of proteins (essential non-homologous virulent enzymes in unique metabolic pathways) that could be a potential target, as shown in Table 2. A total of 1073 proteins were identified as essential proteins. They are required for the survival of *M. tuberculosis*. A total of 315 essential proteins were found in unique metabolic pathways. These proteins are indispensable for the survival and growth of the pathogen without changing the host's metabolic pathways (Melak and Gakkhar, 2015; Khan *et al.*, 2022). A total of 135 essential proteins in unique metabolic pathways showing sequence similarity with humans were removed so as to eliminate the risk of causing human toxicity (Melak and Gakkhar, 2015). Therefore, a total of 180 essential non-homologous proteins were selected. Non-homologous proteins are proteins of the pathogen that have no similar amino acid sequences in humans (Duguma and Dinka, 2024). Furthermore, a total of 50 proteins were identified as essential non-homologous and virulent proteins. Targeting the virulent protein is an interesting technique, because they determine the pathogenesis that enables them to cause infection (Liu *et al.*, 2019).

Among 50 essential non-homologous virulent proteins, thirty of them were found to be cytoplasmic proteins. They are more suitable for inhibitor targets, because the proteins found in the cell membrane are difficult to purify (Duguma and Dinka, 2024). Moreover, cytoplasmic proteins are frequently utilized as therapeutic targets (Khan and Uddin, 2022).

3.3. A hub-bottleneck protein identification

The refined lists of proteins were generated by the PPI network to prioritize potential targets (Fig. 3). The STRING results showed different nodes (proteins) and edges (interactions) of each protein. They also showed that the prioritized target may act as a hub node.

Table 1. List of proteins in unique metabolic pathways of *M. tuberculosis*.

No.	Metabolic pathways name	Pathway_ID	Protein number	No.	Metabolic pathways name	Pathway_ID	Protein number
1	Mycolic acid biosynthesis	mtu00074	36, 28	37	Caprolactam degradation	mtu00930	23, 1
2	Secondary bile acid biosynthesis	mtu00121	1, 1	38	Degradation of flavonoids	mtu00946	1, 0
3	Monobactam biosynthesis	mtu00261	7, 7	39	Steroid degradation	mtu00984	13, 13
4	Lysine biosynthesis	mtu00300	13, 9	40	“Biosynthesis of various plant secondary metabolites”	mtu00999	2, 1
5	Penicillin and cephalosporin biosynthesis	mtu00311	2, 2	41	“Biosynthesis of siderophore nonribosomal peptides”	mtu01053	9, 9
6	Carbapenem biosynthesis	mtu00332	2, 2	42	“Biosynthesis of secondary metabolites”	mtu01110	341, 236
7	Chlorocyclohexane and chlorobenzene degradation	mtu00361	5, 5	43	“Microbial metabolism in diverse environments”	mtu01120	219, 35
8	Benzoate degradation	mtu00362	40, 40	44	Degradation of aromatic compounds	mtu01220	12, 0
9	Fluorobenzoate degradation	mtu00364	2, 0	45	Nitrogen cycle	mtu01310	6, 0
10	Novobiocin biosynthesis	mtu00401	3, 3	46	Beta-Lactam resistance	mtu01501	7, 5
11	Cyanoamino acid metabolism	mtu00460	6, 6	47	Vancomycin resistance	mtu01502	6, 2
12	Streptomycin biosynthesis	mtu00521	11, 11	48	Cationic antimicrobial peptide (CAMP) resistance	mtu01503	2, 2
13	Polyketide sugar unit biosynthesis	mtu00523	7, 1	49	Two-component system	mtu02020	58, 36
14	Acarbose and validamycin biosynthesis	mtu00525	4, 0	50	Quorum sensing	mtu02024	24, 11
15	Lipopolysaccharide biosynthesis	mtu00540	3, 3	51	Bacterial secretion system	mtu03070	14, 5
16	O-Antigen nucleotide sugar biosynthesis	mtu00541	19, 13	Summation of protein			1113, 573
17	O-Antigen repeat unit biosynthesis	mtu00542	1, 1	Remark: Regular fonts represent all proteins. Bold fonts represent the unique protein.			
18	Exopolysaccharide biosynthesis	mtu00543	2, 1	Table 2. Stepwise applied in this study.			
19	Peptidoglycan biosynthesis	mtu00550	17, 15	Step used in this study			<i>M. tuberculosis</i>
20	Teichoic acid biosynthesis	mtu00552	2, 0	Essential proteins from DEG			2072
21	Lipoarabinomannan (LAM) biosynthesis	mtu00571	13, 13	Non-redundant essential proteins			1073
22	Arabinogalactan biosynthesis - Mycobacterium	mtu00572	14, 10	Proteins in unique metabolic pathways			573
23	Dioxin degradation	mtu00621	4, 0	Non-redundant essential proteins in unique metabolic pathways			315
24	Xylene degradation	mtu00622	4, 0	Non-redundant essential non-homologous proteins in unique pathways			180
25	Toluene degradation	mtu00623	2, 0	Virulence proteins			50
26	“Chloroalkane and chloroalkene degradation”	mtu00625	10, 7	Subcellular localization:			
27	“Naphthalene degradation”	mtu00626	4, 0	- Cytoplasmic			30
28	Aminobenzoate degradation	mtu00627	30, 9	- Cytoplasmic membrane			14
29	Styrene degradation	mtu00643	5, 0	- Periplasmic			-
30	“C5-Branched dibasic acid metabolism”	mtu00660	9, 9	- Outer membrane (Cell wall)			-
31	“Methane metabolism”	mtu00680	21, 18	- Extracellular			-
32	“Carbon fixation by Calvin cycle”	mtu00710	11, 0	- Unknown			5
33	“Other carbon fixation pathways”	mtu00720	37, 0	- Unknown (multiple sites)			1
34	Limonene degradation	mtu00903	5, 2	STRING is claimed to be a web resource database dedicated to experimentally known and predicted PPI, including both physical and functional interactions (Melak and Gakkhar, 2015; Khan <i>et al.</i> , 2022). This network composed of 21 nodes (protein) and 15 edges (interaction). The node hisC1 was a hub-bottleneck node, presenting			
35	Carotenoid biosynthesis	mtu00906	1, 1				
36	“Pinene, camphor and geraniol degradation”	mtu00907	23, 0				

high degree and betweenness (Yu *et al.*, 2007; Nithaya *et al.*, 2023). This node is very important to the cell (Yu *et al.*, 2007). Deletion of this node is lethal to the cell, leading to it being a potential target (Nithaya *et al.*, 2023). Furthermore, Raman *et al.* (2008) reported that hisC1 is a potential target of *M. tuberculosis*.

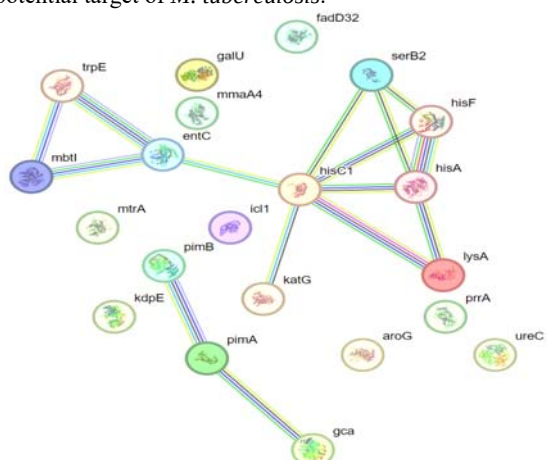


Figure 3. Protein-protein interaction network for 21 proteins by STRING.

3.4. Significance of the opted potential protein (hisC1)

The hisC1 is a histidinol-phosphate aminotransferase (EC: 2.6.1.9). It is classified as required for optimal growth (Lunardi *et al.*, 2013). It is the seventh enzyme in the L-histidine biosynthetic pathway, converting imidazole-acetol-P to L-histidinol-P (Kulis-Horn *et al.*, 2014). Thus, inhibition of this enzyme leads to the blockage of histidine biosynthesis, ultimately disrupting bacterial growth and survival, because histidine is an essential amino acid for cellular processes (Singh *et al.*, 2025). The histidine biosynthesis pathway comprises ten enzymatic steps that convert phosphoribosyl pyrophosphate (PRPP) and ATP to L-histidine, and it appears to be conserved in many living things (Kulis-Horn *et al.*, 2014). Importantly, this pathway is absent in mammals, making it a promising target (Lunardi *et al.*, 2013). In other words, this inhibition is a key strategy for developing novel antibiotics against pathogenic bacteria. Finally, the x-ray structure of this enzyme with PDB_ID: 4R8D has also supported its targetability and use for molecular docking (Fig. 4A and 4B).

A



B

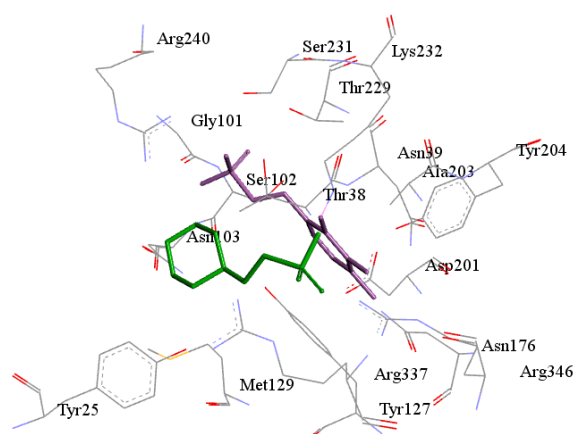


Figure 4. X-ray structure of hisC1 enzyme. (A) Sphere is the binding pocket around the ligand (MES: green) and cofactor (PLP: pink) within a radius of 4 Å. (B) A close-up view of the binding pocket, created with Discovery Studio.

3.5. Molecular docking of 2D similarity compounds

None of the compounds was obtained from NPASS and SuperNatural_3. A total of 12 compounds were obtained from KEGG, showing similarity with the original ligand (MES). Four compounds were obtained from KnapSACk, whereas eight compounds were obtained from the compound target database. The similarity scores were in the range of 0.40-0.60. The RMSD value of redocking was 1.1035 Å (Fig. 5). Thus, this technique is valid and could be implemented in compound docking. The docked screening led to the identification of 12 compounds with binding energies ranging from -2.76 to -6.37 kcal/mol (Table 3). Only the top 5 compounds were selected with binding energies spanning between -5.97 and -6.37 kcal/mol, which was similar to those of redocking (-7.10 kcal/mol). The lower the value of the binding free energy, the stronger the attachment between the ligand and

receptor (Jain *et al.*, 2021). Moreover, the results also revealed that the taurocyamine compound could inhibit this enzyme as well as a specific inhibitor (MES) based on the estimated inhibition constant (K_i) value of 26.18 $\mu\text{mol/L}$ and 6.22 $\mu\text{mol/L}$, respectively. Increased inhibition arises from less dissociation, as evidenced by a lower K_i value (Pandey and Verma, 2020).

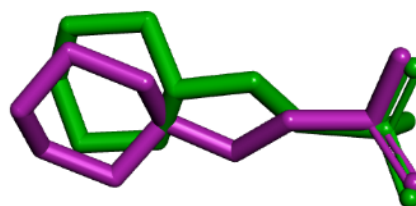


Figure 5. The superimposed co-crystallized MES (in pink) over redock (in green), created by Discovery Studio.

3.6. Post docking analysis

LigPlot⁺ version 2.3.1 was used for binding mode analysis (Laskowski and Swindells, 2011). For redocking complex, the results showed that Tyr25, Thr38, Asn39, Asn103, Tyr127, Met129, Asn176, Arg337, and Arg346 were common residues in the binding pocket (Fig. 6). It showed 6 hydrogen bonds with 5 residues, such as Asn39, Tyr127, Asn176, Arg337, and Arg346. It also displayed hydrophobic interaction with 4 residues, such as Tyr25, Thr38, Asn103, and Met129. These interactions determine the drug-receptor affinity (Sahu *et al.*, 2008). These results are similar to the results of Naris *et al.* (2016). Amino acids involved in binding between the MES ligand with histidinol-phosphate aminotransferase are Tyr25, Thr38, Asn39, Tyr127, Met129, Asn176, Pro260, Tyr261, Arg 337, and Arg346. Moreover, the residues involved in the active site region are Asn103, Tyr127, Met129, Lys232, Arg337, and Arg346 (Nasir *et al.*, 2016).

Table 3. Molecular docking of compounds posing the 2D structure similar to MES.

No.	Compound_ID	Binding energy (kcal/mol)	Ki value (μmol/L)
Lig_1	C00050208	-6.27	25.54
Lig_2	C00048188	-3.85	1500.00
Lig_3	C00000767	-5.42	107.32
Lig_4	C00001530	-5.94	43.88
Lig_5	C01616	-6.37	21.50
Lig_6	C05353	-6.10	33.81
Lig_7	C14452	-3.47	2850.00
Lig_8	C01959	-6.25	26.18
Lig_9	C22977	-5.03	204.14
Lig_10	C06771	-2.76	9410.00
Lig_11	C19283	-4.85	278.49
Lig_12	C00519	-3.95	1280.00
Lig_13	MES (redocking)	-7.10	6.22

Remark: Compounds with bold font were selected for binding pattern analysis.

For hydrogen bonds, the pattern is identical, while hydrophobic interactions are also similar with 3 residues, such as Tyr25, Thr38, and Met129. These results, therefore, also confirmed that the docking technique was valid. Among ligand candidates, only Lig_8 (2-(diaminomethylideneamino) ethanesulfonic acid: taurocyamine) could identically form hydrogen bonds with redocked results (Fig. 7), while the remaining compound could not form an interaction pattern similar to the redocked molecule.

Hydrogen bonds help in proper orientation of the compound, and they are primary contributors to the specificity of molecular recognition to make maximum interaction (Sahu *et al.*, 2008; de Freitas and Schapira, 2017). Moreover, Lig_8 could form hydrophobic interactions with Tyr25, Thr38, and Met129. Hydrophobic binding type is essential in ligand-protein interaction due to their predominant and major contribution (Sahu *et al.*, 2008). Therefore, the taurocyamine was selected as the best candidate.

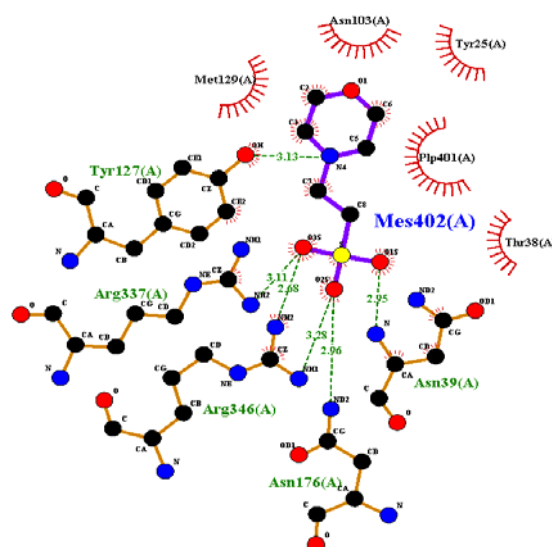


Figure 6. The binding interactions between enzyme and Lig_13. Hydrogen bonds are shown in green dotted lines, whereas hydrophobic interactions are shown in spikes. This figure was created by LigPlot⁺.

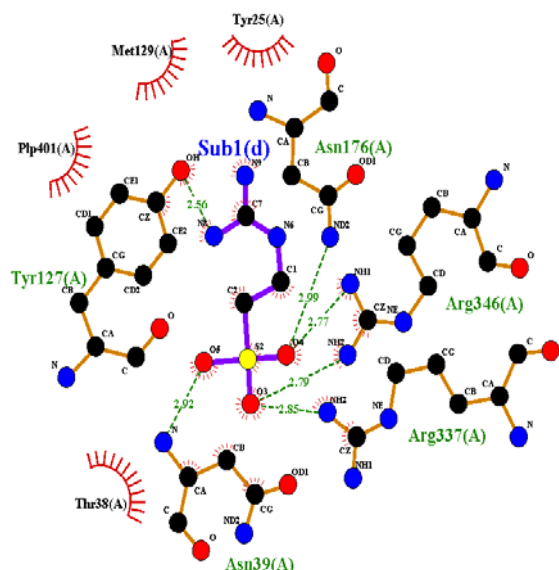


Figure 7. The binding interactions between enzyme and Lig_8. Hydrogen bonds are shown in green dotted lines, whereas hydrophobic interactions are shown in spikes. This figure was created by LigPlot⁺.

The taurocyamine has a molecular formula of $C_3H_9N_3O_3S$ with a molecular weight of 167.19 g/mol based on the KEGG database. It consists of a guanidine group attached to an ethanesulfonic acid molecule. Remarkably, the guanidine is a functional group of antibacterial agents due to hydrogen bond interaction with the target (Kim *et al.*, 2021). Based on the author's knowledge, the taurocyamine is not reported to be an antibacterial agent.

4. Conclusion

In this study, comparative and subtractive proteomics combined with many bioinformatics resources led to obtaining the histidinol-phosphate aminotransferase as a potent enzyme target. Then the taurocyamine compound was discovered as a potent inhibitor. These results may lead to stopping the spread of this pathogen that

contaminates the environment. However, the proposed inhibitor and target need to be studied further in depth and experimentally validated both *in vivo* and *in vitro*. Notably, applying the computational platform from this study may enable swift and economical environmental remediation in the future with the discovery of effective natural compounds against various other pathogens contaminating the environment before they become widespread.

Acknowledgements

The author would like to thank the owner of the academic databases and tools.

Funding

The author declares that no fund was received during the preparation of this manuscript.

Conflicts of interest

The author declares there are no conflicts of interest.

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Biosynthesis and characterization of lipase produced by *Bacillus subtilis* DB104 and its potential in biodiesel production

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Received: October 1, 2025; Revised: December 21, 2025; Accepted: February 6, 2026

Abstract

Lipases hydrolyse fats into glycerol and free fatty acids and extensively used in various industries, including food, pharmaceuticals, biodiesel production, and detergents. The present study aimed to optimize fermentation conditions for lipase production by *Bacillus subtilis* DB104 in shake flask cultivations using one factor at a time (OFAT) method. The optimal conditions identified included the supplementation of basal salt medium with 0.5% waste cooking oil (WCO) and 15 mM ammonium nitrate at an initial medium pH 7.0 and resulted in the highest lipase activities of 8.97 U/mL. In enzyme stability tests, the lipase exhibited optimal activity at pH 7.0 and 37°C. Metal ions, CaCl₂ and MnCl₂, enhanced lipase activity, as did non-ionic detergents such as Tween 80, and Tween 20. Lipase produced retained its stability (>70%) in the presence of 1% inhibitors like EDTA, and PMSF, showing its robustness. Also, it maintained full activity (100%) in 1% of isopropanol, DMSO, and hexane. SDS-PAGE and Zymogram analyses showed the molecular weight of lipase obtained was in the range from 33-35 kDA. Lipase catalysed- transesterification of WCO has resulted in FAME conversion of 68.8%. Further production and optimization in a bigger scale fermenter and lipase purification are ongoing to meet the mass biodiesel production demand in Indonesia.

Keywords; *Bacillus subtilis* DB104, biodiesel, fatty acid methyl ester, lipase production, optimization.

1. Introduction

Indonesia, a country rich in natural resources, has seen a significant rise in its biodiesel production over the past decade. This increase has been driven by government policies, the growing demand for renewable energy, and efforts to reduce greenhouse gas emissions (Wirawan *et al.* 2024). Since the implementation of the B30 program in 2020, which requires a 30% blend of biodiesel in diesel fuel, this program has encouraged domestic biodiesel use and decreasing the country's reliance on fossil fuel imports (Mokhtar *et al.* 2023). Indonesia has set renewable energy goals by aiming for 23% of its energy must come from renewable sources by 2025 (Budiarto *et al.* 2021; Erdiwansyah *et al.* 2021). As one of the world's leading producers of palm oil, Indonesia has steadily increased its biodiesel production capacity (Purwoko and Wibowo, 2020). The government's consideration of adopting even higher biodiesel blends, such as B40 and possibly B50, shows the commitment to further expanding the biodiesel sector.

Biodiesel production from lipase-catalyzed process has attracted attention due to the enzyme's effectiveness in facilitating the transesterification process, which converts fats and oils into biodiesel (Wancura *et al.* 2023). Various

feedstocks, such as non-edible oils (Zambare *et al.* 2021), waste cooking oils (Alshehri *et al.* 2024), and microalgae for biodiesel synthesis (Fattah *et al.* 2020) are gaining popularity suiting circular bioeconomy concepts. Lipase-catalyzed transesterification works well for feedstocks with high free fatty acid content, which can be challenging for chemical catalysts (Wancura *et al.* 2023; Alshehri *et al.* 2024; Akhter *et al.* 2022). The use of lipases was reported to be able to decrease the need for energy-intensive processes and harsh chemicals, which may lower production costs. Enzyme-catalyzed methods are also seen as more environmentally friendly, producing fewer by-products and waste compared to traditional chemical methods (Ali *et al.* 2023).

Lipase production from waste cooking oil (WCO) by *Bacillus* species has gained considerable research interest due to the enzyme's effectiveness in biocatalytic processes and the chance to use sustainable waste materials (Binhayeeding *et al.* 2020). Using WCO as a feedstock for lipase production may address environmental issues related to waste disposal and offers a cost-effective option for enzyme production (Akhter *et al.* 2022). Various pretreatment methods to remove impurities and enhance the suitability of waste cooking oil for microbial fermentation have been reported. Several *Bacillus* strains with exceptional lipase production have been isolated from

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different environments (Suci *et al.* 2018; Akhter *et al.* 2022; Khattab *et al.* 2022).

Improving the operational stability of *Bacillus* lipases under industrial settings remains a big challenge. Transitioning laboratory-scale achievements to large-scale production necessitates overcoming various technical and economic hurdles. Research on optimizing fermentation conditions such as pH, temperature, nutrient composition, and aeration, to maximize lipase yields are progressing (Suci *et al.* 2018; Akhter *et al.* 2022; Moschona *et al.* 2024). Current research is aiming to develop more robust enzymes through genetic modifications and immobilization techniques. Optimizing fermentation conditions and utilizing low-cost substrates are also among the initiatives to reduce production costs (Nawani *et al.* 2006).

Due to the problems or issues stated above, the present study aimed to optimize the lipase production using WCO under different cultivation conditions using one factor at a time (OFAT) by *B. subtilis* DB104. Partially purified lipase was characterized, and the stability of lipase was tested in terms of thermal stability; substrate specificity was carried out. Potential application of lipase produced by *B. subtilis* DB104 from WCO for biodiesel conversion was also reported in the present work.

2. Materials and Methods

2.1. Preparation of bacterial cell culture

B. subtilis DB104 was obtained from the Indonesian National Research and Innovation Agency (BRIN) Culture Collection and stored as a glycerol stock at -80 °C. This strain was then streaked onto a nutrient agar (NA) plate and incubated at 37 °C for 24 h. To prepare the seed culture, a single colony of *B. subtilis* DB104 was inoculated into a sterile test tube containing 5 mL of nutrient broth (NB) and incubated at 37 °C, 170 rpm for 15-16 h or until reaching OD 0.6-0.8 measured using UV spectrophotometer at 600 nm.

2.2. Medium and fermentation conditions for lipase production

Mineral salt media (MSM) containing following components per liter of distilled water: NH_4Cl (2.0 g/L), Na_2HPO_4 (6.0 g/L), MgSO_4 (0.1 g/L), NaCl (5.0 g/L), KH_2PO_4 (3.0 g/L) was prepared as proposed by Zhang *et al.* (2012). The MSM was autoclaved at 121°C for 20 min, separately from the carbon source prior to a 10% inoculum addition to media. For the optimization study, *B. subtilis* DB104 was incubated under different conditions, including temperature, initial medium pH, concentrations of carbon sources, nitrogen sources, and concentrations of the chosen nitrogen source, for 48 h with an agitation speed at 170 rpm.

2.3. Optimization conditions for lipase production

2.3.1. Type of carbon sources

Various carbon sources (glucose, glycerol, WCO, palm cooking oil, and soybean oil) at a 1% concentration were evaluated. *B. subtilis* DB104 was inoculated into MSM containing each carbon source, and the initial pH of the medium was adjusted to 7.0 using acid or base. The

cultures were incubated at 37 °C for 48 h with agitation at 170 rpm.

2.3.2. Concentrations of carbon sources

The optimal carbon source, WCO, identified in the experiment described in section 2.3.2 was further investigated for its concentrations on the growth and lipase production by *B. subtilis* DB104. The cultures were supplemented with 0.5-2.0% of WCO, while only MSM served as the control. The initial medium pH was adjusted to 7.0 using an acid or base. All cultures were incubated at 37 °C for 48 h with an agitation speed at 170 rpm.

2.3.3. Type of nitrogen sources

The cultures were supplemented with 0.5% WCO and different nitrogen sources (ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium sulphate, peptone, yeast extract and urea) at a concentration of 15 mM. The initial medium pH was set at 7.0, and the cultures were incubated at 37 °C for 48 h with agitation speed at 170 rpm.

2.3.4. Concentrations of NH_4NO_3

The cultures were cultivated in MSM containing 0.5% WCO and NH_4NO_3 at varying concentrations from 15 to 100 mM. This experiment was performed to investigate the effects of different NH_4NO_3 concentrations on the growth and lipase production by *B. subtilis* DB104. The initial medium pH was adjusted to 7.0 using either 1M NaOH or 1M HCl, and the cultures were incubated at 37 °C for 48 h with agitation at 170 rpm.

2.3.5. Initial medium pH

B. subtilis DB104 was cultivated in an optimized MSM supplemented with 0.5% WCO, 15 mM of NH_4NO_3 and the initial medium pH was adjusted to the selected pH range of 6.5 to 8.5 using either 1M NaOH or 1M HCl. The culture was then incubated at 37°C for 48 h with agitation at 170 rpm.

2.4. Analyses

2.4.1. Extraction of crude enzyme

The crude lipase was extracted from the supernatant of the fermentation broth. The fermentation mixture was centrifuged at 12,000 rpm at 4 °C for 5 min to separate the enzyme-containing supernatant (Falony *et al.* 2006).

2.4.2. Partial purification using acetone precipitation

Protein precipitation using acetone was performed according to the method described by Ewelina *et al.* (2010) with slight modifications. The crude enzyme extract was gradually mixed with chilled acetone at a ratio of 2:1 (acetone:enzyme, v/v) and incubated at -20 °C overnight. The mixture was then centrifuged at 10,000 rpm for 20 min. The resulting precipitate was allowed to air-dry for 20 min to remove residual acetone and subsequently resuspended in phosphate buffer.

2.4.3. Lipase activity assay

A standard titrimetric method was employed to assay lipase activity. The substrate for the enzyme assay was prepared by emulsifying olive oil with Arabic gum in 5 mL of phosphate buffer at pH 7 and 2 mL of 0.6% CaCl_2 solution. During estimation, 500 μL of the crude enzyme extract was added, and the substrate-enzyme mixture was

incubated in a rotary shaker at 150 rpm and 30°C for 15 min. A solution containing 3 mL of ethanol and 3 mL of acetone was used to terminate the reaction and extract the fatty acids. Phenolphthalein was used as an indicator dye during the titration of the fatty acids with 0.1M NaOH at pH 10.5. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 µmol of fatty acids per minute from the triglyceride substrate (tributyrin) (Kojima *et al.* 2003).

2.4.4. Lipase activity test

The lipase activity was measured using a spectrophotometric assay with *p*-nitrophenyl palmitate as the substrate (Silva *et al.* 2005). The substrate was prepared by mixing two solutions: Solution A containing 30 mg of *p*-nitrophenyl palmitate in 10 mL of isopropanol, and Solution B containing 100 mg of gum Arabic and 400 mg of Triton X-100 in 90 mL of 50 mM Tris-HCl buffer. The substrate was created by combining these two solutions evenly. Next, 100 µL of lipase was mixed with 900 µL of the substrate. Blank samples were prepared by replacing the lipase with 50 mM Tris-HCl buffer. The lipase-substrate and blank mixtures were incubated for 30 min at 37°C in a water bath shaker. Then, the absorbance was then measured using a UV-Vis spectrophotometer at a wavelength of 410 nm. The enzyme activity was calculated as the amount of *p*-nitrophenol released enzymatically from the substrate per minute. The absorption measurement results were converted into the standard curve equation of *p*-nitrophenol, which was used to determine the lipase activity.

2.4.5. Protein content

Protein content of *B. subtilis* DB 104 was determined using Bradford assay (Bradford, 1976) with slight modification. Protein concentrations were measured by adding a 1:50 dilution of the sample to the Bradford reagent, incubating for 30 min, and the absorbance was recorded at 595 nm. The specific activity was then calculated by dividing the enzyme activity by the measured lipase protein content.

$$\text{Specific activity} = \frac{\text{Lipase activity} \left(\frac{\text{U}}{\text{ml}} \right)}{\text{Protein content} \left(\frac{\text{mg}}{\text{ml}} \right)}$$

2.5. Lipase stability tests

2.5.1. pH and temperature

The experiment on the effect of pH and temperature on lipase stability was performed according to method proposed by Lu *et al.* (2010). The effect of pH on lipase activity was carried out in the pH range from pH 5 – pH 10 using following buffers; pH 5 (acetate); pH 6-8 (phosphate); and pH 9-10 (Glycine-NaOH).

Meanwhile, the effect of temperature on lipase stability was determined at 35 °C, 37 °C, 40 °C, 45 °C, and 50 °C.

2.5.2. Effect of ions

The effect of several ions on lipase stability was determined by measuring lipase activity after incubation for 30 min at optimum temperature and pH in a buffer containing 5 mM of CaCl₂, MgCl₂, ZnCl₂, MnCl₂, FeCl₃, KCl, and NaCl (Kotogan *et al.* 2018).

2.5.3. Effect of detergents

The effect of different detergents on lipase stability was determined by measuring lipase activity after incubation for 30 min at optimum temperature and pH in a buffer containing 1% (w/v) of SDS, Triton X, Tween 80 and Tween 20 (Guncheva *et al.* 2007).

2.5.4. Effect of inhibitors and activators

The influence of inhibitors and activators on lipase stability was determined by measuring lipase activity after incubation for 30 min at optimum temperature and pH in a buffer containing 1% of DTT (Dithiotreitol), PMSF (Phenyl Methyl Sulfonyl Fluoride), β Mercaptoethanol and EDTA (Ethylene Diamine Tetra Acetic Acid) (Kumar *et al.* 2020).

2.5.5. Effect of organic solvents

The effect of organic solvents on lipase stability was investigated by measuring the lipase's catalytic performance following a 30 min incubation at the optimal temperature and pH in the presence of 1% (v/v) of DMSO, methanol, chloroform, n-hexane, isopropanol, and acetone (Vahidi *et al.* 2020).

2.5.6. SDS Page and Zymogram

SDS-PAGE was conducted according to protocol suggested by Laemmli (1970) using 12% polyacrylamide gels. After electrophoresis, the gel was stained with Coomassie brilliant blue. Subsequently, the proteins were renatured by incubating the gel in a renaturation buffer (0.05 mM phosphate buffer (pH 7) and 2.5% Triton X-100) for 30 min. The gel was kept at 4°C for 15 min and then soaked in 0.1% Congo red for 30 min. Then, the gel was soaked in 1 M NaCl until the bands became visible, with the lipolytic activity shown as a red band (Bora and Bora, 2012).

2.5.7. Biodiesel production

Biodiesel production via enzymatic route was carried out by reacting WCO with methanol at a molar ratio of 1:5 and lipase amounting of 10% (v/v) relative to the WCO was added into the mixture. The reaction was conducted at 40°C with continuous stirring at 500 rpm for 24 h. The samples were centrifuged at 12,000 rpm for 5 min at 15°C to obtain the upper layer and solution from this layer was then used to determine the percentage and composition of biodiesel produced from the synthesis reaction (Husar *et al.* 2021).

2.5.8. Analysis of biodiesel content

The biodiesel content of fatty acid methyl ester (FAME) was determined using gas chromatography (GC-17A, Shimadzu, Japan) with a flame ionization detector (GC-FID). The GC unit was equipped with a Zb-5TH column (0.25 mm x 15 m) along with an auto sampler. The injector and detector temperatures were set at 140°C and 280°C, respectively, and helium gas was used as the carrier at a flow rate of 35 mL/min. The FAME composition for each reaction was determined by peak area normalization and calculating as a percentage through comparison with authentic FAME standards including methyl myristate, methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate. Quantification was further supported by the use of standards comprising methyl myristate, methyl

palmitate, methyl stearate, arachidate, behenate, tetracosanoate, hexacosanoate, octacosanoate, and triacontanoate.

3. Results and Discussion

3.1. Optimization for lipase production

3.1.1. Effect of carbon sources

Using waste cooking oil (WCO) as a substrate greatly influenced lipase production, which is an enzyme that helps break down fats. *B. subtilis* DB104, achieved the highest lipase activity at 4.49 U/mL when using WCO as a substrate. In comparison, soybean oil produced 3.37 U/mL and palm oil resulted in 3.17 U/mL (Table 1).

Table 1 Effect of carbon sources on the growth and lipase production by *B. subtilis* DB 104.

Carbon sources	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
Glucose	1.02 ± 0.07	0.03 ± 0.00	0.42 ± 0.02
Glycerol	0.13 ± 0.01	0.06 ± 0.00	1.42 ± 0.00
Waste cooking oil	1.34 ± 0.01	0.07 ± 0.00	4.49 ± 0.01
Palm cooking oil	0.75 ± 0.06	0.07 ± 0.00	3.17 ± 0.15
Soybean cooking oil	0.52 ± 0.03	0.14 ± 0.00	3.37 ± 0.06

Lower lipase activity was obtained when the culture media was supplemented with glucose and glycerol, with activity recorded at 0.42 U/mL and 1.42 U/mL, respectively. A substantial increase in lipase activity with lipid carbon sources (WCO, palm and soybean cooking oil) is likely due to the availability of fatty acids during growth on these compounds. Similarly, Carvalho *et al.* (2022) also found the lipase production from fatty acid esters is stronger than that observed with glucose or other non-lipid substrates. *Bacillus* species was reported to be efficiently utilized WCO, with high lipase yields (Fattah *et al.* 2020; Carvalho *et al.* 2022). The present work shows the triglycerides and free fatty acids found in WCO was an ideal substrate for lipase induction, promoting increased enzyme production.

3.1.2. Effect of WCO concentration

Mineral salt medium with supplementation of 0.5% WCO showed the best growth and lipase activity, measuring 1.67 OD and 5.35 U/mL, respectively (Table 2). Similar results were obtained from a study reported by Baltaci *et al.* (2019), which also found the highest lipase activity was obtained at 0.5% WCO as a substrate. WCO is a cost-effective substrate because it contains free fatty acids, glycerides, and various impurities that *B. subtilis* DB104 can use as a carbon source.

Table 2 Effect of WCO concentrations on the growth and lipase production by *B. subtilis* DB 104.

WCO concentration(%)	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
0	0.29 ± 0.08	0.02 ± 0.00	0.40 ± 0.01
0.5	1.67 ± 0.07	0.08 ± 0.02	5.35 ± 0.17
1.0	1.62 ± 0.08	0.13 ± 0.02	4.31 ± 0.27
1.5	0.65 ± 0.13	0.05 ± 0.00	1.19 ± 0.06
2	0.59 ± 0.04	0.04 ± 0.00	1.16 ± 0.06

Using WCO as a carbon source in fermentation process is a good choice as it helps in waste management and turning a waste product into useful biological products. The excess supply of WCO in the fermentation medium may result in reduction of microbial growth and lipase activity. The decrease in growth and lipase activity can be explained due to the buildup of toxic by-products, feedback inhibition caused by free fatty acids released during hydrolysis, and impurities that may inhibit microbial growth or lipase activity (Khodadai *et al.* 2020). Furthermore, high WCO concentrations can create a viscous environment that hinders oxygen transfer in aerobic fermentation processes (Ismail *et al.* 2018).

3.1.3. Effect of nitrogen sources

Types and concentration of nitrogen are necessary for microbial growth and lipase production, though its optimal concentration varies with the microbial strain and fermentation conditions (Al Mohaini *et al.* 2022). *B. subtilis* DB104 produced the highest lipase at 8.97 U/mL when using NH_4NO_3 as the nitrogen source and followed by other nitrogen sources ($\text{NH}_4)_2\text{SO}_4$ and NH_4Cl at 8.63 U/mL and 7.51 U/mL, respectively (Table 3). Inorganic nitrogen sources are preferable for lipase production than organic ones. Similar findings were reported by Akter *et al.* (2022) and Ali *et al.* (2023), which found that no growth was observed with yeast extract in *B. cereus* NC7401. NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ are fully soluble in water and readily available for microbial growth, and high ionic strength of ammonium ions from these sources help protein synthesis by being easily incorporated into amino acids (Ismail *et al.* 2018). However, it can be inferred that the nitrogen sources significantly affect lipase production, and the optimal nitrogen concentrations vary by species.

Table 3 Effect of nitrogen sources on the growth and lipase production by *B. subtilis* DB104.

Nitrogen sources (15 mM)	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
Ammonium chloride	1.43 ± 0.11	0.32 ± 0.00	7.51 ± 0.25
Ammonium sulfate	5.34 ± 0.05	0.11 ± 0.01	8.63 ± 0.31
Ammonium nitrate	5.03 ± 0.14	0.13 ± 0.01	8.97 ± 0.42
Urea	1.89 ± 0.16	0.19 ± 0.14	6.10 ± 0.07
Peptone	1.33 ± 0.28	0.53 ± 0.03	6.81 ± 0.21
Yeast extract	5.35 ± 0.16	0.42 ± 0.02	5.59 ± 0.40

3.1.4. Effect of NH_4NO_3 concentrations

Table 4 Effect of NH_4NO_3 concentrations on the growth and lipase production by *B. subtilis* DB104 using WCO.

$(\text{NH}_4)_2\text{NO}_3$ (mM)	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
0	1.45 ± 0.11	0.21 ± 0.05	1.40 ± 0.11
15	4.96 ± 0.15	0.27 ± 0.02	8.94 ± 0.21
25	1.80 ± 0.14	0.23 ± 0.03	8.50 ± 0.70
35	0.99 ± 0.17	0.14 ± 0.03	7.88 ± 0.55
45	1.35 ± 0.06	0.13 ± 0.03	7.57 ± 0.11

MSM was supplemented with NH_4NO_3 at concentrations from 0 to 45 mM after identifying the optimal nitrogen source for growth and lipase production (Table 4). The best growth and lipase activity was obtained at 15 mM NH_4NO_3 , with values of 4.96 OD and 8.94 U/mL. Sufficient nitrogen is essential for synthesizing amino acids, nucleotides, and other cellular components, leading to strong microbial growth and metabolism, which can boost lipase production (Pham *et al.* 2021). However, increasing nitrogen levels further reduced growth and resulted in a plateau for lipase activity. Akhter *et al.* (2022) reported that high nitrogen levels may suppress the production of lipase, by shifting microbial metabolism to primary growth rather than secondary processes like lipase production. Thus, the present study suggests that finding the right nitrogen concentration is important for ensuring the growth of *B. subtilis* DB104 and balanced metabolism, ultimately promoting more lipase production. Obtaining the right nitrogen source, type of nitrogen used, carbon/nitrogen ratio, and overall nutrient balance are key to maximizing lipase production in fermentation.

3.1.5. Effect of initial medium pH

Table 5 Effect of initial medium pH on the growth and lipase production by *B. subtilis* DB 104 using WCO.

Initial medium pH	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
6.5	1.27 ± 0.31	0.55 ± 0.18	3.28 ± 0.11
7.0	4.97 ± 0.14	1.12 ± 0.10	8.55 ± 0.22
7.5	3.09 ± 0.14	0.79 ± 0.12	8.55 ± 0.22
8.0	1.35 ± 0.34	0.63 ± 0.13	3.66 ± 0.22
8.5	1.00 ± 0.34	0.42 ± 0.02	2.99 ± 0.22

The highest lipase activity, 8.55 U/mL, was obtained when the culture grew at initial medium pH 7.0. Lipase activity varied significantly across different pH levels (pH 6.5 to 8.5) with lipase activity recorded from 2.99 U/mL to 8.55 U/mL (Table 5). *B. subtilis* PCSIRNL-39 was reported to produce the highest lipase at neutral pH (Mazhar *et al.*, 2017). In other reports, *Bacillus* sp. LBN2 achieved maximum lipase production within the alkaline range of 8.0 to 10.0 (Bora and Bora, 2012). Obtaining an optimal pH is vital for nutrient absorption, bacterial growth, and boosting enzyme production through signaling pathways, including the release of extracellular enzymes via proteolytic mechanisms (Ismail *et al.* 2018). Maintaining the right pH throughout cultivation process is essential for maximizing lipase yield and often needs strain-specific optimization and careful control during fermentation (Ebrahimipour *et al.* 2017).

3.1.6. Lipase stability under selected conditions pH

Lipase was very sensitive to pH changes. *B. subtilis* DB104 lipase work best at pH 7 and 8 with lipase activity of 9.03 U/mL and 8.36 U/mL, respectively (Fig. 1). Outside this range, the enzyme's activity dropped significantly from 9.02 U/mL to 7.39 U/mL and 7.29 U/mL at pH 5 and pH 10, respectively. This can be explained in that pH affects the ionization state of amino acid residues at the active site and the overall shape of the enzyme protein, impacting its ability to catalyze reactions (Di Russo *et al.* 2012). Maintaining high enzyme activity and stability all the time with suitable pH range is important, especially during processes like enzymatic transesterification for biodiesel production (Moschona *et al.* 2024).

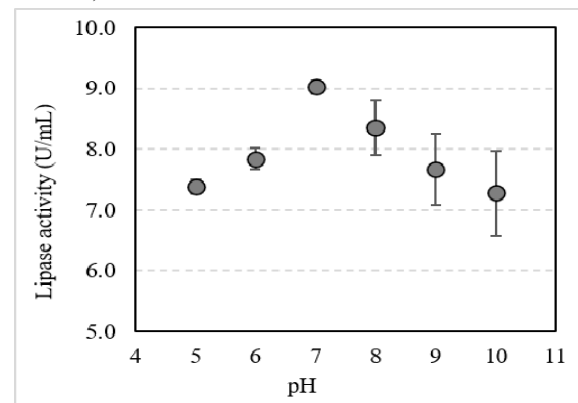


Figure 1 Comparison of Lipase activity of *B. subtilis* DB 104 under different pH

Temperature

B. subtilis DB104 strain had optimal lipase activity at a temperature of 37 °C (Fig. 2). Gradual decrease in lipase activity was observed when lipase was exposed to higher temperature (up to 50 °C). This can be explained in that when temperature increased, the molecules gained more kinetic energy, which resulted in more frequent collisions between the enzyme and substrate molecules (Ali *et al.* 2023), and eventually enzyme reached its optimal temperature. However, going beyond this temperature caused a sharp decrease in the enzyme's activity due to thermal inactivation. High temperatures could break essential hydrogen bonds, ionic bonds, and hydrophobic interactions that keep the enzyme's three-dimensional structure intact (Kumar *et al.* 2020).

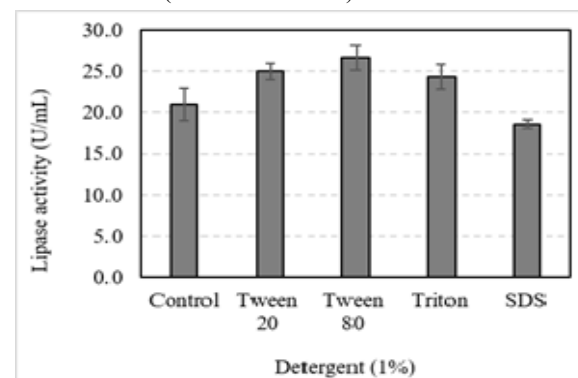


Figure 2 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different detergents

Effect of metal ions

Some metal ions acted as cofactors, enhancing enzyme activity, while others inhibited or destabilized the enzyme. This study showed that NaCl, KCl, FeCl₃, MnCl₂, ZnCl₂, MgSO₄, and CaCl₂, all at a concentration of 5 mM, increased lipase activity (Fig. 3). The ranking of lipase activity from highest to lowest was: CaCl₂ > MnCl₂ > Control > ZnCl₂ > NaCl > KCl > MgSO₄ > FeCl₃. Neha *et al.* (2023) reported that CaCl₂ boosted lipase activity, with activity increasing as CaCl₂ concentrations rose. Other metal ions like Ca²⁺ and Mg²⁺ are known to enhance lipase activity by stabilizing the enzyme structure or taking part in catalytic processes (Lu *et al.* 2018). These ions helped maintain the enzyme's shape, making it more effective in catalysis. On the other hand, heavy metal ions like Cu²⁺ and Zn²⁺ inhibited lipase activity by binding to the enzyme and disrupting its active site, which decreased its ability to catalyze reactions (Pham *et al.* 2021). However, the concentration of metal ions, microbial source of lipases, and the pH of the environment might influence the activity and stability of lipase.

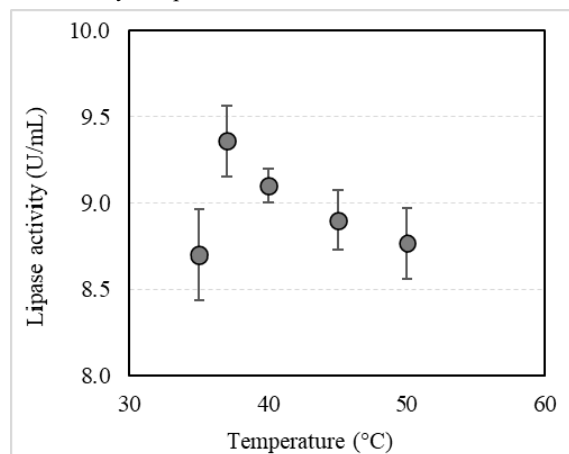


Figure 3 Comparison of Lipase activity of *B. subtilis* DB 104 under different temperature

Effect of detergents

Detergents can act either as inhibitors or activators, depending on their concentration and type. Non-ionic detergents like Tween 80, Tween 20, and Triton X-100 affected lipase activity, with values of 26.67 U/mL, 25 U/mL, and 24.33 U/mL, respectively (Fig. 4). Paz *et al.* (2023) reported that non-ionic detergents such as Triton X-100 and Tween 20 might boost lipase activity at low concentrations by improving substrate solubility or changing the enzyme's shape favorably. Also, the increase in lipase activity could result from higher availability of substrates (oil) or the detergent acting as an emulsifier to boost substrate uptake for lipase production (Al Mohaini *et al.* 2022). Lipase of *B. subtilis* DB104, when treated with SDS, showed lower activity but maintained at least 90% of its activity compared to the control. However, SDS was often reported as strong detergent and anionic surfactant to denature proteins that lead to a loss of enzyme activity (Ali *et al.* 2023).

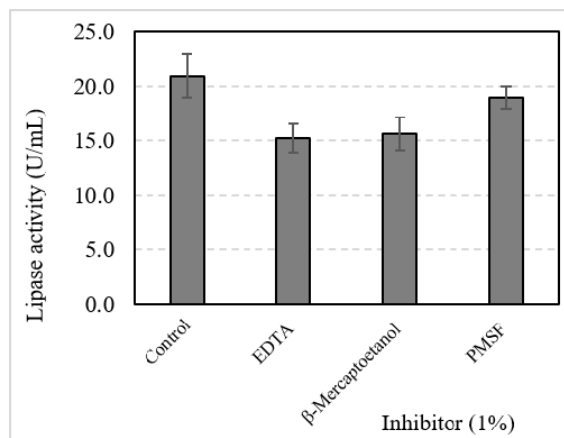


Figure 4 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different inhibitors

Effect of inhibitors

Various inhibitors were tested on lipase from *B. subtilis* DB104 with the aim to provide insight into enzyme regulation and applications. It was observed that the lipase treated with 1% concentrations of EDTA, β-mercaptoethanol, and PMSF retained at least 75-90% of its activity (Fig. 5). The highest inhibition on lipase activity was observed from EDTA (15 U/mL) since EDTA is a chelating agent and might sequester essential metal ions needed for the enzyme to function (Ali *et al.* 2023). PMSF, on the other hand, a serine protease inhibitor, might target serine residues at the active site and eventually reduce enzyme activity (Pham *et al.* 2021). Overall, the resistance of *B. subtilis* DB104 lipase shown in the present study towards these inhibitors suggests its potential use in various industrial applications.

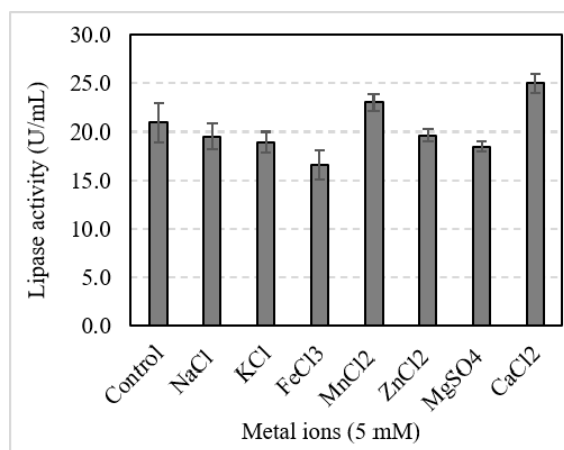


Figure 5 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different metal ions

Effect of organic solvents

Lipase from *B. subtilis* DB104 maintained 100% of its activity after treatment with 1% concentrations of isopropanol, DMSO, n-hexane, methanol, and DMSO (Fig. 6). However, its activity was reduced (18 U/mL and 19 U/mL) after exposure to acetone and chloroform, respectively might be due to the disruption of the lipase structure or unfavorable interactions with the active site. Organic solvents are often used in biocatalysis but can affect enzymatic activity and stability in various ways. Moschona *et al.* (2024) and Lotti *et al.* (2015) reported that

lipase activity can be boosted by low concentrations of methanol and ethanol since these solvents may increase substrate solubility or favorably altering the enzyme's shape. Ismail *et al.* (2018) reported that the presence of hydrophobic solvents like toluene and hexane at high concentrations might reduce enzyme activity due to the denaturation or partial unfolding of the enzyme. Water-immiscible solvents can hinder enzyme activity by limiting their shape, but adding water to such solvents can significantly enhance enzymatic activity in enzyme suspensions (Ali *et al.* 2023).

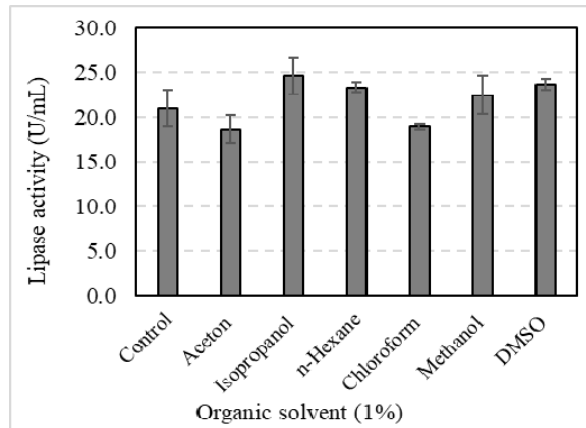


Figure 2 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different organic solvent

3.2. SDS-PAGE and Zymogram

SDS-PAGE analysis of *B. subtilis* DB104 lipase revealed a protein band profile with molecular weights ranging from about 15 to 180 kDa and several thick protein bands appeared at 33 and 65 kDa (Fig. 2). Lipases from various *Bacillus* species were reported to have specific bands with high lipolytic activity around 35 kDa (Ali *et al.* 2023; Paz *et al.* 2023). Same molecular weight of 35 kDa was observed from *B. pumilus* V1 lipase, using fructose and yeast extract as carbon and nitrogen sources (Kandasamy *et al.* 2023). A slightly higher molecular weight lipase (38 kDa) was obtained from *B. halotolerans*

VSH 09 when grown on olive oil for 72 hours (Mahnashi *et al.* 2022). *B. cereus* ATA179 and *B. safensis* produced greater molecular weights of 47 kDa and 55 kDa respectively when grown on sucrose and olive oil (Demirkan *et al.* 2021; Patel *et al.* 2022). From this study, it can be concluded that the molecular weight of lipases produced from *Bacillus* sp. is species dependent and heavily depending on substrate and other cultivation factors.

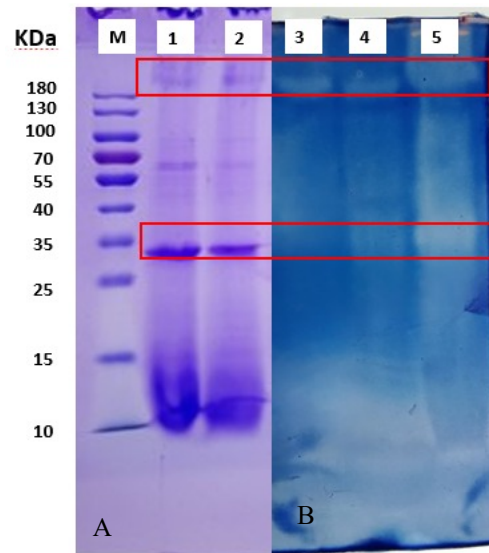


Figure. 7. SDS-PAGE protein profiling (A) and Zymogram lipolytic detection (B) of lipase produced by *B. subtilis* DB104 using WCO.

Table 6 summarizes recent lipase production from selected *Bacillus* species under specific fermentation conditions. It is worth noting that lipase production depended on the species and relied heavily on certain growth and nutrient needs. The growth requirements and lipase activity from this study were also compared with other *B. subtilis* species (Nadaf *et al.* 2020; Soeka *et al.* 2020; Kaur *et al.* 2024).

Table 6 Lipase-producing *Bacillus* species under optimal fermentation conditions.

No.	Microorganism	Fermentation conditions	Lipase activity	References
1.	<i>B. subtilis</i> DB104	Substrate: WCO Nitrogen: 15 mM (NH ₄) ₂ SO ₄ pH: 7.0 Temperature: 37 °C Fermentation: Submerge SDS-PAGE: 33-35 kDA Duration: 48 h	8.94 U/mL 21.0 U/mL (partial purified)	This study
2.	<i>B. subtilis</i> KUBT4	Substrate: 1% sucrose Nitrogen: 1% tryptone pH: 7.5 Temperature: 36 °C Fermentation: Submerge Duration: 48 h	46-49 U/mL	Nadaf and Thimmappa (2020)
3.	<i>B. subtilis</i> C2	Substrate: 0.1% olive oil Nitrogen: pH: 9.0 Temperature: 30 °C Fermentation: Submerge Duration: 120 h	1.76 umol/ mL	Soeka and Sulistiani (2020)
4.	<i>B. subtilis</i> TTP-06	Substrate: 2.75% glucose Nitrogen: 1.6% peptone pH: 7.5 Temperature: 30 °C Fermentation: Submerge Duration: 24 h	8.89 U/mL	Kaur and Gupta (2024)
5.	<i>B. subtilis</i> B-1-4	Substrate: WCO Nitrogen: 1.6% tryptone and 1.6% yeast extract pH: 8.0 Temperature: 45 °C Fermentation: Submerge Duration: 24 h	1.7 U/ mL	Al Shehri <i>et al.</i> (2024)
6.	<i>B. cereus</i> ATA179	Substrate: Sucrose Nitrogen: (NH ₄) ₂ HPO ₄ pH: 6.0 Temperature: 55 °C Fermentation: Submerge SDS-PAGE: 47 kDA Duration: 24 h		Demirkan <i>et al.</i> (2021)
7.	<i>B. cereus</i> NC7401	Substrate: Maltose Nitrogen: tryptone pH: 8.0 Temperature: 55 °C Fermentation: Submerge	40 U/mL	Akhter <i>et al.</i> (2022)
8.	<i>B. cereus</i> I ₁₃	Substrate: Glucose and olive oil Nitrogen: Yeast extract pH: 7.0 Temperature: 20 °C Fermentation: Submerge Duration: 96 h	11.42 U/mL	Kattathayil <i>et al.</i> (2024)
9.	<i>B. licheniformis</i> SMIA-3	Substrate: 0.5% orange flour and 0.5% WCO Nitrogen: - pH: 7.5 Temperature: 50 °C Fermentation: Submerge Duration: 42 h	0.35 U/mL	Carvalho <i>et al.</i> (2022)
10.	<i>Bacillus</i> sp. VITL8	Substrate: 6% olive oil Nitrogen: 0.7% peptone pH: 7.5 Temperature: 36 °C Fermentation: Submerge Duration: 25 h	325.0 U/ mL	Balaji <i>et al.</i> (2020)

11.	<i>B. halotolerans</i> VSH 09	Substrate: Olive oil pH: 7.0 Temperature: 55 °C Fermentation: Submerge SDS-PAGE: 38 kDA Duration: 72 h	41.66 IU/mL	Mahnashi <i>et al.</i> (2022)
12.	<i>B. safensis</i>	Substrate: 2% olive oil Nitrogen: 2g/L peptone pH: 7.0 Temperature: 37 °C Fermentation: Submerge SDS-PAGE: 55 kDA Duration: 48-72 h	13.71 U/mg	Patel and Parikh (2022)
13.	<i>B. salmalaya</i> 139SI	Substrate: Olive oil Nitrogen: - pH: 8.0 Temperature: 37 °C Fermentation: Submerge Duration: 48 h	11.0 U/ mL	Al Mohaini <i>et al.</i> (2022)
14.	<i>B. amyloliquefaciens</i>	Substrate: soybean extract Nitrogen: Yeast extract pH: 7.0 Temperature: 40 °C Fermentation: Solid state Duration: 72 h	41.20 U/mL	Mazhar <i>et al.</i> (2017)
15.	<i>B. aryabhatai</i> BA03	Substrate: 25% olive mill wastewater Nitrogen: Yeast extract pH: 8.0 Temperature: 27 °C Fermentation: Submerge	28.34 U/mL	Paz <i>et al.</i> (2023)
16.	<i>B. pumilus</i> V1	Substrate: Fructose Nitrogen: Yeast extract pH: 8.0 Temperature: 37 °C Fermentation: Submerge SDS-PAGE: 35 kDA	16.6-27.8 ug/mL	Kandasamy <i>et al.</i> (2023)
17.	<i>B. halotolerans</i> VSH 09	Substrate: 2.5% molasses Nitrogen: 2% peptone pH: 7.0 Temperature: 35 °C Fermentation: Submerge Duration: 72 h	26.04 IU/mL	Hombalimath and Gurumurthy (2024)

3.3. Lipase catalyzed-biodiesel production

Lipase-catalyzed biodiesel synthesis showed that the transesterification reaction successfully converted waste cooking oil (WCO) into FAME. The chromatogram profile at retention times 5, 6, 7, 8, 10, 16, and 23 matched the internal standard chromatogram profile. The enzymatic

synthesis of biodiesel using *B. subtilis* DB104 lipase yielded FAME compositions of 0.7% methyl myristate, 22% methyl palmitate, 17.9% methyl stearate, 23.7% methyl oleate, 4.1% methyl linoleate, and 0.4% methyl linolenate (Fig. 8b).

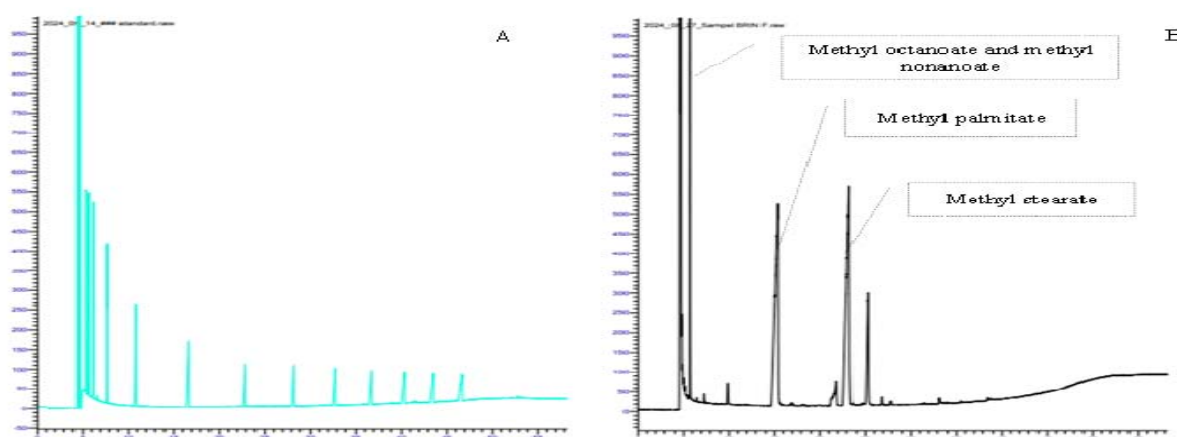


Figure. 8. Chromatogram of FAME standard (A), Enzymatic biodiesel synthesis (B)

The total FAME conversion from WCO was 68.8%, which fell short of the required standards for FAME content. According to the EN 14103 (GC) testing method, a minimum FAME content from the transesterification reaction should be 96.5% (m/m) (Husar *et al.* 2021). *B. subtilis* DB104 lipase showed both hydrolysis and transesterification activities. Biodiesel production through lipase-catalyzed transesterification is affected by several key factors, such as the ratio of WCO to methanol, lipase purity, lipase volume and activity, and the resulting biodiesel conversion efficiency.

Theoretically, oil-to-alcohol molar ratio in the transesterification reaction is 1:3, although this ratio varies based on the type of oil and alcohol used (Susanty *et al.* 2013). Enzymatic transesterification using *Pseudomonas fluorescens* lipase was performed with oil and methanol molar ratios of 1:3, 1:6, and 1:9. The highest FAME percentage (4.55%) was achieved with a molar ratio of 1:6. Methanol was reduced during the reaction due to evaporation at a 1:3 ratio, while at a 1:9 ratio, lipase enzyme inhibition occurred. Enzymatic transesterification using lipase extracted from *Leonotis nepetifolia* (Christmas candlestick) seeds was successfully performed at a molar ratio of 1:3 for oil and methanol (Vazquez *et al.*, 2023).

Even though FAME conversion using lipase extracted from *B. subtilis* DB104 was below the minimum requirement for biodiesel production, there is a room for the improvement since this study is still in the optimization of cultivation stage. The mineral salt medium (MSM) used with WCO as sole carbon source showed that lipase can be produced at minimal and cost-effective conditions.

4. Conclusion

The production of lipase by *B. subtilis* DB104 reached its optimum of 8.97 U/mL activity when the initial medium had pH 7.0 and contained 0.5% of WCO and 15 mM of NH₄NO₃ as the most effective carbon and nitrogen sources, respectively. The optimal activity of partial purified lipase was observed at pH 7 – 9, and a temperature of 37 °C. An enhancement of enzyme activity was shown with the addition of CaCl₂ Tween 80, Tween 20, and Triton X-100. Meanwhile, treatment with EDTA, β-mercaptoethanol, and PMSF, and organic solvent such as isopropanol, DMSO, n-hexane, and methanol showed a stable activity, indicating high stability and robustness for industrial applications. The lipase from *B. subtilis* DB104 exhibited a molecular weight of 33 – 35 kDa and effectively catalyzed transesterification reaction, resulting in a 68.8% biodiesel yield.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

The authors would like to thank the National Research and Innovation Agency which has provided financial support for the research grant and Manejemen Talenta for the Visiting Researcher Program offered.

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Evaluation of Antioxidant and Photoprotective Activities of Extract and Fractions from *Ulva lactuca*

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Received: November 28, 2025; Revised: January 21, 2026; Accepted: February 10, 2026

Abstract

Background. Exposure to sunlight, the main component of which is ultraviolet radiation, can cause various damage to the skin, including sunburn, premature aging, and more serious conditions such as increased symptoms of autoimmune diseases and skin cancer. Therefore, there is currently a growing interest in developing more effective photoprotective agents in protecting the skin from UV exposure as well as antioxidants that can overcome the oxidative stress it causes. This study aims to evaluate the potential photoprotective and antioxidant activities of various *Ulva lactuca* (*U.lactuca*) fractions and determine the relationship between the parameters tested.

Methods. *U. lactuca* samples were extracted using 70% ethanol solvent, then fractionated in stages using *n*-hexane, ethyl acetate, and water solvents. Analysis was carried out to determine the yield, total phenols, photoprotective activity, and antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, and ferric reducing antioxidant power (FRAP) methods on the extract and each fraction of *U. lactuca*.

Results. The ethyl acetate fraction showed the highest photoprotective activity with an SPF value of 42.38 ± 0.87 , followed by the ethanol extract (32.60 ± 1.10), aqueous fraction (16.26 ± 0.21), and hexane fraction (9.00 ± 0.17). Consistently, the ethyl acetate fraction also exhibited the strongest antioxidant activity in all assays, with IC₅₀ values of 17.23 ± 0.27 µg/mL in the DPPH assay, 4.15 ± 0.02 µg/mL in the ABTS assay, and 2.15 ± 0.02 µg/mL in the FRAP assay. The ethanol extract showed moderate antioxidant activity with IC₅₀ values of 19.38 ± 0.23 µg/mL (DPPH), 6.31 ± 0.12 µg/mL (ABTS), and 6.88 ± 0.75 µg/mL (FRAP), followed by the aqueous fraction with IC₅₀ values of 27.79 ± 0.88 µg/mL (DPPH), 9.13 ± 0.07 µg/mL (ABTS), and 6.83 ± 0.07 µg/mL (FRAP). In contrast, the hexane fraction exhibited the lowest antioxidant activity, with IC₅₀ values of 38.91 ± 0.43 µg/mL (DPPH), 42.55 ± 0.63 µg/mL (ABTS), and 32.33 ± 0.14 µg/mL (FRAP). Correlation analysis showed a positive relationship between yield, phenolic compound content, antioxidant activity, and SPF value.

Conclusion. Bioactive compounds of *U. lactuca* as a potential source of natural photoprotective and antioxidant ingredients for cosmetic formulations.

Keywords: Antioxidants, Marine bioactives; Sun protection factor; *Ulva lactuca*.

1. Introduction

Exposure of human skin to ultraviolet radiation can trigger a variety of short-term and long-term biological responses (Wu *et al.*, 2024). Common short-term effects include skin redness (erythema), pigmentation changes, and decreased immune function. Meanwhile, prolonged exposure can lead to photocarcinogenesis and premature skin aging (Rubio *et al.*, 2025). These mechanisms are related to changes at the molecular and cellular levels, including DNA damage, increased formation of reactive oxygen species (ROS), release of inflammatory mediators, activation of melanogenesis, and apoptosis (Menakh *et al.*,

2024). Antioxidants play a vital role in maintaining human health. These compounds work by neutralizing ROS produced by both exogenous sources such as UV radiation and endogenous cellular processes (Russo *et al.*, 2008). In recent years, increasing attention has been directed toward natural photoprotective agents derived from biological sources, as these compounds may provide both antioxidant and UV-protective effects with fewer adverse reactions compared to synthetic ingredients (Schneider *et al.*, 2022). As skin cancer cases increase globally each year, dermatologists emphasize the importance of regular sunscreen use to reduce the harmful effects of the sun's ultraviolet radiation (Hanif *et al.*, 2025). The level of sunscreen protection against erythema or sunburn is

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measured using the Sun Protection Factor (SPF). The SPF value represents the ratio of the minimum UVR dose required to cause erythema on protected and unprotected skin under artificial sunlight conditions.

Demand for sunscreen products continues to rise as consumers become more aware of the importance of facial skincare. However, one major issue is the use of synthetic ingredients in many over-the-counter cosmetic products on the market, which can potentially have detrimental effects on skin health. An alternative to address the damage caused by UV radiation is to look for molecules that have antioxidant and photoprotective potential. Therefore, the development of natural sunscreen ingredients is a promising alternative, especially from *Ulva lactuca* which is rich in bioactive compounds with natural photoprotective abilities.

The green seaweed *Ulva lactuca* (Chlorophyta), widely known as “sea lettuce.” These algae have a thin, wavy leaf structure, reaching 20–30 cm in length, and are easily recognized by their bright green color. *U. lactuca* grows in coastal areas, especially in tidal zones, attached to rocks or coral, and is often found in tropical and temperate climates, thus playing an important role in maintaining the balance of aquatic ecosystems. As a photosynthetic organism, *U. lactuca* is an important part of the food chain in marine ecosystems and is known to have various biological activities (Kaur *et al.*, 2025).

U. lactuca contains various commercially valuable compounds that have potential applications in the cosmetics, pharmaceutical, food, chemical and bioenergy industries (Kusmiyati *et al.*, 2020). The composition consists of carbohydrates up to 60%, protein 10–47%, lipid 1–3%, and mineral ash 7–38% (Ambarsari *et al.*, 2018). The main bioactive compounds that have industrial value include phenolics, pigments (chlorophyll and carotenoids), and polysaccharides (Merdekawati *et al.*, 2019). Recent studies have shown that *U. lactuca* has antioxidant, antimicrobial, and anticancer activities (Pradhan *et al.*, 2023). Therefore, *U. lactuca* is increasingly receiving attention both from a health perspective and for its industrial potential (Putra *et al.*, 2024). Several studies have reported the antioxidant potential of *U. lactuca* extracts. Methanolic extracts were shown to exhibit strong DPPH radical scavenging activity, with an IC₅₀ value of 0.45 mg/mL (Korkmaz, 2025). In addition, ethanol extracts demonstrated antioxidant activity with IC₅₀ values of 0.88 mg/mL in the DPPH assay and 1.12 mg/mL in the ABTS assay, along with a total phenolic content of 20.5 mg GAE/g (Putu *et al.*, 2024). However, most previous investigations have primarily focused on single crude extracts, with limited attention given to solvent-partitioned fractions and their association with photoprotective efficacy. Therefore, systematic evaluation of different solvent fractions and their relationship with both antioxidant capacity and photoprotective activity remains limited. The aim of this study was to evaluate the potential photoprotective and antioxidant activities of various fractions of *U. lactuca* obtained from 70% ethanol extract, and to determine the relationship between the parameters tested to assess the potential of this alga as a source of natural active ingredients for sunscreen formulations and skin care products.

2. Methods

2.1. Material

U. lactuca L. samples were obtained from Teluk Kode, West Pemenang Village, Pemenang District, North Lombok Regency, Indonesia with coordinate points 8°26'27.6"S 116°6'7.2"E. The samples were then placed in plastic containers filled with sterile seawater and stored in a coolbox to maintain the freshness (Kusmita *et al.*, 2021; Nugraheni *et al.*, 2010). Botanical determination was performed to ensure the authenticity of the materials used in this study. The determination process was carried out at the Indonesian Institute of Sciences (LIPI), Marine Bioindustry Center, North Lombok Regency, and the results were stored as authentic specimens with collection number B-164/III/DI.02/9/2021.

After collection, the samples were washed with seawater to remove epiphytes, sand, and dirt, then rinsed with distilled water. The cleaned samples were air-dried in a shaded area at room temperature, then dried in a circulating air oven at 40°C for 72 hours. The dried material was then ground into a fine powder and stored in a tightly closed container for further extraction and analysis.

The chemicals used were 100% ethanol (Merck), *n*-hexane (Merck), ethyl acetate (Merck), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA), FeCl₃, ammonium acetate; K₂S₂O₈, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); ascorbic acid, trolox; dimethyl sulfoxide (Merck).

2.2. Extraction of *U. lactuca*

500 grams of dried *U. lactuca* powder was weighed and extracted using 70% ethanol, each volume of 5 L. The method used was maceration for 72 hours with occasional stirring. The filtrate was filtered using Whatman No. 1 filter paper and evaporated with a rotary evaporator at 40–50°C until a thick extract was obtained.

2.3. Preparation of *U. lactuca* Fraction

The dried resulting extract was then fractionated using the liquid-liquid partitioning method with solvents of increasing polarity (*n*-hexane, ethyl acetate, and water) according to the protocol described by Mfopa (Mfopa *et al.*, 2017). In brief, 50 g of crude extract was dissolved in 100 mL of distilled water (water fraction) and placed in a separating funnel. 100 mL of *n*-hexane was then added, the mixture was shaken vigorously for 3 minutes, then left for 5 minutes until two layers were formed. The water fraction (lower layer) and the *n*-hexane fraction (upper layer) were separated and collected separately. The water fraction obtained was then fractionated again with ethyl acetate (1:1) to obtain the ethyl acetate fraction and the water fraction.

2.4. Determination of Total Phenolic Content (TPC)

The total phenolic content in *U. lactuca* extract was measured spectrophotometrically using the Folin-Ciocalteu reagent, as described by Mutmainah *et al.* (2019). Specifically, 7.9 mL of distilled water and 0.1 mL of sample were placed in a glass vial and homogenized by vortexing. 0.5 mL of Folin-Ciocalteu reagent was added and homogenized again, followed by the addition of 1.5 mL of 20% (w/v) Na₂CO₃ solution. The final mixture was

then incubated for 30 minutes in a water bath at 40 °C, and the absorbance was measured at 765 nm using a UV-VIS spectrophotometer (SHIMADZU), then compared with the gallic acid calibration curve. Total phenol value was expressed as gallic acid equivalents (mg/g dry weight). Measurements were carried out three times (triplicate).

2.5. Photoprotection Activity Test

The Sun Protection Factor (SPF) value of *U. lactuca* extract was determined using a Shimadzu UV-Vis spectrophotometer (Aparicio-álvarez *et al.*, 2023). The extract and its fraction were dissolved separately in ethanol at a concentration of 100 mg/mL and their absorbance was measured at a UV-B wavelength (290–320 nm) at 5 nm intervals. Ethanol p.a. (pro analysis grade) was used as a blank solution. The SPF value was calculated using the equation:

$$SPF = CF \times \sum_{\lambda=290}^{320} \frac{EE(\lambda)}{I(\lambda) \times Abs(\lambda)}$$

where CF represents the correction factor, EE (λ) denotes the erythral effectiveness of radiation at a specific wavelength λ, I(λ) is the solar intensity spectrum (considered constant), and Abs (λ) corresponds to the absorbance value of the sample at wavelength λ.

2.6. Antioxidant activity test using the DPPH method

Antioxidant activity test used the DPPH method (Pramesti *et al.*, 2025). The test was performed by dissolving the sample in methanol, then making serial dilutions. A 2 mL sample solution was reacted with 2 mL of 0.1 mM DPPH solution and incubated for 30 minutes in the dark. Absorbance was then measured using a UV-Vis spectrophotometer at a wavelength of 517 nm.

2.7. Antioxidant activity test using the ABTS method

The radical scavenging potential of *U. lactuca* extracts and fractions against ABTS radicals was determined using a spectrophotometer (Untea *et al.*, 2018). ABTS radicals were produced by mixing two stock solutions, namely 7 mM ABTS and 2.4 mM potassium persulfate, in equal proportions, then incubated in the dark for 12 hours at room temperature. The ABTS⁺ solution that had been formed was then diluted with ethanol until the absorbance value reached 0.70 at a wavelength of 734 nm. A total of 6 mL of ABTS⁺ solution was reacted with 40 μL of sample, then the absorbance was measured at a wavelength of 734 nm using a UV-Vis spectrophotometer with ethanol as a blank. The ABTS radical scavenging activity of the sample was stated based on the calibration curve.

2.8. Antioxidant activity test using the FRAP method

The ferric reducing antioxidant capacity (FRAP) of *U. lactuca* extract was determined based on the method described by Wijayanti *et al.* with some modifications (Wijayanti *et al.*, 2024). Fresh reaction solution was prepared by mixing 10 mM TPTZ (2,4,6-Tripyridyl-s-

triazine) in 40 mM HCl and 20 mM ferric chloride in 300 mM acetate buffer (pH 3.6) at a ratio of 5:5:25 (v/v), then stored at 37 °C for 0.5 h. A 0.15 g sample was mixed with 2850 μL of FRAP reagent. The mixture was incubated in the dark for 0.5 h, then the absorbance was measured at a wavelength of 539 nm. Trolox was used as a positive control. The FRAP activity of each sample was determined at different concentrations, and the IC₅₀ value (μg/mL) was calculated from the concentration–response curve. All tests were performed in triplicate.

2.9. Data analysis

Statistics were calculated using GraphPad Prism version 9.0. Results are presented as mean ± SD (standard deviation).

3. Results

With the aim of evaluating the effect of the fractionation process on the bioactive potential of *U. lactuca*, this study focused on determining the antioxidant and photoprotective activities of the ethanol extract and the resulting solvent fractions. Furthermore, this study also aimed to determine the relationship between the tested parameters to understand the contribution of each fraction to the overall biological activity of *U. lactuca*.

3.1. Yield

The use of solvents with different levels of polarity aims to extract bioactive compounds from *U. lactuca* based on their polarity properties. The yield of *U. lactuca* extracts and fractions is presented in Table 1.

Table 1. Extraction and fractionation Results of *U. lactuca*

Sample	Average extract weight (g) ± SD	Average extract yield (%) ± SD
Ethanol Extract	58.86 ± 0.88	11.77 ± 0.18
Hexane Fraction	5.43 ± 0.69	10.85 ± 1.38
Ethyl Acetate Fraction	28.28 ± 0.53	56.55 ± 1.07
Aqueous Fraction	16.30 ± 0.98	32.60 ± 1.95

3.2. Total Phenolic Content (TPC)

Differences in solvent polarity produce extracts with varying photoprotective and antioxidant activities (Bagiana *et al.*, 2025). This is due to the selective ability of each solvent in extracting chemical compounds, especially phenolic compounds that have high antioxidant capacity. In this study, extracts and fractions of *U. lactuca* obtained using three different types of solvents - *n*-hexane, ethyl acetate, and water - were evaluated to assess their effectiveness in extracting bioactive compounds that have the potential to provide natural photoprotective and antioxidant activities (Figure 1).

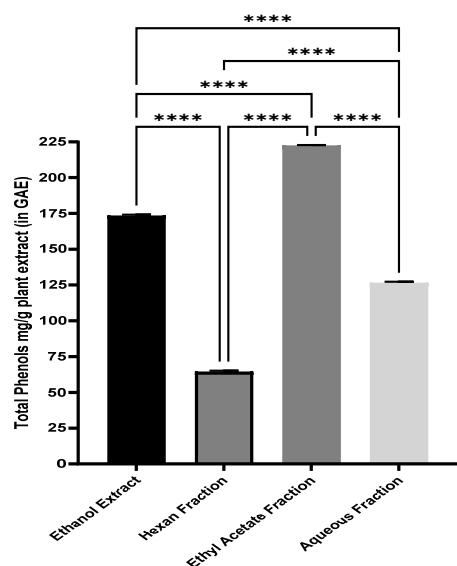


Figure 1. Total amount of plant phenolic compound of extract and fractions of *U. lactuca*. The data were mean $\pm$ SD ($n=3$). The data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

3.3. Photoprotection Activity Test

The crude extract, *n*-hexane, ethyl acetate, and water fractions of *U. lactuca* were tested for their Sun Protection Factor (SPF) activity using UV-Vis spectrophotometry (Figure 2).

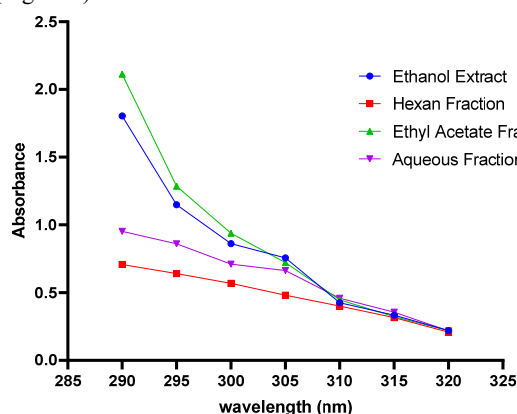


Figure 2. Spectrophotometric absorption spectra (290-320 nm) of ethanol extract, hexane, ethyl acetate, and aqueous fractions of *U. lactuca* (100 mg/mL).

The chemical ability of a compound to absorb UV radiation is greatly influenced by the nature of the solvent used. The photoprotective ability of *U. lactuca* extracts and fractions is expressed in SPF values (Table 2).

Table 2. Sun protection factor values of extract and fractions of *U. lactuca*

Sample	Sun protection factor	Category
Ethanol Extract	32.60 $\pm$ 1.10	High protection
Hexane Fraction	9.00 $\pm$ 0.17	Minimal protection
Ethyl Acetate Fraction	42.38 $\pm$ 0.87	High protection
Aqueous Fraction	16.26 $\pm$ 0.21	Moderate protection

3.4. Antioxidant test

The antioxidant activity of *U. lactuca*, including ethanol extract and *n*-hexane, ethyl acetate, and water fractions, was evaluated using three assay methods: DPPH (Figure 3), ABTS (Figure 4), and FRAP (Figure 5). Ascorbic acid was used as a positive control for the DPPH method, while Trolox was used as a positive control for the ABTS and FRAP methods.

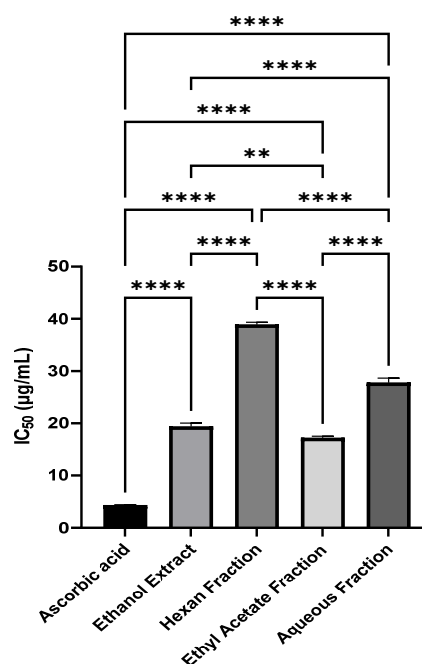


Figure 3. Free radical scavenging activity results of extract and fractions of *U. lactuca* against DPPH. The data were mean $\pm$ SD ($n=3$). The data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

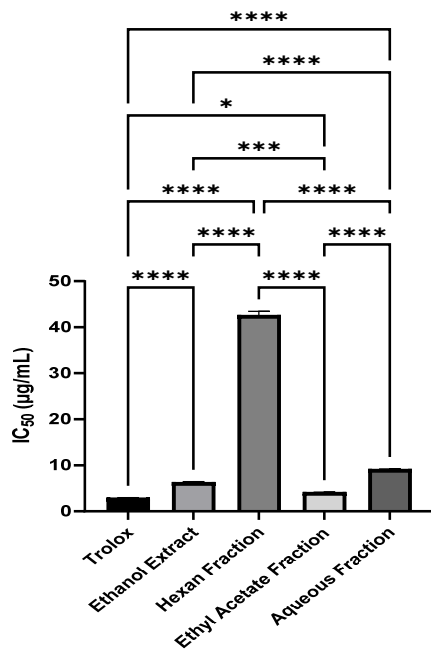


Figure 4. Free radical scavenging activity results of extract and fractions of *U. lactuca* against ABTS. The data were mean $\pm$ SD (n =3). The data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test (*p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001).

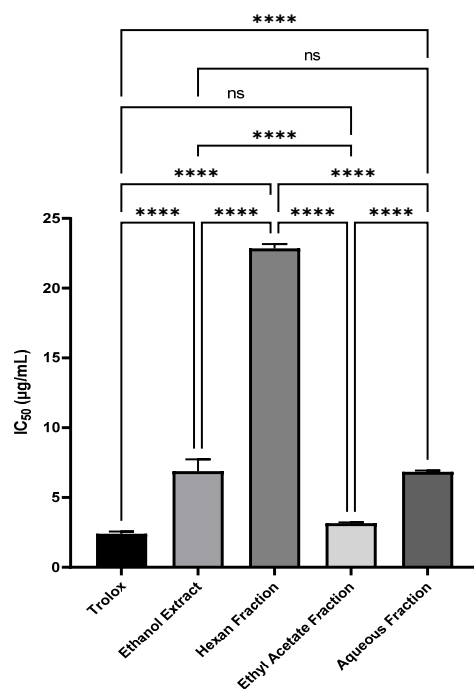


Figure 5. Free radical scavenging activity results of extract and fractions of *U. lactuca* against FRAP. The data were mean $\pm$ SD (n =3). The data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test (ns= not significant; *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001).

3.5. Statistical study

To evaluate the strength and direction of the relationship between the variables studied, a correlation analysis was conducted using the Pearson linear correlation coefficient test. This analysis produced the correlation coefficient (R^2) values presented in Table 3.

Table 3. Pearson correlation coefficients among yield, total phenolic compound, SPF values and antioxidant capacities of fractions of *U. lactuca*.

Parameters	Parameters					
	Yield	TPC	SPF	DPPH	ABTS	FRAP
Yield	1.00	1.00	0.96	-1.00	-0.91	-0.93
TPC	1.00	1.00	0.98	-0.99	-0.86	-0.89
SPF	0.96	0.98	1.00	-0.95	-0.75	-0.79
DPPH	-1.00	-0.99	-0.95	1.00	0.93	0.95
ABTS	-0.91	-0.86	-0.75	0.93	1.00	1.00
FRAP	-0.93	-0.89	-0.79	0.95	1.00	1.00

4. Discussion

In this study, *U. lactuca* was selected as a potential source of natural compounds with photoprotective and antioxidant activities that can be utilized in the field of dermocosmetics, particularly as active ingredients in sunscreen formulations. The yield of *U. lactuca* fractions varied depending on the type of solvent used (Table 1). Ethanol extraction produced a yield of $11.77 \pm 0.18\%$, demonstrating the effectiveness of ethanol as a solvent capable of extracting active compounds of various polarity levels. The fractionation results showed that the ethyl acetate fraction had the highest yield followed by the water fraction and the *n*-hexane fraction. The high yield of the ethyl acetate fraction indicates the dominance of semi-polar compounds, such as flavonoids and phenolic compounds, which have the potential to provide the main biological activity (Zaatout *et al.*, 2019). Conversely, the low yield of the *n*-hexane fraction indicates that it contains relatively few nonpolar compounds. Overall, this distribution pattern reflects the diversity of secondary metabolites based on the polarity of the solvent used.

The extraction procedure and the type of solvent used play an important role in dissolving endogenous compounds found in plants (Siddhuraju & Becker, 2003). Plant components can be polar or non-polar. Phenolic compounds are more soluble in polar organic solvents due to the presence of hydroxyl groups (Alara *et al.*, 2021). Figure 2 shows the total phenol content (expressed in mg GAE/g plant extract) of the ethanol extract and three fractions of *U. lactuca* obtained using solvents with different polarities, namely *n*-hexane, ethyl acetate, and water. The analysis results showed a significant difference (****, p < 0.0001) between all groups. The ethyl acetate fraction had the highest total phenol content (~220 mg GAE/g), followed by the ethanol extract (~175 mg GAE/g), the water fraction (~125 mg GAE/g), and the *n*-hexane fraction (~65 mg GAE/g). The ethanol extract also showed high phenol content due to its amphiphilic nature, capable of dissolving both polar and semi-polar compounds. Ethanol extraction of natural products is effective because it has a wide solubility range as a neutral solvent, capable of extracting various compounds, including water-soluble hydrophilic components and lipophilic components such as fats (Lee *et al.*, 2024). The high phenol content in the ethyl acetate fraction indicates that most of the phenolic compounds in *U. lactuca* are semi-polar, making them more easily extracted with ethyl

acetate. In contrast, the non-polar *n*-hexane fraction is only able to dissolve a small amount of phenolic compounds.

The photoprotective activity of *U. lactuca* extracts and fractions was evaluated by calculating the Sun Protection Factor (SPF) value using a spectrophotometric method. Figure 3 shows the spectrophotometric absorption profiles of *U. lactuca* extracts and fractions. It was observed that both the extracts and fractions displayed characteristic absorption bands in the UVB region, indicating a potential photoprotective effect. Measurements were made in the UV-B region, which is the region with the highest intensity during the day and the longest exposure to human skin (Putri *et al.*, 2025).

Based on the SPF value classification, the SPF range of 2–12 is included in the minimal protection category, SPF 12–30 is included in moderate protection, and SPF ≥ 30 is categorized as high protection (Mansur *et al.*, 1986). The results in Figure 3 show that the ethyl acetate fraction has the highest SPF value (42.38 ± 0.87), followed by the ethanol extract (32.60 ± 1.10), both of which are included in the category of high protection against UV radiation. These values indicate that both samples have the potential as candidates for natural sunscreen active ingredients. Meanwhile, the water fraction shows an SPF value of 16.26 ± 0.21 which is included in the category of moderate protection, while the *n*-hexane fraction has the lowest SPF value (9.00 ± 0.17) and is included in the category of minimal protection. This difference in SPF values is likely caused by variations in the content of active compounds that play an important role in photoprotective activity through their ability to absorb UV radiation and neutralize free radicals.

Antioxidants are compounds that help the body fight oxidative stress associated with tissue damage and the activity of highly reactive free radicals (Abu-Lubad *et al.*, 2024). Exogenous antioxidant intake from food can effectively enhance the body's natural antioxidant system by stimulating the production of antioxidant enzymes and playing a role in protecting the body from excessive free radical accumulation (Anam *et al.*, 2023). Antioxidant activity strength is generally categorized as very strong (<50 ppm), strong (50–100 ppm), moderate (101–150 ppm), and weak (>150 ppm). Based on this classification, MKE's antioxidant activity is included in the very strong category because it has an IC_{50} value of <50 mg/L (Flieger *et al.*, 2021). In this study, extracts and fractions of *U. lactuca* were used as research subjects whose antioxidant activity will be determined through in vitro free radical scavenging methods using several tests, namely DPPH, ABTS and FRAP.

The antioxidant activity of *U. lactuca* extracts, including ethanol extract, *n*-hexane fraction, ethyl acetate, and water, was tested using the DPPH free radical scavenging method. Ascorbic acid was used as a positive control. The DPPH method is based on the ability of antioxidant compounds to donate hydrogen atoms or electrons to stabilize DPPH free radicals (Nuraini *et al.*, 2025). This reaction causes a decrease in the absorbance value accompanied by a change in the color of the solution from dark purple to light yellow or pale purple, which can then be measured quantitatively using a spectrophotometer (Tue *et al.*, 2024).

The results of the antioxidant activity test of *U. lactuca* extracts and fractions using the DPPH method showed

variations in potential influenced by the type of solvent used (Figure 4). Ascorbic acid as a positive control showed very strong antioxidant activity with an IC_{50} value of 4.34 ± 0.05 $\mu\text{g/mL}$. Among the test samples, the ethyl acetate fraction showed the highest antioxidant activity with an IC_{50} value of 17.23 ± 0.27 $\mu\text{g/mL}$, followed by the ethanol extract (19.38 ± 0.23 $\mu\text{g/mL}$), the water fraction (27.79 ± 0.88 $\mu\text{g/mL}$), and the *n*-hexane fraction (38.91 ± 0.43 $\mu\text{g/mL}$). Statistical analysis showed that all extracts and fractions had significant differences in IC_{50} values compared to ascorbic acid. In addition, significant differences were also observed between fractions (Figure 4), which indicates that solvent variations affect the antioxidant capacity of *U. lactuca* fractions.

The results of the antioxidant activity test of *U. lactuca* extracts and fractions using the ABTS method showed varying abilities in neutralizing ABTS⁺ radicals (Figure 5). Trolox was used as a positive control and showed the highest activity with an IC_{50} value of 2.94 ± 0.02 $\mu\text{g/mL}$. Among the test samples, the ethyl acetate fraction had the strongest antioxidant activity ($IC_{50} = 4.15 \pm 0.02$ $\mu\text{g/mL}$), followed by the ethanol extract (6.31 ± 0.12 $\mu\text{g/mL}$), the water fraction (9.13 ± 0.07 $\mu\text{g/mL}$), and the *n*-hexane fraction (42.55 ± 0.63 $\mu\text{g/mL}$). Based on the classification of antioxidant potential, all extracts and fractions except the *n*-hexane fraction are classified as having very strong antioxidant activity ($IC_{50} < 50$ $\mu\text{g/mL}$). The ethyl acetate fraction showed an ability close to the positive control, indicating that the active compounds contained therein have high effectiveness in neutralizing ABTS⁺ radicals. Statistical analysis using one-way ANOVA followed by Tukey's test showed a significant difference between samples ($****p < 0.0001$). The strong ABTS radical scavenging activity in the ethyl acetate fraction indicates that compounds with medium polarity play a dominant role in the antioxidant activity of *U. lactuca*.

The antioxidant activity test of *U. lactuca* using the FRAP method showed variations in the Fe^{3+} ion reduction ability between fractions (Figure 5). Trolox as a positive control had the highest activity ($IC_{50} = 0.23 \pm 0.02$ $\mu\text{g/mL}$). Among the samples, the ethyl acetate fraction showed the strongest activity ($IC_{50} = 2.15 \pm 0.02$ $\mu\text{g/mL}$), followed by the water fraction (6.83 ± 0.07 $\mu\text{g/mL}$) and the ethanol extract (6.88 ± 0.75 $\mu\text{g/mL}$). The *n*-hexane fraction had the lowest activity ($IC_{50} = 32.33 \pm 0.14$ $\mu\text{g/mL}$), but was still considered strong. These results indicate that the dominant active compounds in *U. lactuca* are polar to semi-polar, such as flavonoids and phenolics, which play a major role in the electron transfer mechanism. Statistical analysis using one-way ANOVA followed by Tukey's test showed significant differences ($****p < 0.0001$) between most samples, except between the ethanol extract and the water fraction (ns) which showed similar activity. The antioxidant activity of the ethyl acetate fraction and Trolox as a positive control was also not significantly different, indicating comparable radical scavenging abilities. The high FRAP reduction activity in the ethyl acetate fraction indicates that *U. lactuca* contains compounds with moderate polarity that contribute predominantly to its antioxidant capacity. The photoprotective and antioxidant effects of *U. lactuca* can be attributed to the presence of specific phenolic acids such as gallic acid and vanillic acid, which act through electron and hydrogen donation mechanisms to neutralize free radicals, as well as through

UV absorption. Total phenolics, flavonoids, and saponins extracted by subcritical water hydrolysis have been shown to enhance radical scavenging and reducing power, which supports the observed high SPF and antioxidant activities. Additionally, sulfated polysaccharides such as ulvan may contribute synergistically to protection against UV-induced oxidative damage (Pangestuti *et al.*, 2021).

Pearson correlation analysis showed a very strong relationship between the parameters tested in the *U. lactuca* fraction. The correlation matrix between the extract yield, total phenols (TPC), SPF value, and antioxidant parameters (DPPH, ABTS, and FRAP) showed a strong relationship between these variables (Table 3). The Pearson correlation coefficient (*r*), which ranges from -1 to +1, was used to evaluate the strength and direction of these relationships, where positive values indicate direct correlations and negative values indicate inverse correlations. A significant positive correlation was found between the yield, TPC, and SPF ($r = 0.96\text{--}1.00$), indicating that the higher the extract yield and the concentration of phenolic compounds, the higher the photoprotective activity was. This relationship indicates that phenolic compounds play an important role in absorbing UV rays and providing a protective effect, thus contributing to the SPF value of the *U. lactuca* extract.

In contrast, a strong negative correlation was recorded between TPC and antioxidant parameters (DPPH, ABTS, and FRAP) with *r* values ranging from -0.75 to -1.00. This inverse relationship reflects the fact that antioxidant capacity is expressed as IC₅₀ values, where lower IC₅₀ values correspond to stronger radical scavenging activity. This inverse relationship arises because lower IC₅₀ values in antioxidant assays indicate stronger radical scavenging activity. Thus, extracts rich in phenolic compounds exhibit higher antioxidant capacity. Among the three antioxidant assays, the DPPH assay showed the strongest negative correlation with TPC ($r = -0.99$) and yield ($r = -1.00$), confirming the sensitivity of this method in detecting phenolic-based antioxidant potential. In addition, high positive correlations were also found between antioxidant methods (DPPH vs. ABTS = 0.93; DPPH vs. FRAP = 0.95; ABTS vs. FRAP = 1.00), indicating that all three assays consistently measure the redox capacity of phenolic compounds in *U. lactuca*. The consistency of the correlation between these tests strengthens the reliability of the research results and confirms the dominant contribution of phenolic compounds to antioxidant and photoprotective activities.

The evaluation of the photoprotective and antioxidant activities of *U. lactuca* in this study provides strong evidence for its potential as a potential UV filter and natural antioxidant source. The results showed that the polarity of the extraction solvent significantly influenced the photoprotective activity and antioxidant effectiveness. The results of the activity test showed a consistent pattern, where the ethyl acetate fraction of *U. lactuca* had the highest activity, followed by the ethanol extract and the water fraction, while the *n*-hexane fraction showed the lowest activity. This solvent-dependent pattern is in line with the basic principles of phytochemistry, where solvents with medium polarity are able to optimally extract various secondary metabolites, such as phenolic acids, flavonoids, and their derivatives in the form of glycosides (Hidayati *et al.*, 2020).

5. Conclusion

Fractionation using solvents with increasing polarity demonstrated a clear linear trend toward higher extract yield, antioxidant activity, and SPF values. Among the tested solvents, ethyl acetate was identified as the most effective in enhancing both antioxidant capacity and photoprotective activity of *U. lactuca*, indicating the predominance of semi-polar bioactive compounds. These findings highlight the significance of solvent polarity in maximizing the biological potential of *U. lactuca* and support its promising application as a natural source of photoprotective and antioxidant agents in the health and cosmetic industries. The results provide practical implications for the selection of optimal fractionation strategies as a foundation for the isolation and further development of active compounds. However, this study is limited to *in vitro* evaluations; therefore, further studies involving compound identification, formulation development, and *in vivo* or clinical assessments are required to confirm efficacy and safety.

Conflict of Interest

The authors have no conflict of interests regarding this investigation.

Acknowledgments

The authors would like to acknowledge the Postdoc World Class University Program, Universitas Diponegoro 2025.

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Antioxidant and anti-inflammatory activities of endophytic *Fusarium* sp. isolate NN-Fus-1

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Received: November 8, 2025; Revised: January 20, 2026; Accepted: February 23, 2026

Abstract

Endophytic fungi are a valuable source of unprecedented bioactive metabolites of medical and pharmaceutical potential. In the current study, the fungal isolate NN-Fus-1 was selected following a screening process for bioactive crude extracts. The isolate was cultivated on a solid rice medium, and the culture was extracted with ethyl acetate. The fungal isolate was identified morphologically and via partial sequencing of the 18S rDNA. The isolate was identified as *Fusarium* sp. isolate NN-Fus-1F, and its sequence was deposited in GenBank. Its crude extract revealed antioxidant activity by scavenging DPPH radicals with an IC_{50} of 109.17 ± 1.30 μ g/ml (6.41 ± 0.08 μ g Trolox/mg crude extract) but not nitric oxide radicals. Although it did not stabilize the membrane of heat-induced RBCs, it showed anti-inflammatory potential by inhibiting heat-induced albumin denaturation ($IC_{50} = 469.05 \pm 3.72$ μ g/ml) and reducing lipooxygenase 5-LOX activity ($IC_{50} = 83.51 \pm 1.36$ μ g/ml) *in vitro*, as well as decreasing rat paw edema ($IC_{50} = 159.76 \pm 3.6$ μ g/g body weight) compared to untreated controls *in vivo*. The extract was ineffective against tested bacterial strains in the agar diffusion assay. *Fusarium* sp. isolate NN-Fus-1F crude extract comprised of alkaloids, phenolic derivatives, flavonoids, and organic acids as main constituents. The presence of these classes of compounds in the crude extract reflects its potential antioxidant and anti-inflammatory activities detected herein. *Fusarium* sp. isolate NN-Fus-1 is considered a candidate for secondary metabolites with medical and pharmaceutical applications.

Keywords: Anti-inflammatory, Antioxidant, Bioactive metabolites, Endophytes, *Fusarium* sp.

1. Introduction

Fungi have drawn the attention of several natural product scientists since the discovery of penicillin by Fleming in 1928 (Saxena *et al.*, 2024). Part of these microorganisms colonize the plant host's internal tissues or intracellular spaces without causing adverse effects (Al-Qaralleh *et al.*, 2021). The *Fusarium* genus is placed in the third level of plant-associated fungi, after the *Penicillium* and *Aspergillus* genera. It is a cosmopolitan filamentous fungus in the class Sordariomycetes (order: Hypocreales, family: Nectriaceae) with more than 70 species (Ahmed *et al.*, 2023). *Fusarium* species are widely distributed in various habitats, not only as phytopathogens but also found in soil, plants, insects, and animal substrates in terrestrial and marine environments (Amuzu *et al.*, 2024). They are reservoirs of novel compounds, including peptides, polyketides, quinones, steroids, terpenoids, alkaloids, flavonoids, and phenolic acids (Wei and Wu, 2020), metabolites that revealed unprecedented activities in different biological assays. Therefore, they are exploited as a rich source of novel bioactive metabolites, including anticancer, antioxidant, antibacterial, antiviral, antidiabetic, anti-inflammatory, and immunomodulatory agents (Singh *et al.*, 2021).

Three hundred ninety compounds from various *Fusarium* species were reported from 1999 to 2022; more

than 50% were isolated throughout 2017-2022, and 92 compounds (23%) were novel (Ahmed *et al.*, 2023), highlighting the importance of this genus as a source for natural bioactive metabolites. Among the reported bioactive metabolites derived from endophytic *Fusarium* species were the cytotoxic azaanthraquinone derivatives and the anti-inflammatory fusopolide A metabolite from *F. solani*, the antimicrobial aminobenzamide derivative fusarithioamide B from *F. chlamydosporum* (Wei and Wu, 2020), and Rohitukine alkaloid from *F. proliferatum* as an anti-cancer, anti-inflammatory, and immunomodulatory agent (Ahmed *et al.*, 2023).

Notably, some endophytes synthesized secondary metabolites previously known from their hosts (Yan *et al.*, 2018; Archana and Nagadesi, 2022), raising questions about the exact biosynthetic origin of these metabolites. For example, the anticancer drug Taxol, which was primarily isolated from the *Taxus brevifolia* plant (Pacific yew), was synthesized by the associated *Taxomyces andreanae* fungus (Stierle *et al.*, 1995). Similarly, the vinca alkaloids vinblastine and vincristine, identified in the Madagascar periwinkle plant *Catharanthus roseus*, are synthesized by some endophytic fungi, including *Fusarium oxysporum*, *Alternaria* sp., and *Nigrospora sphaerica*, which are fungal species associated with the same plant (Ayob *et al.*, 2017). These findings highlighted the potential of endophytes as prolific producers of

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pharmacologically active compounds, often in greater quantities than their host plants.

Inflammation is a protective response triggered by microbial infections, oxidative stress, or tissue injury (Mang *et al.*, 2018). It involves changes in vascular permeability, pain induction via inflammatory mediators (such as prostaglandins, kinins, and histamine), and tissue damage due to the release of lysosomal enzymes and protein denaturation (DiStasi and Ley, 2009). However, prolonged or excessive inflammation can lead to chronic disorders. Managing inflammation is achieved using non-steroidal anti-inflammatory drugs (NSAIDs); however, their side effects limit their therapeutic utility (Panchal and Sabina, 2023). This underscores the need for safer, naturally derived anti-inflammatory and antioxidant agents (Al-Zereini *et al.*, 2023). Accordingly, this study aims to screen endophytic fungi for crude extracts with potential antioxidant and anti-inflammatory properties.

2. Materials and Methods

2.1. Plant sample collection and isolation of the endophytic fungus NN-Fus-1

A healthy *Marrubium vulgare* (white horehound, family Lamiaceae) specimen was collected from the Al-Jami'ah sub-district in Western Amman, Jordan (GPS 32.0015346, 35.8449004). The plant was taxonomically authenticated by Dr. Feryal Kherissat in the Department of Biological Sciences at Mutah University-Jordan, where a voucher specimen was deposited for future reference. Isolation of the endophytic fungus NN-Fus-1 from the plant stem was performed as described in Hazalin *et al.* (2009). Briefly, the plant aerial parts were aseptically surface sterilized by submersion in 70% ethanol for 2 min, then in 3% sodium hypochlorite solution for 1 min, followed by submersion in 70% ethanol for 30 seconds. This was followed by rinsing in sterilized distilled water for 5 min. The sterilized parts were cut aseptically into segments of 1 cm long and placed on potato dextrose agar plates (Oxoid, UK) supplemented with 100 µg/mL of streptomycin and penicillin G (Bio Basic Inc, Canada). Aliquots of the sterilized rinsing water were plated on a PDA plate to ensure the sterilization effectiveness and the absence of epiphytic microorganisms. The agar plates were incubated at 27°C for 2-4 weeks to observe mycelial growth. The grown mycelia with different morphological appearances were picked and transferred to malt extract agar (Oxoid, UK). Pure cultures of NN-Fus-1, cultivated on malt extract agar slants, were maintained at 4°C for long-term storage.

2.2. Cultivation of NN-Fus-1 and preparation of crude extracts:

The fungal isolate NN-Fus-1 was inoculated in a 500 ml Erlenmeyer flask containing sterilized rice medium (50 g of rice submerged in 75 ml of distilled water, autoclaved at 121°C for 20 min). Five agar blocks (1 x 1 cm) from a well-grown fungal culture on potato dextrose agar (PDA) were transferred aseptically to the rice flask. The flask was incubated statically at 27°C for 30 days, allowing complete colonization of the medium by fungal mycelia (Chen *et al.*, 2020). Post-incubation, the biomass and rice matrix were homogenized and exhaustively extracted with ethyl acetate (2 x 200 ml, 24h each). The pooled organic phases were

filtered through Whatman No. 1 paper containing anhydrous sodium sulfate for drying. The water-free organic solvent was concentrated *in vacuo* at 50°C using a rotary evaporator. The resulting crude extract was dissolved in methanol to prepare a 100 mg/ml stock solution, which was stored at 4°C till bioassays (Al-Qaralleh *et al.*, 2021).

2.3. Morphological and molecular identification of the NN-Fus-1

The fungal isolate NN-Fus-1 was characterized morphologically as mentioned in Watanabe (2010). The fungal isolate was cultured on PDA plates at 27°C for 14 days. Colony features (e.g., pigmentation, texture, margin) were documented using a stereomicroscope at 40x, while microscopic structures (hyphae, conidiophores, conidia) were examined under a compound microscope at 400x magnification. For molecular identification, genomic DNA was extracted using the fungal DNA Mini Kit (Omega BioTek, USA), and the internal transcribed spacer (ITS1-5.8S-ITS2) was amplified via PCR with the universal primers ITS1 and ITS4. The PCR reaction constituents and conditions were previously described (Al-Qaralleh *et al.*, 2021). The amplified product was sequenced and compared to the NCBI GenBank database via BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic analysis was conducted in MEGA X software using the Maximum Likelihood Algorithm to confirm taxonomic affiliation (Kumar *et al.*, 2018).

2.4. Evaluation of antioxidant and anti-inflammatory activities

2.4.1. Determination of antioxidant activity

The radical scavenging potential of the NN-Fus-1 isolate's crude extract was assessed against nitric oxide (NO) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals, as outlined by Al-Qaralleh *et al.* (2023). For the DPPH assay, extract concentrations (50-500 µg/ml) were mixed with 0.1 mM methanolic DPPH solution at 37°C for 30 min in the dark. The reduction in the color of the DPPH radical solution was measured at 517 nm using a UV/Vis spectrophotometer (Novaspec Model 80-2088-64, Pharmacia Biotech, UK). In the NO scavenging assay, the same concentrations of the crude extract were reacted with 10 mM sodium nitroprusside in 0.2 M phosphate-buffered saline (pH 7.4) for 3 h at 25°C. An equal volume of Griess reagent (Sigma-Aldrich, Germany) was added, and the absorbance of the resulting chromophore was read at 540 nm. Trolox (Sigma-Aldrich, Germany) was employed as a positive control. All experiments were conducted in triplicate, and the scavenging activity (%) was calculated compared to untreated controls.

2.4.2. Assessment of anti-inflammatory activity

2.4.2.1. Albumin denaturation test

The inhibition of heat-induced bovine serum albumin denaturation (BSA) was tested following the method of Sakat *et al.* (2010), with slight modifications. Ten microliters of the crude extract were added to 2 ml of 1% aqueous BSA (pH 5.3±0.2) at final concentrations (50-1000 µg/ml) with incubation at 37°C for 20 min. The preparation was heated at 56°C for 20 min. After cooling, the inhibition of protein agglutination was measured at 660 nm using a spectrophotometer, relative to untreated control

samples. Indomethacin (5-25 µg/ml) in 1% BSA was used as a reference standard. All assays were conducted in triplicate. The percentage of inhibition in albumin denaturation was calculated using the following equation: Albumin denaturation inhibition (%) = $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$ [1]

Where A_{control} : absorbance of denatured albumin without treatment, and A_{sample} : absorbance of denatured albumin including the test sample.

2.4.2.2. Red blood cells (RBCs) membrane stability test

Venous blood samples (~ 5-10 ml each) in anticoagulant-containing blood tubes were generously obtained from the clinical center at Mutah University/Karak-Jordan; samples were collected from healthy individuals attending the center for routine blood count checks. The blood tubes were centrifuged at 3,500 rpm for 10 min (Combi-514R, Hanil, South Korea), and the resulting cell pellets were washed three times and suspended in normal saline at a concentration of 10% (v/v). Following the method of Tantary *et al.* (2017), the erythrocyte membrane stabilization assay involved incubating 10% human RBC suspensions with 50-500 µg/ml of the test crude extract at 56°C for 30 min, followed by cooling under running tap water and centrifuging at 2500 rpm for 5 minutes. The decrease in the released hemoglobin was quantified at 560 nm. As a negative control, normal saline was added instead of the RBC suspension. The assay was carried out in triplicate.

2.4.2.3. Lipoxigenase inhibitor assay

The ability of the fungal crude extract to inhibit the lipoxigenase enzyme (LOX) activity was conducted as described by Gopi and Jisho (2018). The tested extract (50-500 µg/ml) was reacted for 5 min with ice-cold LOX enzyme (2000 U/ml in 0.2 M borate buffer). After that, 1 ml of ice-cold 0.6 M linoleic acid substrate, prepared in the same buffer, was added to the reaction mixture. The decrease in absorbance of the formed hydroperoxy-linoleic acid product was checked for 2 min at 234 nm to determine the % LOX activity inhibition. Nordihydroguaiaretic Acid (NDGA) was used as a reference standard, and the test was repeated three times.

2.4.2.4. Carrageenan-induced paw edema inflammation in rats

In vivo carrageenan-induced paw edema model was employed using 25 albino rats (aged 7-8 weeks, 180-200 g body weight). The rats were kept in cages in a 12 h light/dark cycle, with food and water, in a temperature-controlled room. The rats were divided into four groups, each of five rats. They were lightly anesthetized with ether before treatment with test samples. Rats were pretreated with the extract or indomethacin for 1 h before injecting 100 µl of 1% λ-carrageenan into the right hind paw. Group 1 was injected with 100 µl buffer saline solution containing 100 µg/g b.w crude extract, Group 2 received the same volume with 200 µg/g b.w extract, Groups 3 and 4 were treated with 10 µg/g b.w and 25 µg/g b.w of indomethacin, respectively, while Group 5 was injected with 100 µl of buffer saline solution into the right paw as an untreated control. The left paw in all rats received 100 µl of buffer saline as a negative control. Paw swelling in the right paw was measured at 0, 1, and 4 h post-injection using a digital caliper and compared to the left paw measurement for each rat in each group. The anti-

inflammatory potency of the tested samples was evident from the difference in paw thickness between the right and the left limb due to treatments with either fungal crude extract or the positive control (indomethacin) relative to that of the untreated Group 5.

2.5. Chemical analysis

2.5.1. Thin-layer chromatography (TLC)

Preliminary chemical screening of the main classes of compounds present in the crude extract was performed on silica gel 60 TLC plate (20 x 20 cm, Alugram® SIL G/UV254, Macherey & Nagel, Düren) following Jork *et al.* (1990). The TLC plate was developed in toluene: acetone: formic acid (70:30:1, v/v/v). The developed bands were visualized under UV light (254 and 366 nm) and were stained with specific spray reagents: 1% ethanolic solution of aluminum chloride for detecting flavonoids; a mixture of 0.5 ml p-anisaldehyde in 50 ml glacial acetic acid and 1 ml 97% sulfuric acid for the detection of phenols; 37% formaldehyde in concentrated sulfuric acid at a ratio (1:10) for the detection of alkaloids and aromatic hydrocarbons, terpenes, sugars, and steroids; mixture of 0.1g Bromocresol green in 500 ml ethanol and 5 ml of 0.1 M NaOH for the detection of organic acids; a solution of 1g sodium nitrite in 100 ml 1M HCl for the detection of thiazoles and indoles.

2.5.2. High-performance liquid chromatography (HPLC)

Chemical constituents of the ethyl acetate crude extract of *Fusarium* sp. NN-Fus-1 was analyzed using an HPLC instrument (Shimadzu Prominence-i LC-2030, Japan). The instrument is equipped with a Shim-pack GISS C18 column with dimensions of I.D. 4 x 125 mm and 5 µm particle size (Shimadzu, Japan) and a photodiode array detector (PDA). Analysis was performed using a mobile phase mixture of solvent A (0.1% formic acid solution) and solvent B (Acetonitrile). The run program was a linear gradient elution as follows: 0-1 min, 0-1% B; 1-11 min, 1-100% B; 5 min, 100% B; decreasing in solvent B from 100% to 1% within 2 min with a flow rate of 1 ml/min. The injection volume was 20 µl of the sample, the PDA detection wavelength was set to 190-600 nm, and the column temperature was 40°C. The sample was filtered pre-analysis through a 0.45 µm disposable PTFE filter.

2.6. Statistical analysis

Data were expressed as means ± standard deviations (SD). Statistical significance ($P < 0.05$) was determined using one-way ANOVA followed by Turkey's post-hoc test in SPSS ver. 26.0 (SPSS Inc., Chicago, IL, USA) software.

3. Results

During screening for endophytic fungi with potent anti-inflammatory activity, fungal isolate NN-Fus-1 was selected for further investigations. The crude extract of this isolate did not exhibit antibacterial activity nor stabilize the membrane of heat-induced RBC. However, it demonstrated pronounced DPPH radical scavenging and lipoxigenase activities starting at 100 µg/ml, along with 80-90% inhibition in the albumin denaturation test at 1 mg/ml, indicating promising anti-inflammatory properties. The fungal isolate NN-Fus-1 forms colonies with fluffy,

cottony aerial mycelia, displaying whitish pigmentation and yellow-colored edges after 3-4 weeks of cultivation on PDA medium. Microscopic examinations revealed lateral hyaline conidiophores with flexible, irregular secondary conidiophore branches. The macroconidia were hyaline, 1-2 celled, ovoid to pyriform shaped (13.6-30.9 μm long and 5.7-8.4 μm wide), and arose from slender phialides. The chlamydospores were yellowish brown, measuring 32-60 μm wide (Fig. 1).

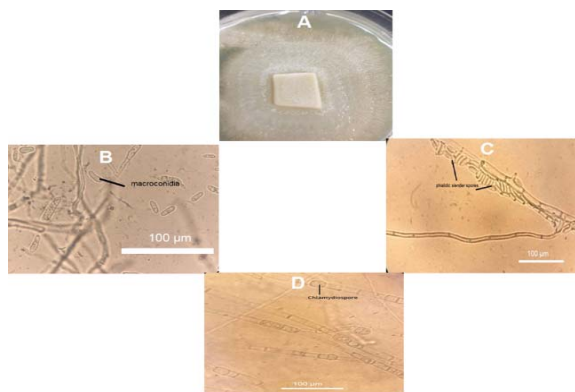


Figure 1. Microphotographs of *Fusarium* sp. isolate NN-Fus-1. A) fungal block grown on PDA medium at 100X magnification, B) macroconidia, C) an aggregate of spores forming the phialide, and D) chlamydospores.

Amplification of the partial 18S rRNA gene partial /sequence yielded a ~515 bp amplicon, which showed 98% similarity to *Fusarium redolens* isolate-FWN2 (Accession no. MN537039.1) upon alignment with sequences in the GeneBank (Fig. 2). The partial 18S rDNA gene sequence of the isolate was deposited in GenBank as *Fusarium* sp. isolate NN-Fus-1 (Accession number MW047084.1) (www.ncbi.nlm.nih.gov/nuccore/MW047084.1).

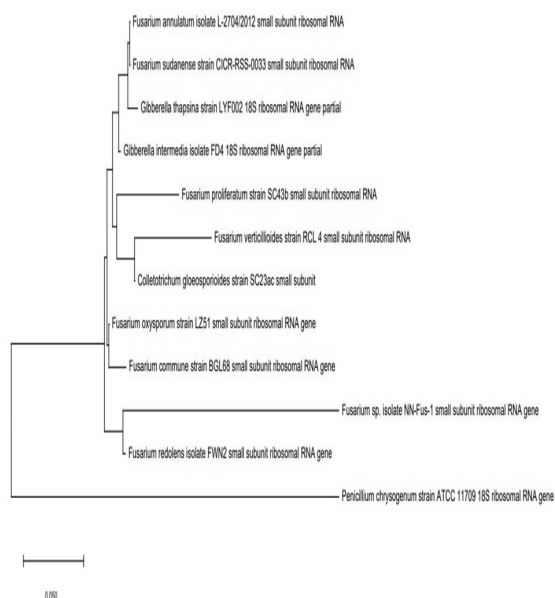


Figure 2. Phylogenetic tree of fungal isolate NN-Fus-1 constructed using the Maximum Likelihood Algorithm in MEGA7 software (Kumar *et al.*, 2018).

3.1. Biological activity

3.1.1. Antioxidant activity

Fusarium sp. isolate NN-Fus-1 crude extract exhibited significant antioxidant activity, neutralizing DPPH radicals with an IC_{50} value of $109.17 \pm 1.30 \mu\text{g/ml}$ ($6.41 \pm 0.08 \mu\text{g}$ Trolox/mg crude extract) (Table 1). However, the extract did not show scavenging activity against the nitric oxide radical at concentrations up to 500 $\mu\text{g/ml}$, suggesting differences in the type of metabolites active in the DPPH assay compared to NO radical scavenging.

3.1.2. Anti-inflammatory activity assay

3.1.2.1. Albumin denaturation inhibition

Fusarium sp. isolate NN-Fus-1 crude extract inhibited heat-induced albumin denaturation with an IC_{50} of $469.05 \pm 3.72 \mu\text{g/ml}$, indicating its ability to abate inflammatory reactions caused by the denaturation of cellular proteins. In comparison, indomethacin showed inhibition with an IC_{50} of $18.35 \pm 1.99 \mu\text{g/ml}$ (Table 2).

Table 1. DPPH radical scavenging activity of *Fusarium* sp. isolate NN-Fus-1 crude extract.

Sample	Concentration ($\mu\text{g/ml}$)	% Scavenging activity $\pm$ SD	IC_{50}	TEAC (μg Trolox/mg crude extract)
crude extract	50	33.73 ± 0.09^a	109.17 ± 1.30	6.41 ± 0.08
	100	57.20 ± 1.85^b		
	300	$76.41 \pm 1.68^{b,c}$		
	500	87.11 ± 1.59^c		

Post hoc Tukey HSD test analysis of scavenging activities, results with similar letters are not significantly different from each other.

Table 2. Inhibiting heat-induced albumin denaturation by *Fusarium* sp. isolate NN-Fus-1 crude extract.

Sample	Concentration ($\mu\text{g/ml}$)	% Inhibition $\pm$ SD	IC_{50} ($\mu\text{g/ml}$)
Crude extract	50	21.302 ± 1.454^a	$469.05 \pm 3.72^*$
	100	23.872 ± 3.15^a	
	300	38.435 ± 3.55^a	
	500	$53.741 \pm 5.98^{a,b}$	
	1000	85.551 ± 4.44^b	
Indomethacin	5	7.91 ± 0.65^c	18.35 ± 1.99
	10	14.22 ± 3.05^c	
	25	58.36 ± 3.98^d	
	50	76 ± 7.63^d	

Post hoc Tukey HSD test analysis of inhibition percentages; results with similar letters are not significantly different from each other. Asterisk indicated a statistically significant difference in IC_{50} value of the tested sample to the positive control ($p < 0.05$).

3.1.2.2. Lipoxxygenase inhibition assay

Lipoxxygenase (LOX) converts fatty acids into peroxide derivatives, including arachidonic acid to leukotrienes, which are inflammatory mediators. Using linoleic acid as a substrate, the crude extract of *Fusarium* sp. NN-Fus-1 inhibited LOX activity in a concentration-dependent manner, with an IC_{50} value of $83.51 \pm 1.36 \mu\text{g/ml}$. In

contrast, NDGA revealed LOX inhibitory activity at an IC_{50} of 0.11 ± 0.004 $\mu\text{g/ml}$ (Table 3).

Table 3. Inhibition of the lipoxygenase enzyme by the *Fusarium* sp. isolate NN-Fus-1 crude extract.

Sample	Concentration ($\mu\text{g/ml}$)	% Inhibition $\pm$ SD	IC_{50} ($\mu\text{g/ml}$)
Crude extract	50	38.77 ± 0.78^a	$83.51 \pm 1.36^*$
	100	56.38 ± 1.46^a	
	300	80.4 ± 1.41^b	
	500	87.77 ± 1.22^b	
NDGA	0.05	31.4 ± 0.32^c	0.11 ± 0.004
	0.1	47.31 ± 1.54^d	
	0.2	58.29 ± 3.95^d	
	0.3	83.9 ± 1.75^e	

Post hoc Tukey HSD test analysis of inhibition percentages; results with similar letters are not significantly different from each other. Asterisk indicated a statistically significant difference in IC_{50} value of the tested sample to positive control ($p < 0.05$).

3.1.2.3. Carrageenan-induced paw edema inflammation in rats

Treatment with the fungal crude extract (100 and 200 $\mu\text{g/g}$ b.w) ameliorates the thickness of the edema due to the carrageenan inflammatory effect, with a maximum

inhibition of 55.5% at 200 $\mu\text{g/g}$ b.w and an IC_{50} of 159.76 ± 3.6 $\mu\text{g/g}$ b.w. Indomethacin (25 $\mu\text{g/g}$ b.w) reduced paw thickness by 28%, with an IC_{50} of 58.05 ± 2.85 $\mu\text{g/g}$ b.w (Table 4).

Table 4. Inhibition of carrageenan-induced paw edema in a rat model ($n=5$).

Sample	Concentration ($\mu\text{g/g}$ b.w)	Inhibition (%) $\pm$ SD	IC_{50} ($\mu\text{g/g}$ b.w)
Crude extract	100	41.887 ± 4.0	$159.76 \pm 3.6^*$
	200	55.453 ± 3.2	
Indomethacin	10	18.176 ± 2.1	58.05 ± 2.85
	25	28.111 ± 3.6	

*Statistically significant difference in IC_{50} value of the tested sample to the positive control ($p < 0.05$).

3.2. Chemical analysis

Chemical analysis of the ethyl acetate crude extract of *Fusarium* sp. NN-Fus-1 revealed the presence of alkaloids, phenolic derivatives, flavonoids, and organic acids as the major detected constituents. The presence of phenolic compounds and flavonoids was highly detected at 280 nm (Fig. 3). In comparison, alkaloids were detected at 230 nm (Fig. 4). These wavelengths were chosen because the principal peaks exhibited maximum absorption, and the sample matrix effect is minimal. The eluted constituents were within the retention times 7.5-13.7 min for flavonoids as well as phenolic compounds, and 6.8-11.5 min for alkaloids.

<Chromatogram> mAU

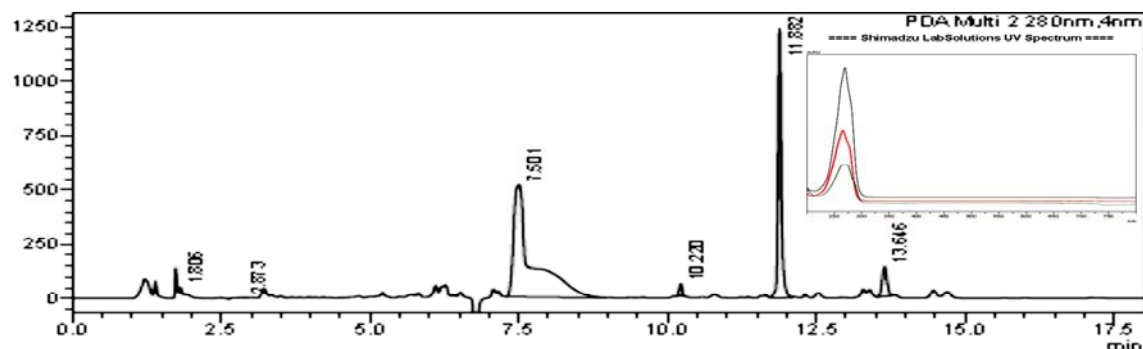


Figure 3. HPLC chromatogram of *Fusarium* sp. NN-Fus-1 ethyl acetate crude extract using an RP-C₁₈ column at 280 nm wavelength. Inset is the UV spectra of the major peaks.

<Chromatogram> mAU

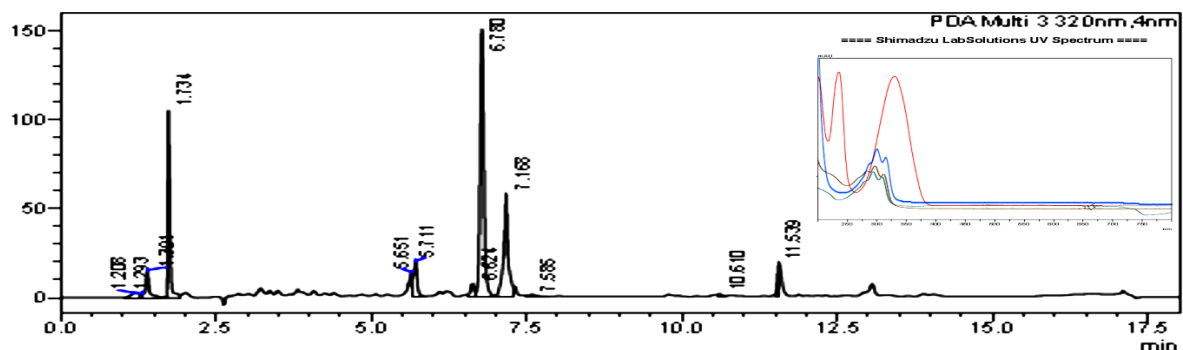


Figure 4. HPLC chromatogram of *Fusarium* sp. NN-Fus-1 ethyl acetate crude extract using an RP-C₁₈ column at 320 nm wavelength. Inset is the UV spectra of the major peaks.

4. Discussion

Human health is endangered by various infections and inflammatory disorders, often intensified by uncontrolled inflammatory responses to various noxious stimuli. Given the adverse effects associated with the consumption of synthetic anti-inflammatory drugs, the search for novel bioactive metabolites from unconventional sources, including microbial compounds, has become a vital issue. Intriguingly, over the years, microbial-derived therapeutic agents have improved human health (Dias *et al.*, 2019), with *Fusarium* species as prolific producers of drug precursors with potential pharmaceutical applications (Kyekyeku *et al.*, 2017).

The host cellular response to many inflammatory reactions triggers the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β), IL-6, tumor necrosis factor-alpha (TNF- α), interferons (INF- α and - β), nitric oxide (NO), prostaglandins, arachidonic acid metabolites, and proteolytic enzymes (Henrotin *et al.*, 2005). These mediators trigger increased vascular permeability, leucocyte infiltration, protein denaturation, membrane destabilization, and edema formation, as key hallmarks of inflammation (Bouriche *et al.*, 2016). Furthermore, oxidative stress resulting from an imbalance in free radical regulation contributes to chronic inflammatory disorders such as rheumatoid arthritis, asthma, allergy, and autoimmune diseases (Al-Qaralleh *et al.*, 2021). Therefore, the potential anti-inflammatory and antioxidant activities of secondary metabolites produced by the *Fusarium* sp. isolate NN-Fus-1 were evaluated herein.

Chemical analysis of the crude extract of *Fusarium* sp. isolate NN-Fus-1F revealed the presence of phenolic compounds, flavonoids, and alkaloids. The eluted constituents were within the retention times 7.5-13.7 min for flavonoids as well as phenolic compounds, and 6.8-

11.5 min for alkaloids, implying their nonpolar to moderate polar nature. Phenolic and flavonoids are successfully visualized in the chromatogram when analyzed at 280 nm (Al-Rimawi, *et al.*, 2024); an absorption band caused by electron transition of the benzoyl group in these groups of compounds (between 240-280 nm), in addition to absorption band corresponding to the cinnamoyl group (300-400 nm) (Feng *et al.*, 2017; Nistor and Butnariu, 2023), depicted in Fig.4 (red spectrum). However, most alkaloids revealed maximum UV absorptions at 210-230 nm, 240-290 nm, and 300-350 nm (Kabulov *et al.*, 1992; Hisiger and Jolicœur, 2007; Aigotti *et al.*, 2022).

The crude extract was identified as a potent source of antioxidant compounds in the DPPH radical scavenging assay (IC₅₀ = 109.16 μ g/ml), comparable to 6.41 $\pm$ 0.08 μ g Trolox equivalents per mg crude extract. However, it was not effective in scavenging NO radicals at concentrations up to 500 μ g/ml. This inconsistency suggests that the bioactive constituents preferentially neutralize hydrophobic radicals (DPPH being prepared in an organic solvent) rather than NO radicals derived from sodium nitroprusside in an aqueous solution, highlighting the hydrophobic nature of the metabolites (Baliga *et al.*, 2003).

The antioxidant potential of *Fusarium* sp. isolate NN-Fus-1F extract renders it a promising source for anti-inflammatory compounds. The extract's anti-inflammatory mechanism appears to involve direct protein stabilization at the site of inflammation, rather than reducing the release of lysosomal constituents from neutrophils that cause inflammation. The extract caused inhibition in heat albumin denaturation (IC₅₀ of 469.05 $\pm$ 3.72 μ g/ml), a process implicated in inflammatory diseases such as rheumatoid arthritis (Moharram *et al.*, 2017), but failed to stabilize RBCs membrane, where erythrocyte membranes model lysosomal membranes (Parvin *et al.*, 2015; Santos *et al.*, 2020). Moreover, the 5-LOX enzyme catalyzes the oxidation of polyunsaturated fatty acids into pro-inflammatory leukotrienes, exacerbating conditions such as rheumatoid arthritis, ulcerative colitis, asthma, and atherosclerosis (Gopi and Jisho, 2018). Intriguingly, the extract inhibited 5-LOX activity with an IC₅₀ of 83.51 $\pm$ 1.36 μ g/ml, likely by scavenging lipid-peroxy-radicals and disrupting the production of lipid hydroperoxides and leukotrienes (Ruma *et al.*, 2013). The dual antioxidant and LOX-inhibitory activity of the crude extract is correlated with *in vivo* anti-inflammatory effects, as demonstrated by a 55.4 % reduction in carrageenan-induced paw edema at 200 μ g/g b.w (IC₅₀ of 159.76 $\pm$ 3.6 μ g/g b.w) compared to indomethacin (28.1% inhibition at 25 μ g/g b.w). This suggests suppression of vascular permeability and leukocyte infiltration, key drivers of edema (Fatima *et al.*, 2021).

Coinciding with our findings, the ethyl acetate extract of the endophytic *Fusarium* sp. and *Aspergillus fumigatus* revealed the presence of phenolic compounds, which endow them with antioxidant properties and a capacity to neutralize DPPH radicals with IC₅₀ values of 20 μ g/ml and 42.25 μ g/ml, respectively. These metabolites also revealed anti-inflammatory potential by inhibiting LOX in a dose-dependent manner with IC₅₀ values of 62 μ g/ml and 63 μ g/ml, respectively (Ruma *et al.*, 2013). Several *Fusarium* species, including *F. redolens*, were isolated from the bulbs of *Fritillaria unibracteata* var. *wabuensis*. They revealed antioxidant activities in various scavenging assays, especially their ethyl acetate and ethanolic extracts, due to their content of phenolic and flavonoid compounds (Pan *et al.*, 2017). Meanwhile, the high flavonoid content in *Xylaria* isolated from the medicinal plant *Conyza blinii* was the primary cause of the radical scavenging activity of their crude extract in DPPH and ABTS assays, with IC₅₀ values of 110 μ g/ml and 200 μ g/ml, respectively (Tang *et al.*, 2020). Interestingly, the phenolic derivative fusagunolics B identified from the endophytic *F. guttiforme* revealed moderate anti-inflammatory activity by inhibiting NO production with an IC₅₀ value of 28.6 μ M (Yue *et al.*, 2024). In addition, the glycosylated flavonoid vitexin from *F. solani* caused a reduction in the pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-33) and enhanced the anti-inflammatory cytokine (IL-10) as a promising anti-inflammatory drug (Ahmed *et al.*, 2023).

However, rohitukine, an alkaloid produced by the endophytic *F. proliferatum*, displayed anti-inflammatory and immunomodulatory properties (Kumara *et al.*, 2012). Moreover, Azaphilone and Pseurotin A, alkaloids from *Penicillium sclerotiorum* cib-411 and *Aspergillus fumigatus*, exert their anti-inflammatory activity by

inhibiting NO production in LPS-induced RAW264.7 macrophages (Shi *et al.*, 2015; Tang *et al.*, 2019). Similarly, Neoechinulin A and Aspersiamides, marine-derived alkaloids, were isolated from *Eurotium* sp. SF-5989 and *A. versicolor*, respectively, inhibited the production of pro-inflammatory mediators (prostaglandins E-2, IL-1 β , NO, and TNF- α) and suppressed the expression of COX-2 and iNOS in lipopolysaccharide (LPS)-stimulated murine RAW264.7 cells (Souza *et al.*, 2020; Bai *et al.*, 2021). Furthermore, the marine isolate *Aspergillus* sp. SCSIO 41024 synthesized cyclopiazonic acid indole alkaloid derivatives, causing inhibition in the DPPH scavenging assay with IC₅₀ values ranging from 31.9–228.4 μ g/ml (Chen *et al.*, 2020).

In line with our results and as documented in all aforementioned studies, crude extracts encompassing alkaloid, phenolic, or flavonoid compounds are considered potential lead antioxidant agents to treat oxidative stress-associated inflammatory disorders, such as cardiovascular, cancer, and neurodegenerative diseases. In general, flavonoid synthesis involves both the acetate pathway to generate ring A and the shikimate pathway to generate the B ring; the two rings condense to form chalcone (a precursor of flavonoids) (Hu *et al.*, 2025), which is linked to the pyran C-ring. The hydrophilicity of glycosylated flavonoids decreases their passage through biological membranes, reducing their bioactivity, but improves their stability in cells. Meanwhile, the aglycone exhibits high bioactivity with poor bioavailability (Dias *et al.*, 2021). Therefore, glycosylated flavonoids undergo enzymatic hydrolysis in biological cells to aglycone congeners, which are degraded enzymatically, producing phenolic compounds of low molecular weight. The impact of flavonoid metabolism exhibited bioactivity rather than the parental flavonoids (Carregosa *et al.*, 2022).

In fact, flavonoids exert their antioxidant activity through radical stabilization, where the hydroxyl group donates electrons and hydrogen through resonance and generates stable phenoxy-radical flavonoids and phenolic acids. They can also activate electrophile-responsive elements to regulate enzymes involved in the generation of free radicals, including NAD(P)H oxidoreductase, mitochondrial succinoxidase, xanthine oxidase, and glutathione S-transferase, thereby resisting oxidative stress (Andrés *et al.*, 2023). The oxo-group of flavonoids participates in the conjugated system to enhance electron delocalization (Hu *et al.*, 2025). The number and position of the hydroxyl groups in the catechol B-ring and their position on the pyran C-ring influence the free radical scavenging ability (Dias *et al.*, 2021). Moreover, they inhibit ROS generation by chelating trace elements such as iron, leading to a reduction in the Fenton reaction kinetics (Lahey-Beitia *et al.*, 2021).

Notably, phenolic compounds and flavonoids can suppress mitogen-activated protein kinase (MAPK), NF- κ B, cyclooxygenase (COX), LOX, and inducible nitric oxide synthase (iNOS), as well as downregulate TNF- α , IL-1 β , and IL-6 (Al-Khayri *et al.*, 2022; Ysrafil *et al.*, 2023); thereby mediating their anti-inflammatory activity. The hydroxylation number on “B” and “C” rings, the presence of a double bond in C2–C3 and/or a ketone in C4 in the “C” ring are structural features that influence their anti-inflammatory potential (Silva *et al.*, 2021). The ability of these metabolites to decrease the production of

arachidonic acid and inhibit the crucial pro-inflammatory enzymes leads to a significant decrease in the production of prostaglandins, leukotrienes, and NO, the key inflammatory substances (Stachelska *et al.*, 2025).

On the other hand, synthesis of most alkaloids starts with amino acid precursors; however, terpenoid and steroidal alkaloids are biosynthesized from isoprenoid and steroidal backbones (Letchuman *et al.*, 2025). They undergo decarboxylation, cyclization, methylation, and oxidation reactions to form the characteristic heterocyclic cores of alkaloids. The nitrogen atoms of alkaloids can engage in hydrogen bonding and ionic interactions that influence their biological activities. They can interact with various biological systems, affecting enzyme activity and cellular signaling pathways (Leclerc and Pierre Fournier, 2024). Alkaloids stabilize the free radicals by donating the hydrogen of their OH and NH functional groups (Dalimunthe *et al.*, 2018) and exert their anti-inflammatory activity by reducing the production of iNOS and COX-2 (Souza *et al.*, 2020; Bai *et al.*, 2021).

5. Conclusion

Fusarium sp. isolate NN-Fus-1 crude extract demonstrates significant antioxidant and anti-inflammatory properties, likely mediated by its content of alkaloids, phenolics, and flavonoids. These metabolites can modulate oxidative stress, inhibiting LOX, reducing protein denaturation, and ameliorating the effect of carrageenan on the induction of paw edema in the mouse model. *Fusarium* sp. isolate NN-Fus-1 is a potential candidate for further pharmaceutical investigations. Future work exploring the mechanistic pathways of its activity and isolation of the lead bioactive compounds is required.

Conflict of interest

The authors declare no conflicts of interest.

Ethical Consent

The study was approved by the Scientific Ethics Committee in the Department of Biology/Mutah University. All animal experiments complied with the ARRIVE guidelines and were performed per the ethical and welfare standards in European Union legislation (EU Directive 2010/63/EU).

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Comprehensive Analysis of *HOXB13* Expression, Prognosis, and Pathogenic Variants in Prostate Adenocarcinoma Across Ethnically Diverse Populations

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Received: October 24, 2025; Revised: February 1, 2026; Accepted: February 23, 2026

Abstract

Background: *HOXB13* is a transcription factor known to play a critical role in prostate development and Prostate Adenocarcinoma (PRAD). However, a systematic evaluation of its expression patterns, prognostic relevance, and pathogenic variants across tissues, cancer types, and populations remains limited. This study hypothesized that *HOXB13* exhibits prostate-specific overexpression in PRAD with prognostic significance across ethnic groups and aimed to comprehensively characterize its expression profiles, prognostic value, and functionally pathogenic variants. **Methods:** We performed a multi-level analysis of *HOXB13* gene expression using bulk RNA-sequencing data from GTEx and TCGA datasets. Expression profiles were evaluated across normal human tissues, multiple cancer types, and stratified by patient race. Differential expression in PRAD was assessed, and survival analysis was conducted using Kaplan–Meier curves based on *HOXB13* expression and Gleason scores. In parallel, functional variant annotation was performed using eight biological scoring criteria (seven in silico prediction tools plus GTEx tissue expression) to identify high-impact SNPs. **Results:** *HOXB13* exhibited tissue-specific expression, with the highest levels observed in normal prostate tissue. In cancer expression profiling, *HOXB13* was markedly overexpressed in PRAD tumor samples compared to normal prostate and other cancer types. This overexpression was consistent across races, with the highest median levels noted in African American patients. Heatmap analysis confirmed strong upregulation of *HOXB13* and other tumor-associated genes (e.g., *TAGLN*, *S100A6*) in PRAD, while some genes (e.g., *ARHGAP10*, *PITPNC1*) were downregulated. Kaplan–Meier survival analysis revealed that patients with high *HOXB13* expression had better survival outcomes, particularly when combined with higher Gleason scores—a paradoxical finding suggesting *HOXB13* may mark less aggressive tumor phenotypes or reflect differentiation status, as its expression reportedly decreases in advanced/neuroendocrine PRAD subtypes associated with poor prognosis. Finally, a scoring system based on 12 annotation tools identified 23 SNPs with a perfect score of 8, including rs1207429780 and rs748782183, indicating strong potential for pathogenicity and functional relevance. **Conclusion:** *HOXB13* is a prostate-specific gene with significant overexpression in PRAD and potential prognostic value. Its expression is consistent across racial groups and correlates with improved survival when highly expressed. The identification of high-scoring pathogenic SNPs further underscores its importance in PRAD biology. These findings support *HOXB13* as a promising biomarker and potential therapeutic target in PRAD.

Keywords: *HOXB13*, Expression, Prognosis, Pathogenic Variants, Prostate Adenocarcinoma

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1. Introduction

Prostate Adenocarcinoma (PRAD) ranks as the second most frequently diagnosed cancer and stands as the fifth leading cause of cancer-related mortality among men worldwide. According to the most recent Global Cancer Observatory (GLOBOCAN 2022) estimates, approximately 1.47 million new cases of prostate cancer were diagnosed globally in 2022, accompanied by nearly 397,000 deaths (Bray *et al.*, 2024; Shah *et al.*, 2025). This substantial global burden underscores PRAD significant impact on men's health across diverse populations and regions. The primary contributors to morbidity and mortality in individuals with PRAD are the long-term and diverse complications arising from the disease, with genomic factors playing a pivotal role. Genetic alterations, including inherited mutations and somatic genomic changes, significantly influence PRAD development, progression, and response to treatment. These genomic factors interact with environmental exposures and other determinants, collectively shaping the clinical outcomes and complications experienced by patients (Chu *et al.*, 2025). Understanding the genomic landscape of PRAD is, therefore, critical for improving prognostication and tailoring personalized therapeutic strategies (Pritchard *et al.*, 2016; Wang *et al.*, 2023).

Single-nucleotide polymorphisms (SNPs) contribute to the development and progression of PRAD by affecting gene expression, messenger RNA (mRNA) stability, and translational efficiency. These genetic variations can alter cellular functions and pathways critical to tumorigenesis. For example, genome-wide association studies have identified over 100 common SNPs linked to PRAD susceptibility, many of which reside in noncoding regions that regulate gene expression (Allemailem *et al.*, 2021). Specific SNPs, such as those in the 8q24 region, have been strongly associated with increased risk and may serve as valuable biomarkers for diagnosis and therapy. Moreover, SNPs influence key biological processes including steroid metabolism, oxidative stress, cell adhesion, angiogenesis, cell cycle regulation, and DNA repair, all of which are implicated in PRAD pathogenesis (Allemailem *et al.*, 2021). Functional studies have demonstrated that certain SNP variants can modulate biochemical activities relevant to tumor growth and metastasis, exemplified by the rs17632542 variant affecting PSA function and clinical outcomes (Srinivasan *et al.*, 2024). Additionally, interactions between SNPs in genes involved in angiogenesis, such as *MMP16*, *ROBO1*, *CSF1*, and *EGFR*, have been linked to aggressive PRAD phenotypes, highlighting the complex genetic architecture underlying disease progression (Lin *et al.*, 2013). Understanding how SNPs impact gene regulation and cellular pathways is essential for improving risk prediction, screening, and personalized management of PRAD. Among the genetic factors implicated in prostate cancer pathogenesis, *HOXB13*, a prostate-lineage transcription factor, has emerged as a key regulator of prostate development and tumorigenesis. *HOXB13* exhibits prostate-specific expression and plays critical roles in cellular differentiation, androgen signaling, and tumor progression (Patel *et al.*, 2024). Recent interactome analyses in prostate cancer cells identified multiple *HOXB13* protein partners (including *TBX3*) and confirmed its functional

relevance for prostate cancer cell growth (Erokhin *et al.*, 2025). Germline mutations in *HOXB13*, particularly the G84E variant, are strongly associated with hereditary prostate cancer predisposition across diverse populations, providing a compelling biological rationale for its systematic investigation in PRAD (Heise *et al.*, 2019). This study focuses specifically on *HOXB13* to comprehensively characterize its expression patterns, prognostic significance, and pathogenic variants across ethnically diverse PRAD cohorts.

The identification of genomic variants associated with cancer has traditionally concentrated on mutations within protein-coding genes, particularly somatic mutations that drive tumor development. Databases such as the Catalogue of Somatic Mutations In Cancer (COSMIC) have been instrumental in prioritizing these somatic variants within coding regions for further study and clinical relevance (Tate *et al.*, 2019). However, it is now recognized that the vast majority of the human genome consists of non-coding regions, which include non-coding RNAs and other regulatory elements that are not yet fully annotated. Importantly, over 90% of genomic variants are located within these non-coding regions (Maurano *et al.*, 2012). This realization highlights the critical need to investigate the functional impact of genomic variants outside of protein-coding sequences, especially germline variants that may predispose individuals to cancer. Understanding how these non-coding variants contribute to cancer susceptibility and progression is essential for comprehensive genomic profiling and the development of personalized medicine approaches. Emerging research has revealed that germline variants—heritable genetic alterations present in normal cells—play a significant role not only in increasing cancer susceptibility but also in influencing tumor progression and patient outcomes across various cancer types, including PRAD. These inherited variants can interact with somatic mutations, shaping the landscape of genomic aberrations that drive oncogenesis. For instance, germline mutations in tumor suppressor genes such as *BRCA1* and *BRCA2* have been shown to predispose individuals to cancer and are often accompanied by loss of the wild-type allele in tumors, consistent with Knudson's two-hit hypothesis (Chatrath *et al.*, 2021; Lichtenstein *et al.*, 2000; Huang *et al.*, 2018).

Genetic variants play a crucial role in the development of cancer and other disease by inducing alterations at the molecular level (Halla and Wathiq, 2025; Usha and Rajeev, 2025; Sözen, 2024). These changes can manifest as single nucleotide mutations or larger chromosomal abnormalities. While the majority of these mutations, known as passenger mutations, accumulate during cancer progression without directly contributing to tumorigenesis, a smaller subset—referred to as driver mutations—actively promotes oncogenesis by conferring growth advantages to cancer cells (Vogelstein *et al.*, 2013). Distinguishing these driver mutations from the extensive background of passenger mutations is essential for understanding cancer biology and identifying therapeutic targets. In this study, we employed bioinformatics approaches to systematically identify putative genomic variants that are associated with increased susceptibility to PRAD. This study addresses these gaps through a comprehensive, multi-level analysis of *HOXB13* expression, prognosis, and pathogenic variants in PRAD across

ethnically diverse populations. We hypothesized that *HOXB13* demonstrates prostate-specific overexpression with complex prognostic significance, systematically characterizing: (1) tissue/cancer expression profiles; (2) race-stratified survival associations with Gleason scores; and (3) high-confidence pathogenic SNPs via eight-criteria bioinformatics pipeline.

2. Method

2.1. Identification and Functional Annotation of *HOXB13*

Despite advances in genomic technologies, interpreting the functional significance of genomic variants, particularly those with potential pathogenic effects, remains a complex challenge. To address this, we developed an integrative bioinformatics pipeline to identify and prioritize potentially pathogenic variants in the *HOXB13* gene, which has been implicated in PRAD.

Variant data for *HOXB13* were retrieved from the ENSEMBL database (accessed: Oct 15, 2025), and analysis was limited to non-synonymous SNPs, especially missense variants, due to their potential to alter protein function. Disease-related annotations were further obtained from PubMed, the Genome-Wide Association Studies (GWAS) catalog (accessed: Oct 18, 2025) (Buniello *et al.*, 2019), and the Phenome-Wide Association Studies (PheWAS) catalog (accessed: Oct 19, 2025) to evaluate prior evidence of clinical or phenotypic associations (Denny *et al.*, 2013).

To functionally assess the impact of variants, we incorporated several widely used in silico prediction tools: (I) Sorting Intolerant From Tolerant (SIFT) predicts whether an amino acid substitution affects protein function based on sequence homology and amino acid properties (Ng and Henikoff, 2003); (II) Polymorphism Phenotyping (PolyPhen) assesses the likely structural and functional consequences of substitutions using physical and evolutionary information (Adzhubei *et al.*, 2010); (III) Combined Annotation Dependent Depletion (CADD) integrates multiple genomic annotations into a composite score estimating the deleteriousness of single nucleotide variants (SNVs) and insertion/deletion (indels) mutations (Kircher *et al.*, 2014); (IV) Rare Exome Variant Ensemble Learner (REVEL) combines predictions from various algorithms to improve the accuracy of missense variant classification (Ioannidis *et al.*, 2016); (V) META-LR uses logistic regression to aggregate outputs from multiple prediction methods for more reliable pathogenicity estimates (Ioannidis *et al.*, 2016; Dong *et al.*, 2015); (VI) Mutation Assessor evaluates functional impact based on evolutionary conservation across species (Reva *et al.*, 2011); and (VII) AlphaMissense applies deep learning models and AlphaFold protein structure predictions to infer how missense variants may influence protein structure and function (Frazer *et al.*, 2021). Additionally, tissue-specific expression was evaluated using the Genotype-Tissue Expression (GTEx) Portal (GTEx Consortium, accessed: Oct 20, 2025), which confirmed that *HOXB13* exhibits its highest expression levels in prostate tissue. This expression profile provided functional validation of gene relevance to prostate-specific biology and was incorporated as a supporting criterion in variant prioritization.

Each variant was scored using a binary system (1 = deleterious, 0 = benign) across *eight biological scoring criteria* comprising seven in silico prediction tools (SIFT, PolyPhen, CADD, REVEL, META-LR, Mutation Assessor, AlphaMissense) plus GTEx prostate tissue expression. The resulting cumulative score (range: 0-8) classified variant pathogenicity, with scores of 8 identifying high-confidence pathogenic candidates. This integrated scoring approach enabled systematic prioritization based on sequence conservation, biochemical effect, structural integrity, disease association, and tissue-specific expression. A schematic overview of this analytical workflow is presented in Figure 1.

2.2. Gene Expression Analysis of *HOXB13*

To evaluate the normal tissue expression of *HOXB13*, RNA-sequencing data were obtained from the GTEx Portal. Transcript abundance was assessed across multiple human tissues to determine baseline expression profiles. This analysis confirmed that *HOXB13* is highly expressed in prostate tissue, reinforcing its potential role in prostate-related pathophysiology. To further examine gene expression in the disease context, we used the UALCAN web resource to access the Cancer Genome Atlas (TCGA) PRAD dataset (accessed: Oct 22, 2025) (Chandrashekar *et al.*, 2022). *HOXB13* expression levels were compared between the tumor and adjacent normal prostate tissues. This comparison enabled the evaluation of differential expression and the identification of potential dysregulation of *HOXB13* in malignancy. Expression patterns were visualized across sample groups to highlight tumor-specific trends.

2.3. Prognostic Evaluation based on *HOXB13* Expression

To assess the prognostic value of *HOXB13*, gene expression data from TCGA-PRAD were further analyzed in relation to clinical outcomes. Using UALCAN's clinical analysis tools (accessed: Oct 25, 2025) (Chandrashekar *et al.*, 2022), we correlated *HOXB13* expression levels with Gleason scores and overall survival. Patients were stratified by high versus low/medium *HOXB13* expression and further categorized based on Gleason grade (ranging from 6 to 10). Kaplan-Meier survival curves were generated to evaluate differences in survival outcomes, with statistical significance determined using log-rank tests. In addition, expression levels of *HOXB13* were compared between racial subgroups, specifically Caucasian and African American prostate cancer patients, to explore potential population-level disparities. Although limited by a small sample size in the African American cohort, this analysis served as a preliminary assessment of race-based expression variation in *HOXB13* within PRAD.

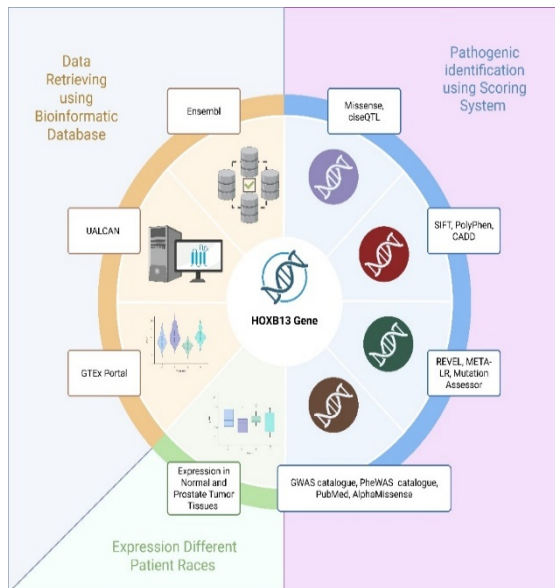


Figure 1. Study workflow and bioinformatics analysis pipeline. The workflow of the present study illustrates the sequential integration of multiple publicly available databases and bioinformatics prediction tools. These included ENSEMBL, PubMed, the Genome-Wide Association Studies (GWAS) Catalog, and the Phenome-Wide Association Studies (PheWAS) Catalog, along with several established variant-effect prediction tools, including SIFT, PolyPhen, CADD, REVEL, META-LR, Mutation Assessor, and AlphaMissense.

3. Result

3.1. Gene expression of *HOXB13* revealed highest expressed on the prostate tissue

Gene expression analysis of *HOXB13* across various human tissues reveals that it is most highly expressed in the prostate (Figure 2). This is clearly illustrated by the significantly elevated expression levels in the prostate compared to all other tissues examined. Moderate expression is also observed in related reproductive and urinary tissues such as the vagina, cervix, bladder, and testis, but these levels are substantially lower than in the prostate. In contrast, most other tissues—including liver, lung, heart, brain, muscle, and blood—show minimal to negligible expression of *HOXB13*. This distinct tissue-specific expression pattern underscores the critical role of *HOXB13* in prostate biology, where it is involved in regulating development, differentiation, and androgen receptor signalling. Given its predominant expression in the prostate and its known association with PRAD risk, *HOXB13* represents an important biomarker and potential therapeutic target. Furthermore, its limited expression in non-prostate tissues suggests that interventions targeting *HOXB13* may have minimal off-target effects, enhancing their clinical appeal. Overall, these findings highlight the specialized function of *HOXB13* in the prostate and its significance in PRAD research and treatment strategies.

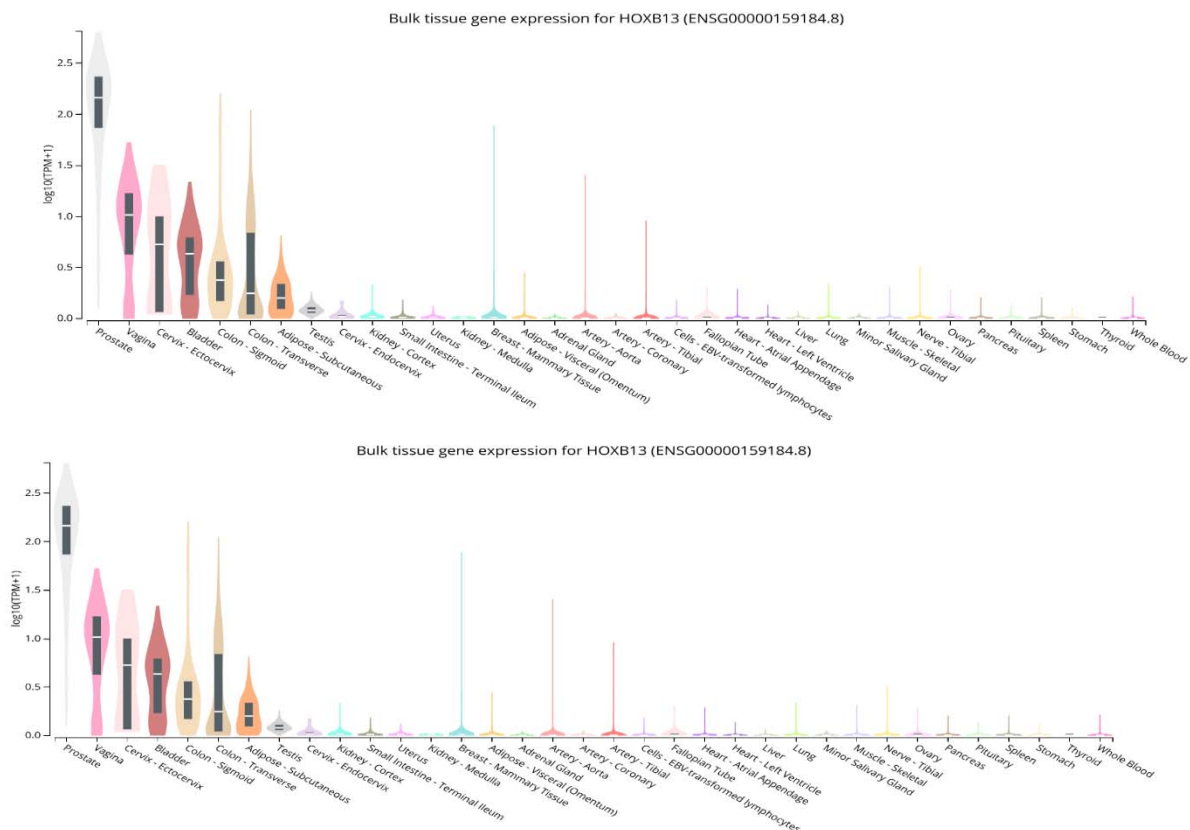


Figure 2. Violin plot depicting the bulk tissue gene expression levels of *HOXB13* gene across multiple human tissues. The y-axis represents the log₁₀-transformed TPM (Transcripts Per Million) values plus one [$\log_{10}(\text{TPM}+1)$], providing normalized expression data. Each violin illustrates the distribution of expression values within a given tissue, with the white dot indicating the median expression and the black bar representing the interquartile range. The plot reveals that *HOXB13* is most highly expressed in prostate tissue compared to all other tissues, highlighting its tissue-specific expression pattern. Moderate expression is observed in select reproductive and urinary tissues, while expres-

sion in other tissues remains low or negligible. This expression profile underscores the gene's specialized role in prostate biology and its relevance to PRAD research.

3.2. Expression profile of *HOXB13* across human cancers

To evaluate the tissue- and cancer-specific expression patterns of *HOXB13*, we analyzed transcriptomic data from The TCGA across 33 tumor types. Gene expression levels were normalized and presented as $\log_2(\text{TPM} + 1)$ for comparative analysis. Figure 3A illustrates *HOXB13* expression in matched tumors (red boxes) and normal tissue samples (blue boxes) across various cancer types. Among all tumor types examined, PRAD exhibited the highest *HOXB13* expression. PRAD tumor tissues demonstrated markedly elevated median expression levels compared to their normal counterparts, as well as to all other tumor types. This strong differential underscores the prostate-specific and cancer-enriched expression of *HOXB13*, supporting its role as a lineage-survival gene and a candidate biomarker for PRAD.

In contrast, for most other cancer types—including bladder (BLCA), breast (BRCA), colon (COAD), lung (LUAD, LUSC), liver (LIHC), and kidney cancers (KICH, KIRC, KIRP)—*HOXB13* expression remains low in both tumor and normal samples, as indicated by the short height of the boxes and low median values. Notably, in some cancers such as colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ), normal tissues show slightly higher *HOXB13* expression compared to their corresponding tumors, but these levels are still much lower than those observed in PRAD. A few other cancer types, such as sarcoma (SARC) and skin cutaneous melanoma (SKCM), show moderate expression in tumor samples, but

again, these do not approach the levels seen in PRAD. The consistently low expression outside of prostate tissues reinforces the gene's tissue-specific regulatory function.

Figure 3B presents *HOXB13* expression exclusively across TCGA tumor samples, allowing a focused comparison of gene activity within the malignant tissue landscape. As expected, PRAD remained the dominant outlier, with uniformly high expression across nearly all tumor samples. This finding aligns with the known biological function of *HOXB13* in prostate development and androgen receptor signaling. Several other tumor types, including rectum adenocarcinoma (READ), pheochromocytoma and paraganglioma (PCPG), uveal melanoma (UVM), and uterine corpus endometrial carcinoma (UCEC), showed moderate expression with wider inter-sample variability, suggesting potential subtype-specific roles or regulatory heterogeneity. Conversely, cancers such as glioblastoma (GBM), lower-grade glioma (LGG), kidney chromophobe (KICH), diffuse large B-cell lymphoma (DLBC), and thymoma (THYM) show very low or near-absent *HOXB13* expression, suggesting the gene has limited or no involvement in these tumor types. Some cancers, like breast cancer (BRCA), bladder cancer (BLCA), and colon adenocarcinoma (COAD) demonstrate moderate expression with wider variation among samples, which may reflect tumor heterogeneity or subtype differences. In summary, this pattern suggests that *HOXB13* is a gene with selective overexpression, most notably in PRAD, and may hold potential as a biomarker or therapeutic target in certain malignancies.

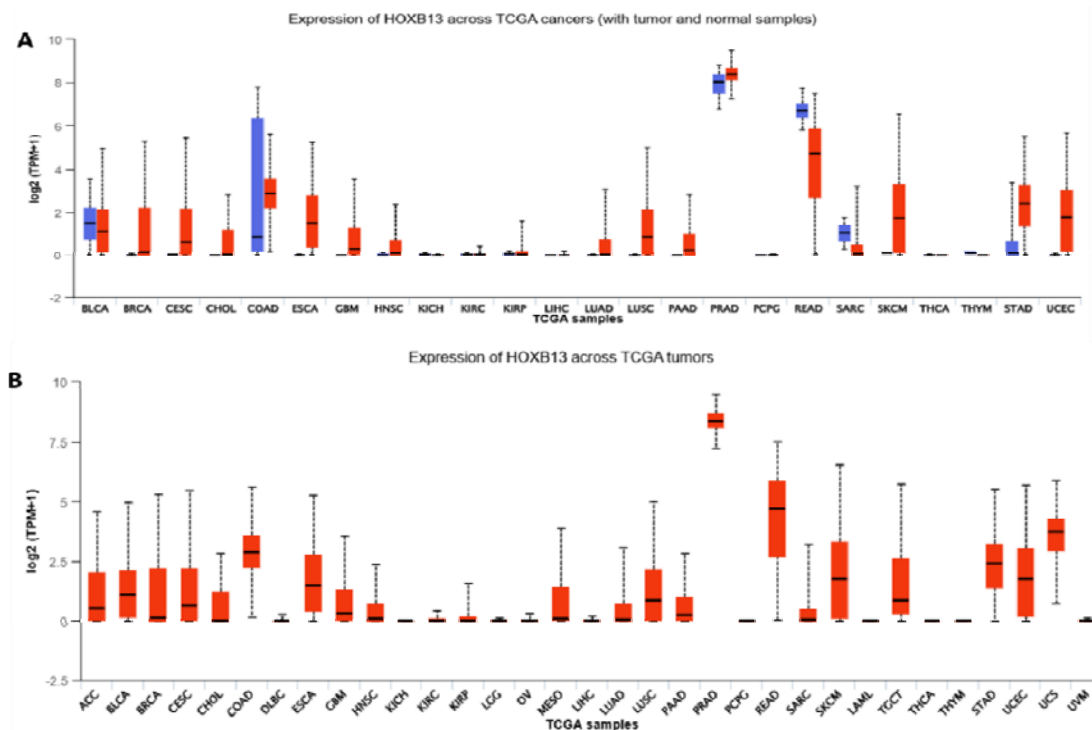


Figure 3. A. Expression of the *HOXB13* gene across TCGA cancers with tumor and normal samples. Box plots represent *HOXB13* gene expression levels measured as $\log_2(\text{TPM} + 1)$ across multiple cancer types from TCGA. Red boxes indicate tumor samples, while blue boxes represent normal tissue samples. Cancer types are labeled along the x-axis using TCGA abbreviation codes (e.g., BLCA: Bladder Cancer, PRAD: Prostate Adenocarcinoma). The plot demonstrates that *HOXB13* is most highly expressed in PRAD compared to other cancer types, with notable expression also observed in rectum adenocarcinoma (READ) and cholangiocarcinoma (CHOL) normal tissues. Each box represents the interquartile range, with the horizontal line indicating the median expression value, and whiskers extending to show data distribution.

tion within 1.5 times the interquartile range. **B.** Expression of *HOXB13* across various TCGA tumor types. Gene expression levels are shown as $\log_2(\text{TPM}+1)$. *HOXB13* is highly expressed in PRAD, with variable expression in other cancers and minimal expression in several tumor types.

3.3. Analysis of *HOXB13* Expression in Normal and Prostate Tumor Tissues Across Different Patient Races

Figure 4A presents a comparison of *HOXB13* gene expression between normal prostate tissues and primary prostate tumor tissues (PRAD) based on TCGA data. Expression levels are measured in Transcripts Per Million (TPM). The results show that *HOXB13* expression is significantly higher in primary tumor samples (n=497) compared to normal tissues (n=52). The median expression in tumor samples is notably elevated, with a narrower distribution range, indicating consistent overexpression across most cancer cases. In contrast, normal tissues exhibit a broader range of expression with several samples showing low or near-zero levels. This suggests that *HOXB13* is markedly upregulated in PRAD and may play a role in tumor development or progression. These findings support the potential use of *HOXB13* as a diagnostic biomarker or therapeutic target in PRAD.

Figure 4B displays *HOXB13* expression in PRAD tumor samples stratified by patient race, again based on

TCGA data. Samples are categorized into three groups: normal tissue (n = 52), Caucasian patients (n = 147), and African American patients (n = 6). The y-axis shows gene expression in TPM. The analysis reveals elevated *HOXB13* expression in both racial groups compared to normal tissues. Median expression is highest in African American patients, followed closely by Caucasian patients, while the normal group shows the lowest expression overall. Interquartile ranges and whiskers indicate variability within each group, with tumor samples displaying greater expression levels and variability than normal samples. Although the African-American group is represented by a small sample size (n = 6), the trend suggests that *HOXB13* overexpression in PRAD may occur across racial groups, potentially with slightly higher expression in African-American patients. These findings warrant further validation with larger sample sizes but highlight the consistent upregulation of *HOXB13* in PRAD and its relevance across different racial backgrounds.

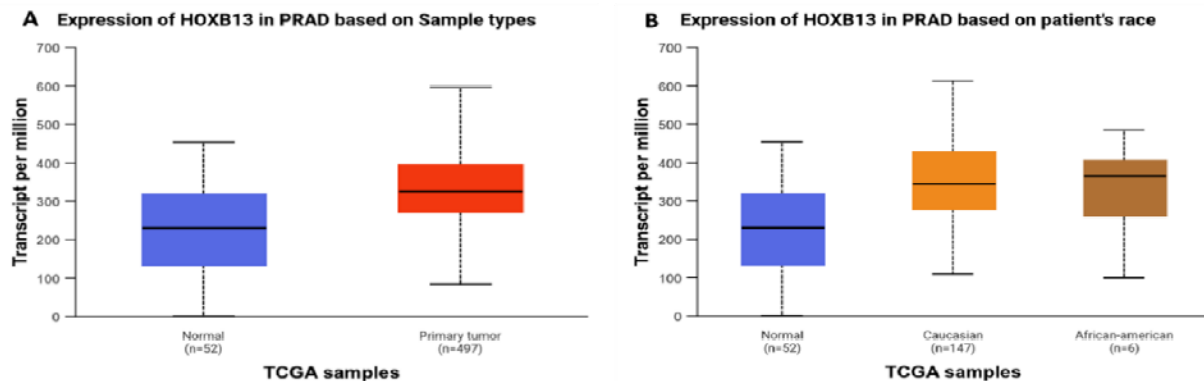


Figure 4. A. Box plot showing *HOXB13* expression (TPM) in normal (n=52) and primary prostate tumor tissues (n=497) from the TCGA PRAD dataset. *HOXB13* is significantly overexpressed in tumors, suggesting a role in PRAD. B. *HOXB13* expression in normal tissue and PRAD tumors from Caucasian (n=147) and African-American (n=6) patients. Elevated expression is observed in both tumor groups, with the highest median in African-American patients. Data accessed on 10 June 2025 from UALCAN.

3.4. Heatmap of Gene Expression Patterns in Normal and Tumor Samples of PRAD

Figure 5A presents a heatmap showing the expression pattern of selected input genes in PRAD, comparing normal and tumor samples from the TCGA dataset. The x-axis represents individual samples categorized into normal and tumor groups, while the y-axis lists the input genes, including *HOXB13*, *SCCPDH*, *TRNT1*, *GDPPD1*, and others. Gene expression levels are visualized using a color scale based on $\log_2(\text{TPM}+1)$, where blue indicates low expression and red indicates high expression. Based on the heatmap, it is evident that *HOXB13* is highly overexpressed in tumor tissues compared to normal tissues, as shown by the strong red signal in the tumor group and minimal expression (blue) in the normal group. This reinforces the earlier finding that *HOXB13* is significantly upregulated in PRAD. Several other genes also show altered expression between normal and tumor samples. For example, *GDPPD1* and *C15orf37* exhibit relatively higher expression in normal samples (deep blue in tumors), suggesting possible downregulation in cancer. In contrast, some genes like *UBE2Z* and *TRPC4AP* show more uniform or

slightly increased expression in tumors. Overall, this heatmap provides a comparative overview of gene expression changes in PRAD, highlighting potential candidate genes, especially *HOXB13*, that may contribute to tumor development or progression. These patterns can inform further investigations into the biological functions and clinical relevance of these genes in PRAD.

Figure 5B further highlights genes with marked differential expression. In addition to *HOXB13*, genes such as *TAGLN*, *S100A6*, and *EMILIN1* are consistently upregulated in tumor tissues, indicated by strong red coloration. These genes may be involved in oncogenic signaling, cytoskeletal remodeling, or stress response pathways relevant to prostate cancer progression. In contrast, genes including *NAAALDL1*, *PITPNC1*, and *ARHGAP10* exhibit reduced expression in tumor samples, suggesting downregulation during malignant transformation and potential roles in maintaining normal cellular homeostasis. Overall, the heatmap analyses in both panels provide a comprehensive view of transcriptional changes between normal and tumor prostate tissues. The observed expression shifts emphasize the deregulation of genes involved in structural organiza-

tion, signaling, and tumor biology. Notably, the prominent upregulation of *HOXB13*, along with several co-regulated genes, highlights a panel of candidate biomarkers and

therapeutic targets that warrant further functional validation in PRAD research.

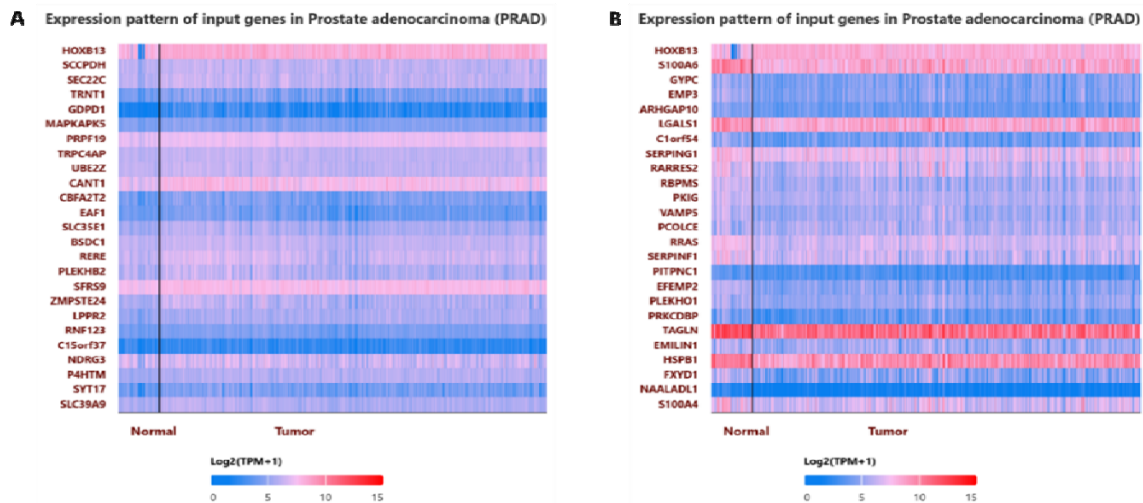


Figure 5. Heatmaps displaying the expression patterns of selected genes in normal and tumor prostate tissues from the TCGA PRAD dataset. Panel A shows gene expression as $\log_2(\text{TPM}+1)$ with each column representing a sample and each row a gene. *HOXB13* is notably overexpressed in tumor samples compared to normal tissue, suggesting its role in Prostate Adenocarcinoma. Panel B highlights additional genes with differential expression: *HOXB13*, *TAGLN*, *S100A6*, and *EMILIN1* are upregulated in tumors, whereas *PTPN1* and *NAAALDL1* are downregulated. These expression patterns identify potential biomarkers and therapeutic targets involved in PRAD progression.

3.5. Survival Analysis of PRAD Patients Based on *HOXB13* Expression and Gleason Score

Figure 6 presents a Kaplan–Meier survival analysis illustrating the combined effect of *HOXB13* gene expression levels and Gleason scores on overall survival in PRAD patients. The x-axis represents time in days, while the y-axis shows survival probability. The patients are stratified into subgroups based on *HOXB13* expression (high vs. low/medium) and Gleason score (ranging from 6 to 10). Each curve represents a different subgroup, and the number of patients (n) in each group is indicated in the legend. A statistically significant p-value of 0.00031 indicates a meaningful difference in survival among the groups. From the plot, it is clear that patients with low or medium *HOXB13* expression and high Gleason scores (particularly 9 and 10) have poorer survival outcomes, as shown by steeper declines in survival probability. For instance, the "Low/Medium expression + Gleason-score10" group (n=4)

and "Low/Medium expression + Gleason-score9" group (n=98) show the most rapid decrease in survival, suggesting a negative prognostic impact of low *HOXB13* expression when combined with high tumor aggressiveness. In contrast, patients with high *HOXB13* expression, even with higher Gleason scores, tend to have better survival probabilities, suggesting a potential protective or prognostic role of elevated *HOXB13* expression in certain contexts.

Overall, current findings highlighted, this analysis underscores the potential of *HOXB13* expression level as a prognostic biomarker in PRAD, especially when interpreted in conjunction with the Gleason score. The findings suggest that patients with high *HOXB13* expression tend to have better survival outcomes, even when their tumors are histologically more aggressive, highlighting the clinical relevance of integrating molecular and pathological data in patient risk stratification.

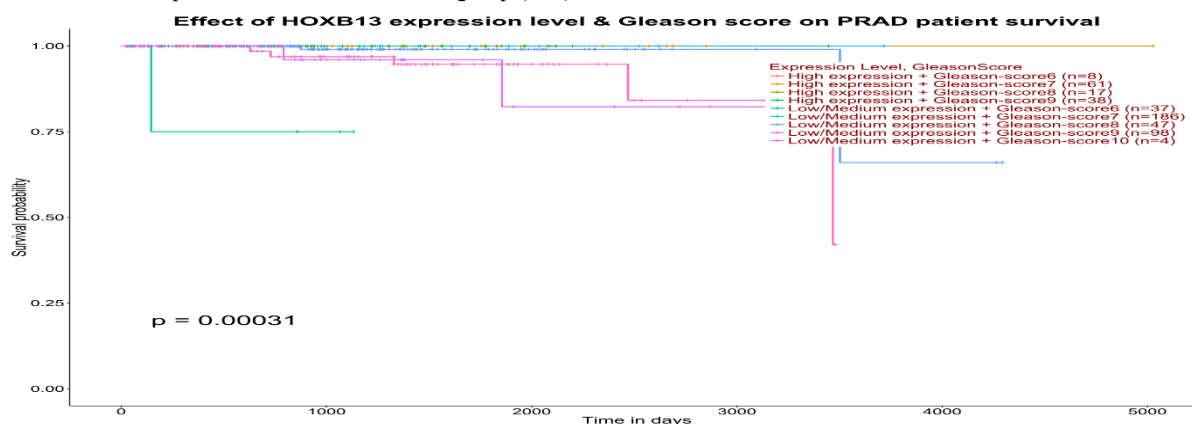


Figure 6. Kaplan–Meier plot showing overall survival of PRAD patients stratified by *HOXB13* expression levels (high vs. low/medium) and Gleason scores (6–10) using TCGA data. Each colored line represents a patient subgroup, with sample size (n) indicated in the legend. Patients with low/medium *HOXB13* expression and high Gleason scores (especially 9 and 10) exhibit significantly poorer survival compared to other groups. In contrast, patients with high *HOXB13* expression generally show better survival outcomes. The log-rank test yields a significant

cant p-value of 0.00031, indicating a statistically meaningful difference in survival across subgroups. These results suggest that *HOXB13* expression may serve as a potential prognostic biomarker in PRAD when combined with Gleason grading.

3.6. Pathogenic variant identification using a functional scoring system

Figure 7 presents a bar chart summarizing the number of *HOXB13* SNPs identified using various biological and computational criteria, reflecting their functional and clinical relevance. The x-axis denotes different tools and databases used for SNP evaluation, while the y-axis represents the number of SNPs identified under each scoring criterion. Among all tools, AlphaMissense identified the largest number of SNPs (843), followed closely by the Missense annotation category (835), emphasizing the focus on coding variants predicted to alter amino acid sequences. Established predictive tools such as PolyPhen and SIFT also yielded a high number of variants (592 and 526, respectively), highlighting their ongoing utility in assessing the potential functional consequences of amino acid substitutions.

Ensemble and integrative tools, including MetaLR (467 SNPs), REVEL (334 SNPs), and CADD (101 SNPs), further contribute to the identification of functionally relevant variants, demonstrating the value of combining multiple predictive approaches. In contrast, tools with limited variant yield included Mutation Assessor (34 SNPs), PubMed (5 SNPs), GWAS (1 SNP), and PheWAS (0 SNPs). The relatively low representation from clinical association databases and literature-based resources reflects the current gap between computational prediction and experimentally validated or population-level data.

The data highlights a computational prioritization framework in which algorithm-based predictions, particularly those from AlphaMissense, play a central role in fil-

tering genetic variants. Although these tools are highly effective at identifying variants with potential functional effects, the limited number of SNPs supported by clinical or literature evidence points to several important needs: expanded experimental validation studies, greater representation of diverse populations in genetic association databases, and the incorporation of multi-omics data beyond purely computational predictions. While this multi-faceted strategy efficiently narrows down candidate variants, it also reveals significant gaps between in silico predictions and variants with confirmed clinical relevance.

The result highlights a group of SNPs that received the highest functional annotation score of 8, indicating their strong potential for biological impact. These SNPs fulfilled all eight evaluation criteria used in the scoring system, namely Missense, SIFT, PolyPhen, CADD, REVEL, META-LR, Mutation Assessor, and AlphaMissense. The SNPs that achieved this top score include: rs1207429780, rs1365586165, rs1371765250, rs155558450, rs1597932515, rs1597932533, rs1597932564, rs1597932808, rs1597932884, rs2038215417, rs2143065331, rs2143066906, rs2143067303, rs747483887, rs748782183, rs749518336, rs754598240, rs757776260, rs778197145, and rs2143065697. Notably, some SNPs such as rs748782183, rs778197145, and rs1597932533 appear more than once in the dataset, suggesting potential occurrences of multiple allelic combinations or repeated annotations. The consistently high scores across multiple predictive tools emphasize the potential significance of these variants in genetic or disease association studies, warranting further functional validation.

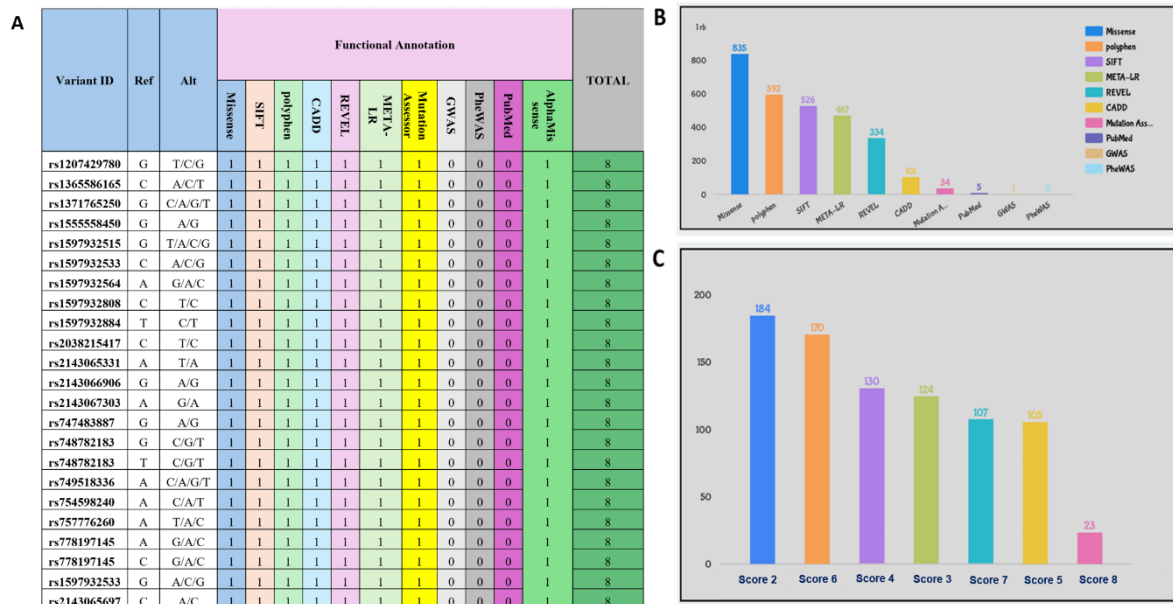


Figure 7. A-B. Bar chart showing the number of Single Nucleotide Polymorphisms (SNPs) identified under various biological scoring criteria used to evaluate their functional significance. The criteria include computational prediction tools (e.g., SIFT, PolyPhen, CADD, REVEL, Mutation Assessor, MetaLR), clinical and literature-based sources (e.g., GWAS, PubMed, PheWAS), and integrated models such as AlphaMissense. AlphaMissense and Missense categories contribute the highest number of SNPs (843 and 835, respectively), highlighting their prominent roles in functional annotation. In contrast, categories like PheWAS and GWAS report minimal or no SNPs, indicating limited coverage or association. This scoring distribution reflects a comprehensive approach to SNP prioritization across multiple biological relevance indicators. C. List of high-confidence SNPs with maximum functional impact scores. The figure displays SNPs that achieved the highest possible score (8 out of 8) across multiple biological annotation tools, including Missense, SIFT, PolyPhen, CADD, REVEL, ME-

TA-LR, Mutation Assessor, and AlphaMissense. These variants are prioritized as likely to have significant functional consequences and are potential candidates for further investigation in disease-related studies.

4. Discussion

This study provides a comprehensive multi-level evaluation of *HOXB13* expression across normal human tissues, multiple cancer types, patient racial backgrounds, and its potential clinical relevance in prostate adenocarcinoma (PRAD). Consistent across GTEx and TCGA datasets, *HOXB13* exhibits a striking prostate-restricted expression pattern, with the highest expression observed in normal prostate tissue and marked upregulation in PRAD tumors. The near absence of *HOXB13* expression in non-prostatic tissues and non-PRAD malignancies reinforces its tissue specificity and establishes *HOXB13* as a robust diagnostic biomarker for confirming prostatic origin, particularly in metastatic tumors of unknown primary.

Our analysis of *HOXB13* expression across cancers using TCGA data confirmed its marked upregulation in PRAD tumor samples compared to normal prostate tissue and other tumor types, consistent with previous studies demonstrating *HOXB13*'s prostate-specific expression and its role in PRAD progression (Patel *et al.*, 2024; Dupont *et al.*, 2021; Zabalza *et al.*, 2015). The negligible expression of *HOXB13* in non-PRAD further supports its tissue-restricted nature and validates its use as a diagnostic biomarker for PRAD, particularly in confirming prostatic origin in metastatic tumors (Patel *et al.*, 2024). Our race-stratified analysis showed elevated *HOXB13* expression in both Caucasian and African-American PRAD patients, with a trend toward higher median expression in African-American men, aligning with reports highlighting *HOXB13*'s universal role across ethnic groups despite some limitations due to smaller sample sizes (Patel *et al.*, 2024). Kaplan–Meier survival analysis revealed that high *HOXB13* expression correlates with improved overall survival, particularly in patients with high Gleason scores, suggesting a complex tumor differentiation role. This paradoxical finding aligns with reports that *HOXB13* expression decreases in advanced/neuroendocrine PRAD subtypes associated with poor prognosis (Kanayama *et al.*, 2024). High *HOXB13* thus identifies Gleason-aggressive but High *HOXB13* expression may therefore identify tumors with high Gleason scores but a more differentiated molecular phenotype, positioning it as a critical complement to histopathological grading (Patel *et al.*, 2024). Conversely, patients with low or medium *HOXB13* expression and high Gleason scores had poorer outcomes, underscoring *HOXB13*'s potential as a dual prognostic marker when combined with histopathological data (Dupont *et al.*, 2021; Zabalza *et al.*, 2015). Collectively, these results reinforce *HOXB13*'s significance as both a diagnostic and prognostic factor in PRAD and highlight the need for further research into its mechanistic role across diverse populations.

Our heatmap analysis revealed significant upregulation of *HOXB13*, along with genes such as *TAGLN* and *S100A6*, in PRAD tumor tissues compared to normal samples, while genes like *ARHGAP10* and *PITPNC1* were downregulated. These findings align with prior studies that have reported *HOXB13*'s involvement in PRAD progression and its co-expression with genes implicated in cytoskeletal organization and tumor invasion (Egevad *et al.*,

2002). For instance, *S100A6* has been recognized for its role in tumor proliferation and metastasis, and *TAGLN* is known to influence cellular motility, both contributing to PRAD aggressiveness (Larkin *et al.*, 2012; Ohuchida *et al.*, 2005). The downregulation of *ARHGAP10* and *PITPNC1* further suggests disruption of signalling pathways critical for maintaining normal prostate epithelial architecture, consistent with observations in other genomic studies of PRAD (Gong *et al.*, 2019). Complementing the expression data, our comprehensive SNP functional annotation identified 23 variants with maximal scores across 12 predictive tools, including rs1207429780 and rs748782183. The convergence of predictions from tools such as AlphaMissense, SIFT, PolyPhen, and CADD supports the potential pathogenicity of these variants. This multi-tool consensus approach has been previously advocated as an effective strategy to prioritize SNPs for functional validation and risk association studies in cancer genomics (Ioannidis *et al.*, 2016; Ng and Henikoff, 2001). While some of these variants have yet to be characterized experimentally, their identification provides promising targets for future research aimed at elucidating the genetic underpinnings of PRAD susceptibility and progression.

HOXB13 is predominantly expressed in prostate tissue, playing a crucial role in prostate development and cancer risk, with limited expression in other tissues. The rare missense variant rs138213197 (G84E) in *HOXB13* is strongly associated with increased PRAD susceptibility, though it is underrepresented in large-scale GWAS and PheWAS databases due to its rarity (Bıyıkoglu *et al.*, 2024; Zhang *et al.*, 2016). Discovered through exome sequencing in primarily Caucasian populations, rs138213197 confers a significantly elevated PRAD risk, with multiple studies confirming odds ratios ranging from 3.3 to over 20 across diverse cohorts (Kote-Jarai *et al.*, 2015). While its association with breast cancer remains uncertain, the variant appears to be a founder mutation originating in Northern Europe around the 1800s, particularly prevalent in Nordic populations. This highlights the importance of rs138213197 in PRAD genetics and the need for focused studies beyond broad population databases (Witte *et al.*, 2013).

These convergent findings position *HOXB13* as a multifaceted biomarker with diagnostic specificity, prognostic utility, and therapeutic relevance in PRAD. The identification of high-impact germline variants further underscores its role in hereditary susceptibility, exemplified by the well-characterized G84E founder mutation (rs138213197). Future research should prioritize experimental validation of prioritized SNPs, single-cell resolution of tumor heterogeneity, and prospective clinical studies integrating *HOXB13* expression with histopathological and genomic profiling. Taken together, this multi-layered analysis reinforces the role of *HOXB13* as a prostate-specific oncogene with diagnostic, prognostic, and therapeutic relevance. Its selective overexpression in prostate tissue, correlation with clinical outcomes, and enrichment of high-impact pathogenic variants highlight its central role in PRAD biology. Nonetheless, further studies are needed to experimentally validate these candidate variants, delineate the mechanisms by which *HOXB13* contributes to tumor progression,

and assess its utility across diverse genetic and clinical contexts.

Strengths of this study include the use of comprehensive bulk RNA sequencing data from large publicly available databases (GTEx and TCGA), race-stratified expression profiling, integration of survival analysis with Gleason scoring, and a multi-algorithm SNP prioritization pipeline. However, several limitations should be acknowledged: the use of bulk RNA-seq data does not capture cell-type-specific expression or tumor heterogeneity; the observational nature of the analysis limits causal inference; and the small representation of certain racial and ethnic groups constrains generalizability. Additionally, while multiple computational tools were used for variant annotation, experimental validation remains essential to confirm biological impact as suggested by a previous study (Admanegara *et al.*, 2025).

5. Conclusion

This study demonstrates that HOXB13 is a prostate-restricted gene with consistent overexpression in PRAD across large transcriptomic datasets and observable associations with clinical outcomes. The identification of high-confidence pathogenic variants further supports its biological relevance in prostate cancer susceptibility and progression. Collectively, these findings suggest that HOXB13 represents a potential diagnostic and prognostic biomarker—and a candidate therapeutic target—in PRAD. However, clinical applicability will require further validation through functional experiments, single-cell analyses, and well-powered, multi-ethnic clinical studies. Continued investigation is, therefore, warranted to fully elucidate the mechanistic and translational significance of HOXB13 in prostate cancer.

The Acknowledgements

None

External funding

The authors gratefully acknowledge the Directorate of Research and Community Service, Directorate General of Research and Development, Ministry of Higher Education, Science, and Technology of the Republic of Indonesia, for providing financial support for this research through grants No. 0209/DST/C3/DT.05.00/2026; No. 132/C3/DT.05.00/PL-Multitahun Lanjutan/2026; and No. 0348.07/LL5-INT/AL.04.03/2026.

Data availability statement

Data will be made available upon request.

Declaration of competing interests

The authors disclose no conflicting interests.

Ethics statement

This study did not require ethical approval as it did not involve human participants, animal subjects, or sensitive data that necessitated ethical review.

Author contribution statement

Lalu Muhammad Irham and Wirawan Adikusuma conceived and designed the study. Lalu Muhammad Irham, Wirawan Adikusuma, Syarifatul Mufidah, and Rahmat Dani Satria performed the computational analysis. Putri Permata Suka Admanegara, Wirawan Adikusuma, Darmawi Darmawi, Rockie Chong, and Lalu Muhammad Irham contributed to writing the manuscript. Lalu Muhammad Irham provided the funding. Lalu Muhammad Irham, Wirawan Adikusuma, Syarifatul Mufidah, Rahmat Dani Satria, Eko Mugiyanto, Darmawi Darmawi, Petrina Theda Philothra, Desti Rahmawati, Siska Widiyastuti, and Rista Yulianti contributed to the revision of the manuscript. Putri Permata Suka Admanegara, Brilliant Citra Wirashada, Sabiah Khairi, Indra Jaya, Baik Heni Rispawati, Rockie Chong, and Ilker Ates also revised the manuscript. All authors have read and approved the final version of the manuscript and have made significant contributions to the study.

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