

Micropropagation and in Vivo Antibacterial Activity of Different Extracts of Rue (*Ruta graveolens* L)

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Abstract

This study investigated the in vitro growth and antibacterial activity of *Ruta graveolens* using callus and extracts obtained from both in vitro and in vivo sources. Shoot induction was conducted on Murashige and Skoog (MS) medium supplemented with different concentrations of BAP, kinetin, and GA₃ (0.0–1.0 mg/L), while root induction utilized MS medium containing 2,4-D (0.0–2.0 mg/L). The antibacterial efficacy of methanolic and ethanolic extracts was assessed against five bacterial strains. *Escherichia coli* and *Salmonella typhimurium* represented Gram-negative bacteria, while *Staphylococcus aureus*, *Bacillus cereus*, and *Micrococcus luteus* represented Gram-positive types. Optimal shoot induction (4.75 shoots, 2.66 cm length) occurred with 0.3 mg/L BAP, and maximum biomass (0.51 g fresh, 0.07 g dry) was achieved at 0.4 mg/L BAP. Kinetin at 0.1 mg/L resulted in the highest shoot number, while 1.0 mg/L improved shoot length and biomass. GA₃ at 1.0 mg/L yielded the highest number of shoots (7.5) and the longest (4.01 cm), with peak biomass at 0.4 mg/L. The best root and shoot development occurred at 0.4 mg/L 2,4-D, while the highest callus biomass (1.02 g fresh, 0.08 g dry) was observed at 2.0 mg/L 2,4-D. All extract types and doses (40 and 80 µL) demonstrated antibacterial activity against the tested bacterial species using an agar-well diffusion assay. These findings suggest that *R. graveolens* may serve as a promising natural source for the development of antibacterial agents.

Keywords: Antibacterial, In vivo, Micropopagatoin, Pharmaceuticals, Rue, *Ruta graveolens*

1. Introduction

Ruta graveolens L., commonly known as "Rue," "Sudab," or "Sadab" in Hindi, is one of the most prevalent medicinal plants in the Rutaceae family (Al-Ajlouni et al., 2022). Native to Europe, it has since spread globally. This perennial evergreen shrub, which can grow up to one meter in height, is widely used both for decorative and therapeutic purposes. Numerous studies on the Rutaceae family have highlighted the potential of its natural products in treating various conditions, including cancer, depression, and Alzheimer's disease. *Ruta* species have also been utilized to address various uterine conditions, such as amenorrhea, menstrual irregularities, and excess menstruation. These species have historically been recommended as an abortifacient and to assist with conception. In addition, *Ruta* species have been used to treat pregnancy-related issues, including placental expulsion and puerperal fever (Pollio et al., 2008). Rue has also been found to have protective properties against genetic abnormalities, such as cancer and mutations, underscoring its potential as a powerful protective agent

(Al-Muffti et al., 2021). Research by Al-Ajlouni et al. (2015) and Kuzovkina et al. (2004) has identified over 120 natural compounds in the roots and apical portions of this plant, including alkaloids, coumarins, essential oils, and flavonoids. These metabolites are of significant interest in biology and pharmacology for their antifungal, anti-leishmanial, antiplasmodial, and antidotal properties (Sampaio et al., 2018).

According to Thomas & Devi (2013), over 50% of clinically used pharmaceuticals are derived from natural products, with approximately 25% originating from plant-based sources. This has motivated continued scientific investigation into plants like Rue, which may serve as sources for novel chemical structures that could aid in the development of new drugs targeting microbial resistance and reducing the adverse effects of specific antibiotics (Chaftar et al., 2016; Haddouchi et al., 2013).

As previously mentioned, Rue produces secondary metabolites (Morton & Telmer, 2014) and is considered one of the best plants for medicinal use. Antimicrobials derived from plants can be valuable in treating resistant microbiological strains, as their mechanisms of action may differ from those of currently used synthetic medications

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(Orlanda & Nascimento, 2015). Numerous studies have shown that chemical components from the Rutaceae family, such as limonoids, flavonoids, coumarins, and alkaloids, exhibit antibacterial, herbicidal, insecticidal, trypanocidal, and antimalarial properties (Luo et al., 2024). The antibacterial activity of these compounds can vary depending on factors such as the solvent, plant parts, harvesting time, and geographic region. For example, ethanolic extracts from the leaves have shown superior antibacterial activity compared to extracts from the stem and root. The leaves contain higher levels of flavonoids and phenols than other plant parts (Krayni et al., 2015). Salman et al. (2018) reported that the primary causes of dental caries, *S. mutans* and *S. sobrinus*, are significantly inhibited by the methanolic extract of *R. graveolens* and its specific components. Additionally, Mohammadpour et al. (2025) demonstrated that alcoholic extracts of *R. graveolens* (both ethanolic and methanolic) possess antibacterial effects. The most effective method for preventing burn infections caused by *S. aureus* and *P. aeruginosa* was found to be the use of complete dilutions of these extracts. However, one challenge farmers face when cultivating *R. graveolens* is slow shoot propagation, as many shoots fail to root easily, and there is considerable seed population variation (Ahmad et al., 2012). Furthermore, *R. graveolens* seeds exhibit poor germination rates, short viability, and susceptibility to pests and diseases in humid conditions (Bohidar et al., 2008). Mass harvesting of *R. graveolens* from its natural habitats has led to rapid depletion of these plant resources, causing the species to become endangered. The conventional propagation method cannot meet the growing demand for plants, as production is limited (Nalawade & Tsay, 2004). Modern biotechnological techniques for growing *R. graveolens* tissues and cells offer new opportunities for preserving and multiplying this important medicinal plant (Shatnawi et al., 2011). Given these challenges, plant tissue culture presents a promising solution to the poor propagation of *R. graveolens*. The objectives of this study were to develop effective methods for micropropagating *R. graveolens* using different types of cytokinin hormones and to investigate the potential antimicrobial activity (both antibacterial and antifungal) of plant extracts from *R. graveolens* grown in vitro and in vivo.

2. Material and Method

The study was conducted at the Tissue Culture Laboratory at Jordan University of Science and Technology. Seeds were collected from approximately five-year-old *R. graveolens* specimens located in Al-Sareeh, Irbid, Jordan, at an altitude of about 600 meters above sea level, with coordinates of 32.3306°N latitude and 35.8951°E longitude.

To initiate the surface sterilization process, the seeds were thoroughly washed under running water for fifteen minutes to remove any residual disinfectant. The seeds were immersed in 100 cc of 4% v/v Chlorex (containing 2.25% sodium hypochlorite) in a laminar flow cabinet for 15 minutes, with continuous shaking. After this, the seeds were rinsed three times with sterile distilled water, each rinse lasting five minutes. The seeds were then cleaned

with 70% ethanol for 30 seconds, followed by immersion in 1% NaOCl for 30 minutes, with two drops of Tween 20 added to reduce surface tension. After sterilization, each seed was washed three times with sterile deionized water and then cut into a single bud under sterile conditions in a laminar flow cabinet.

The media used to cultivate the shoots is known as Murashige and Skoog (MS) medium, developed in 1962. This medium consists of 3% sucrose, 0.5 mM myo-inositol, 0.34 mM thiamine hydrochloride, 2.4 mM pyridoxine hydrochloride, 4.1 mM nicotinic acid, and 2.4 mM thiamine hydrochloride. Each 250 mL flask was supplemented with 60 mL of medium. Cotton wool was placed at the opening of each flask to allow for gas circulation, and the flasks were autoclaved at 121°C for 20 minutes. The cultures were incubated under a photoperiod of 16 hours and a PPF of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, using cool white fluorescent lamps, with a temperature of $24 \pm 2^\circ\text{C}$. After the first six weeks, microshoots were transferred to hormone-free Mississippi soil to obtain sufficient plant material. The photoperiod for the soil-grown plants was maintained at 16:8 light:dark, also at $24 \pm 2^\circ\text{C}$. (Al Shhab et al., 2022; Al-Ajlouni et al 2023)

The MS-enriched base medium included 3% sucrose and 0.8% agar (Sigma, USA), and all components were prepared as concentrated stock solutions stored in a refrigerator. The necessary microsalts were dissolved in one liter of distilled water to serve as the stock solution for the experiment. Growth hormone stock solutions were prepared in the same manner. Cytokinins were dissolved in a small quantity of 1M HCl, and the remaining volume was filled with distilled water and stored at 4°C. All growth regulators were obtained from Sigma (USA). Once all the components were added to the conical flask in the correct proportions, the final volume was brought to a boil in distilled water, and the pH was adjusted to 5.8 with 0.1M HCl. The medium was solidified using 0.8% (w/v) agar. For liquid medium, agar was omitted.

After the medium was prepared, it was transferred into flasks with capacities of either 60 mL or 250 mL. These flasks were carefully sealed with cotton and muslin lids and labeled accordingly. Subsequently, the flasks were autoclaved at 126 degrees Celsius for twenty minutes at a pressure of fifteen pounds per square inch. Once sufficient plant material was available, microshoots measuring 10 millimeters in length, including apical meristems, were subcultured onto fresh MS media. The microshoots were grown on MS medium treated with varying concentrations of benzylaminopurine (BAP), kinetin, and GA₃, specifically at levels of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg/L, each tested individually.

A total of twenty separate replicates of each treatment were conducted, and the trials were repeated twice. In a second experiment, microshoots measuring 10 millimeters in diameter were grown in MS media supplemented with varying concentrations of 2,4-Dichloromethoxyacetic acid (2,4-D) at levels of 0.0, 0.4, 0.8, 1.2, 1.6, 1.8, and 2.0 mg/L. Additionally, 0.5 grams of charcoal was added to the medium. The growth conditions in the second experiment were similar to those in the first experiment.

In vitro-rooted plantlets were carefully removed from the growth medium and cleaned in a water bath at 25–30°C to aid acclimatization. The plantlets were then transferred to 6 x 6 x 6 cm plastic pots filled with a sterile mixture of peat and perlite. A clear plastic bag measuring 15 x 20 cm was placed over each pot. To gradually reduce humidity, holes of 0.5 cm in diameter were made in the plastic bag, with additional holes added every three days for two to three weeks. After five weeks, the number of acclimatized plants that remained alive was recorded. This study involved five bacterial species: *Escherichia coli*, *Salmonella Typhimurium*, *Staphylococcus aureus*, and *Bacillus cereus* (all gram-positive bacteria), and *Micrococcus luteus* (a gram-negative bacterium). These species were identified in the microbiology lab at Yarmouk University, Jordan. (Al-Ajlouni et al 2023)

3. Experimental design and statistical analysis.

To assess whether there were differences in the means among the individuals, we conducted a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. The significance threshold was established at 0.05. A completely randomized design (CRD) was employed for all studies, with each treatment replicated ten times.

4. Results and Discussion

4.1. Effect of cytokinin on shoot proliferation

4.1.1. 6-Benzylaminopurine(BAP)

The multiplication rates were lowest in the medium without hormones (Table 1). Increasing the BAP concentration from 0.0 to 1.0 mg/L significantly enhanced the number of multiplied plants. Optimal shoot production was observed in the medium containing 0.3 mg/L BAP, which resulted in 4.75 shoots (Table 1). The maximum shoot length (2.66 cm) was achieved in the same medium (Figure 1; Table 1). Both fresh and dry weights increased significantly when BAP was raised from 0.0 to 0.4 mg/L. The fresh weight increased from 0.06 g to 0.51 g, and the dry weight rose from 0.01 g to 0.07 g (Table 1). Previous studies have well-documented the effectiveness of BAP over other cytokinins in tissue culture systems, including research on various explants, such as *Ocimum basilicum* (Jakovljević et al., 2022). BAP is recognized as a potent hormone for inducing multiple shoots in various plant taxa (George & Sherrington, 1984). Numerous studies have highlighted BAP's significant role in plantlet proliferation and multiplication. For example, Bohidar et al. (2013) and Bohidar et al. (2008) reported that BAP was the most effective compound for shoot induction, proliferation, and elongation. Similarly, the highest in vitro multiplication rate in *Vitis vinifera* was observed at 0.8 mg/L BAP (Shatnawi, 2013). In the present study, as BAP concentrations increased beyond 0.3 mg/L, a decline in shoot number, length, fresh weight, and dry weight was observed. Interestingly, when BAP concentrations were kept at or below 0.3 mg/L, both the number of new shoots and their length increased. However, concentrations exceeding 0.4 mg/L resulted in a reduction in these parameters.



Figure 1: After six weeks, shoot multiplication on (0.3 mg/L BAP).

Table 1: Mean impact of BAP concentrations after six weeks on various characteristics.

Concentration (mg/L)	Number of new shoots	Maximum shoot length (cm)	Fresh weight (g)	Dry weight (g)
0.0	2.70 ^b	1.71 ^d	0.06 ^c	0.01 ^b
0.1	3.05 ^{ab}	1.87 ^{cd}	0.13 ^{bc}	0.02 ^b
0.2	3.55 ^{ab}	1.97 ^{bcd}	0.09 ^c	0.02 ^b
0.3	4.75 ^a	2.66 ^a	0.50 ^a	0.07 ^a
0.4	4.35 ^{ab}	2.36 ^{ab}	0.51 ^a	0.07 ^a
0.5	4.05 ^{ab}	2.17 ^{bc}	0.41 ^{ab}	0.07 ^a
1.0	4.05 ^{ab}	2.37 ^{ab}	0.33 ^{abc}	0.04 ^{ab}

Based on the LSD test at $P < 0.05$, the same letter means they are not significantly different.

4.1.2. Kinetin

In vitro shoot lengths exhibited a favorable response to kinetin, with the maximum measurement at 0.4, 0.5, and 1.0 mg/l kinetin. The most significant fresh weight (FW) and dry weight (DW) were achieved with 1.0 mg/l of kinetin in the medium (Table 2). The current findings align with those of Al Shhab et al. (2021), who indicated that 2.0 mg/l of kinetin produced the highest quantity of new shoots of *R. graveolens*, whereas the optimal shoot length was achieved with 1.0 mg/l. Conversely, Hussain and Nathar (2020) indicated that a medium enriched with kinetin decreased the quantity of shoots exhibiting elongated internodes. Bhat et al. (1995) observed that kinetin is less efficient than BAP in *Quercus shumardii* and *Piper spp* cultures, respectively.



Figure 2: Multiple *Ruta graveolens* shoots after six weeks on MS + 0.1 mg/L kinetin. Bar = 0.5 cm.

Table 2: Mean impact of kinetin concentrations after six weeks on various characteristics.

Concentration (mg/L)	Number of new shoots	Maximum shoot length (cm)	Fresh weight (g)	Dry weight (g)
0.0	2.50 ^b	1.71 ^b	0.06 ^b	0.01 ^c
0.1	3.95 ^a	2.28 ^{ab}	0.18 ^{ab}	0.03 ^{abc}
0.2	3.55 ^{ab}	2.22 ^{ab}	0.20 ^{ab}	0.03 ^{abc}
0.3	2.70 ^{ab}	1.90 ^{ab}	0.12 ^{ab}	0.02 ^{bc}
0.4	3.20 ^{ab}	2.28 ^a	0.17 ^{ab}	0.02 ^{bc}
0.5	3.70 ^{ab}	2.33 ^a	0.19 ^{ab}	0.03 ^{ab}
1.0	3.80 ^{ab}	2.30 ^a	0.24 ^a	0.04 ^a

Based on the LSD test at $P < 0.05$, the same letter means they are not significantly different.

4.1.3. Gibberellins (GA₃)

The current study investigated the effect of GA₃ on microshoot development (Table 3). Increasing GA₃ from 0.1 to 1.0 positively influenced the number of new micro shoots produced from each explant (Table 3, Figure 3). GA₃ at 1.0 mg/l produced the highest number of shoots/explant (7.5) and maximum shoot length (4.01 cm) compared to the other GA₃ concentrations tested (Table 3). The maximum fresh and dry weights were produced at 0.4 mg/l (0.42 g and 0.11 g, respectively). Present results confirmed that GA₃ stimulates the elongation of internodes and has been proven necessary for meristem growth, as in various plant species (Jayantibhai, 2020). Moreover, previous studies indicated that GA₃ is conducive to *in vitro* shoot regeneration (Al-Alouni et al., 2016) or for promoting growth, biomass production, and xylem fiber length (Eriksson et al., 2000).



Figure 3: Six-week *Ruta graveolens* shoot formation on MS + 1.0 mg/L GA₃. 0.5 cm bar

Table 3: Mean impact of gibberellins (GA₃) concentrations after six weeks on various characteristics.

Concentration (mg/L)	Number of new shoots	Maximum shoot length (cm)	Fresh weight (g)	Dry weight (g)
0.0	2.70 ^d	1.71 ^d	0.06 ^c	0.01 ^c
0.1	5.60 ^{abc}	3.56 ^{ab}	0.40 ^a	0.06 ^b
0.2	3.25 ^{cd}	2.75 ^c	0.14 ^{ab}	0.02 ^{bc}
0.3	6.70 ^{ab}	3.63 ^{ab}	0.41 ^a	0.06 ^{ab}
0.4	6.75 ^{ab}	3.18 ^{bc}	0.42 ^a	0.11 ^a
0.5	4.50 ^{bcd}	2.56 ^c	0.26 ^{ab}	0.04 ^{bc}
1.0	7.50 ^a	4.01 ^a	0.37 ^a	0.07 ^{ab}

Based on the LSD test at $P < 0.05$, the same letter means they are not significantly different.

4.1.4. Effect of 2,4-Dichlorophenoxyacetic acid (2,4-D) on root formation

The number of roots formed per microshoot was positively affected by the concentration of 2,4-D (Table 4). Maximum root number, root length, shoot number, and shoot length were obtained from MS media supplemented with 0.4 mg/L 2,4-D followed by 0.2 mg/L where the means of new root and root length were 3.25 and 0.96 cm, respectively. Callus was induced in MS media with different concentrations of 2,4-D MS media. The higher callus fresh and dry weight was 1.02 g and 0.08 g, respectively, when added media with 2.0 mg/L of 2,4-D. Followed by 1.6 mg/L of 2,4-D, where the fresh weight was 0.93 g, and the dry weight was 0.08 g. Using a higher concentration of 2,4-D (2.0 mg/L) resulted in low rhizogenesis and root formation (Figure 4).

This can be explained by the inhibitory effects of auxins (2,4-D) on chlorophyll formation and excessive callus formation (George et al., 2008). A similar observation was reported by (Soh et al., 1998) and (Duan et al., 2012) where there is little or no root formation in a medium containing 2,4-D. Furthermore (Nesrine et al.) reported no root response for *Cicer arietinum* L. on different concentrations of 2,4-D.



Figure 4: Callus development on MS medium with 2.0 mg/L 2,4-D after six weeks of growth

Table 4 Mean impact of different 2,4-D concentrations after six weeks on various characteristics.

Concentration (mg/l)	Number of new shoots	Maximum shoot length (cm)	Number of new Roots	Maximum root length (cm)	Callus fresh weight (g)	Callus dry weight (g)
0.0	2.70 ^{bc}	1.71 ^b	0.00 ^e	0.00 ^d	0.00 ^c	0.00 ^e
0.2	3.35 ^b	2.88 ^a	2.20 ^{ab}	0.92 ^a	0.16 ^c	0.01 ^{de}
0.4	4.95 ^a	2.77 ^a	3.25 ^a	0.96 ^a	0.23 ^c	0.02 ^{cde}
0.8	1.95 ^c	1.75 ^b	0.70 ^{de}	0.30 ^{bcd}	0.24 ^c	0.03 ^{cd}
1.2	1.80 ^c	1.42 ^b	0.90 ^{cde}	0.30 ^{bcd}	0.79 ^{ab}	0.07 ^{ab}
1.6	3.35 ^b	1.70 ^b	1.95 ^{bc}	0.55 ^b	0.93 ^a	0.08 ^a
1.8	2.70 ^{bc}	1.60 ^b	0.40 ^{de}	0.11 ^{cd}	0.65 ^b	0.05 ^{bc}
2.0	2.05 ^c	1.28 ^b	1.25 ^{bcd}	0.34 ^{bc}	1.02 ^a	0.08 ^a

Based on the LSD test at $P < 0.05$, the same letter means they are not significantly different.

5. Hardening

Because the regeneration in the culture settings has been cosseted in an environment with extremely high humidity, fluctuating light, and temperature conditions, it is not feasible to directly transfer plants grown in tissue culture to the wild or the field. This is because of the high mortality rate (Deb & Imchen, 2010). Direct exposure to sunlight is another factor that causes plants to wither and leaves to char (Hiren et al., 2004; Lavanya et al., 2009) (Deb & Imchen, 2010). Therefore, hardening off is essential for improving the chances of survival and effective establishment. Indeed, during tissue culture, the stages of hardening and acclimation are considered the most crucial. This research found that the survival rate of rooted plants reached 100% under greenhouse acclimatization settings, with the surviving specimens exhibiting green, healthy, and robust characteristics. All surviving plants exhibited consistent growth, typical leaf development, and no observable morphological difference (Figure 5).

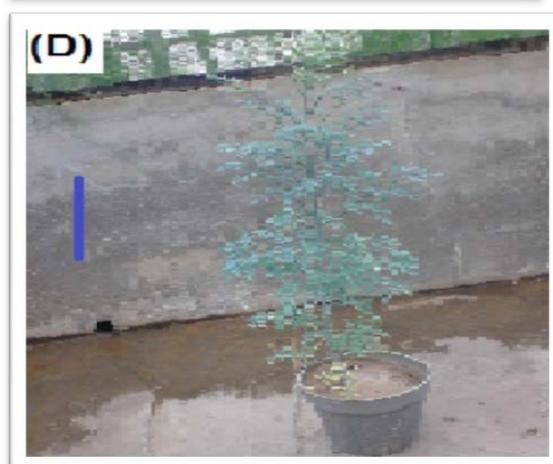
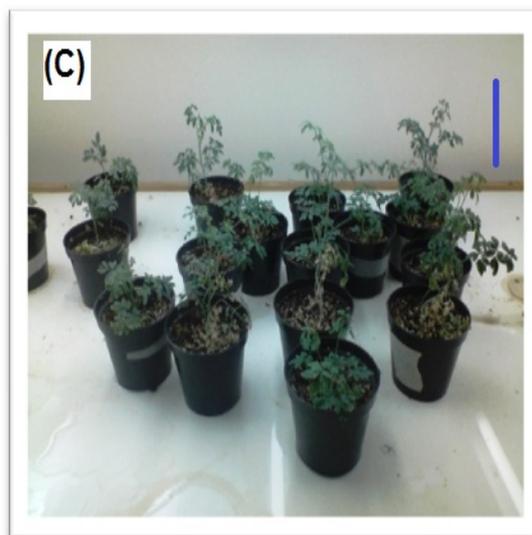


Figure 5: Plantlets were placed in a plastic bag, *R. graveolens* was rooted in a plastic container using a sterile peat moss-perlite (1:1) mixture, and the plant was planted four weeks later. *R. graveolens* growing in soil *in vivo*. There is a 5 mm error bar.

6. Antibacterial Activity

The extracts are made using either methanol or ethanol and are divided into three categories: callus (undifferentiated plant cells, *in vitro* (plant tissues cultivated in a controlled environment), and *in-vivo* (plants grown spontaneously). The extraction technique and solvent employed can affect the size of the inhibitory zones, as methanol and ethanol can extract bioactive chemicals differently, thereby influencing their effectiveness against the bacteria. This test helps evaluate *R. graveolens*' potential as an antibacterial agent against *M. latus*. Various plant materials, including callus, *in vitro*, and *ex-vivo*, were extracted using a variety of solvents, including methanol and ethanol (AL-ajlouni et al., 2023). To evaluate the antibacterial property of each extract, a range of different quantities, namely 40 μ l and 80 μ l, were used. The findings indicate that each of the three extracts of *R. graveolens* has various antibacterial properties, which are effective against the five tested bacteria. It has been shown that the antibacterial activity of extracts is superior to that of the standard antibiotic (positive), oxytetracycline. By utilizing dimethyl sulfoxide (DMSO) alone, the control (negative) experiment could not inhibit

the development of all the bacteria included. Therefore, the experiment produced negative findings. For callus, it was reported that *R. graveolens* extract by methanol and ethanol demonstrated a zone of inhibition in both volume and concentrations against the five bacteria. This was seen in both *in vitro* and *ex vivo* settings. This study's findings agree with those of (Al-Ajlouni et al., 2023).

6.1. *Staphylococcus aureus* (*S. aureus*)

It is a standard part of the body's microbiota, commonly found on the skin and in the upper respiratory tract. It is a gram-positive, spherical bacterium that belongs to the Bacillota. It is a common source of skin infections, but can also become an opportunistic pathogen. One of the most common diseases linked to antibiotic-resistant strains, including methicillin-resistant *S. aureus* (MRSA), and mortality from drug resistance is *S. aureus*. In clinical medicine, *S. aureus* is a global issue (Yamazaki et al., 2024).

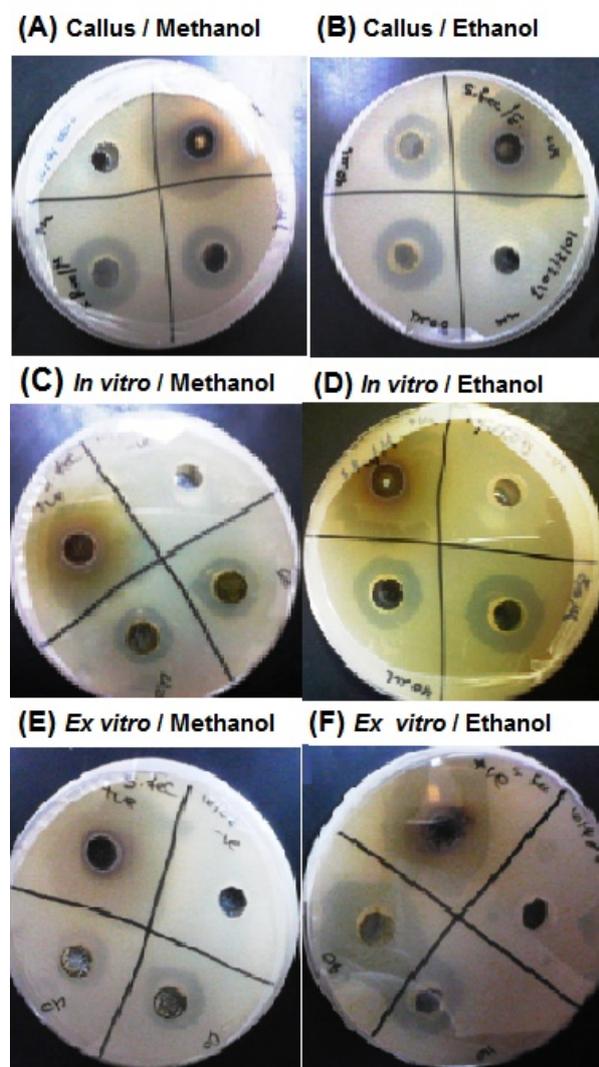


Figure (6): Agar diffusion assay of *R. graveolens* extracts against *S. aureus*: (A) activity of callus extracted with methanol, (B) activity of callus extracted with ethanol, (C) activity of *in vitro* extracts with methanol, (D) activity of *in vitro* extracts with ethanol, (E) activity of *In Vivo* extracts with methanol, and (F) activity of *In Vivo* extracts with ethanol. An inhibition zone was detected around the well.

Figure (6) shows an agar well-diffusion experiment to assess the antibacterial activity of *Ruta graveolens* extracts against *S. aureus*. In the test, wells are made on an agar plate infected with the bacteria, and several *R. graveolens* extracts are then added to the wells. Since the extract stops *S. aureus* from growing, the appearance of an inhibition zone surrounding the wells suggests that the extract has antimicrobial action.

Inhibition zones surrounding the wells are shown in the data, suggesting that the extracts exhibit antibacterial qualities against *S. aureus*. The concentration and kinds of active chemicals extracted from the plant material can be affected by the extraction technique and solvent utilized, which can also affect the size of the inhibitory zones. The efficacy of methanol and ethanol, two popular solvents for extracting bioactive substances, varies according to how soluble the substances are in each solvent (Al-Ajlouni et al., 2023).

6.2. *Bacillus cereus* (*B. cereus*)

It is a rod-shaped, Gram-positive bacterium commonly found in food, soil, and marine sponges. The term "Cereus," meaning "waxy" in Latin, describes the appearance of colonies formed on blood agar (Williams & Weir, 2024). Because they produce spores, some strains are dangerous to people and can lead to foodborne illnesses, but other strains can help animals as probiotics and even show mutualism with some plants. *B. cereus* bacteria can generate protective endospores and be facultative anaerobes or aerobes (Akinsemolu et al., 2024). Li et al. (2025) discovered that quorum sensing regulates various virulence factors in these bacteria, such as phospholipase C, cereulide, sphingomyelinase, metalloproteases, and cytotoxin K.

The antibacterial activity of *Ruta graveolens* extracts against *Bacillus cereus* (*B. cereus*) was evaluated using the agar well-diffusion assay (Figure 7). In this test, several *R. graveolens* extracts are applied to wells made on an agar plate infected with the bacterium. Since the extract stops *B. cereus* from growing, the appearance of an inhibition zone surrounding the wells suggests it has an antimicrobial action. Inhibition zones surrounding the wells are shown in Figure 7, indicating that the extracts exhibit antibacterial qualities against *B. cereus*. The concentration and kinds of active chemicals extracted from the plant material can be affected by the extraction technique and solvent utilized, which can also affect the size of the inhibitory zones.

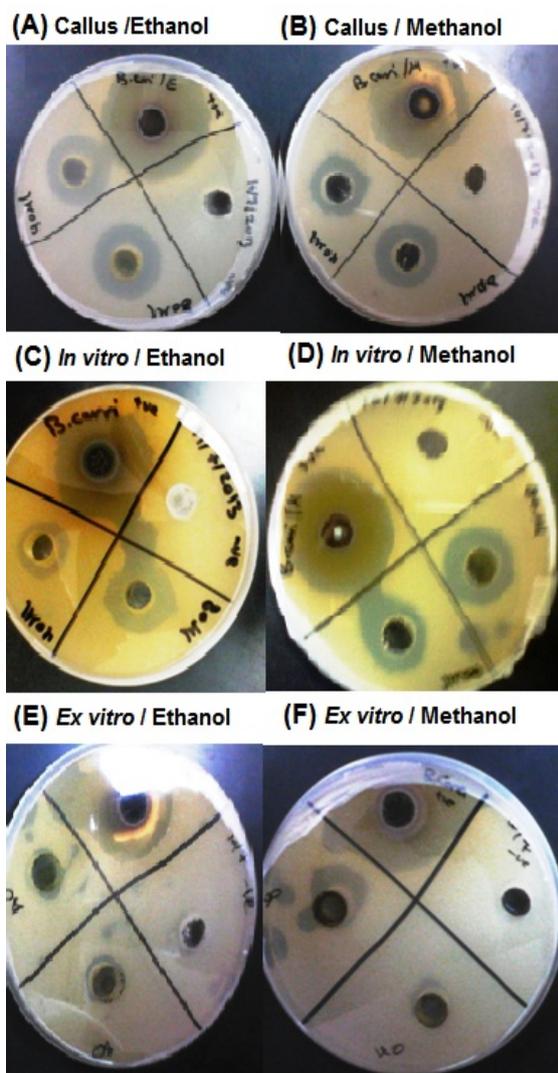


Figure (7): Agar diffusion assay of *R. graveolens* extracts against *B. cereus*: (A) activity of callus extracted with ethanol, (B) activity of callus extracted with methanol, (C) activity of *in vitro* extracts with ethanol, (D) activity of *in vitro* extracts with methanol, (E) activity of In Vivo extracts with ethanol, and (F) activity of In Vivo extracts with methanol. An inhibition zone was detected around the well.

6.3. *Micrococcus luteus* (*M.latus*)

The bacterium *Micrococcus luteus* is a saprotrophic coccus in the family Micrococcaceae that is nonmotile, tetrad-arranging, pigmented, and Gram-positive to Gram-variable. It is positive for catalase and urease. *M. luteus*, an obligatory aerobe, is present in soil, sediment, water, and air, as well as in the ordinary microbiota of the mammalian epidermis (Suresh et al., 2025). The bacterium also colonizes the Mucosae, oropharynx, upper respiratory tract, and human mouth. *Micrococcus luteus* can develop into an opportunistic pathogen despite its generally benign nature in individuals who are immunocompromised or have indwelling catheters. Soria-Camargo et al. (2025) suggest it resists antibiotic treatment by inducing unique genes and delaying key metabolic processes. Figure (8) shows an agar well-diffusion experiment to evaluate the antibacterial activity of *Ruta graveolens* extracts against *Micrococcus latus* (*M. latus*). In this test, several *R. graveolens* extracts are applied to wells made on an agar plate that has been infected with the bacterium. Since the

extracts prevent *M. latus* from growing, the appearance of inhibition zones surrounding the wells suggests antimicrobial action.

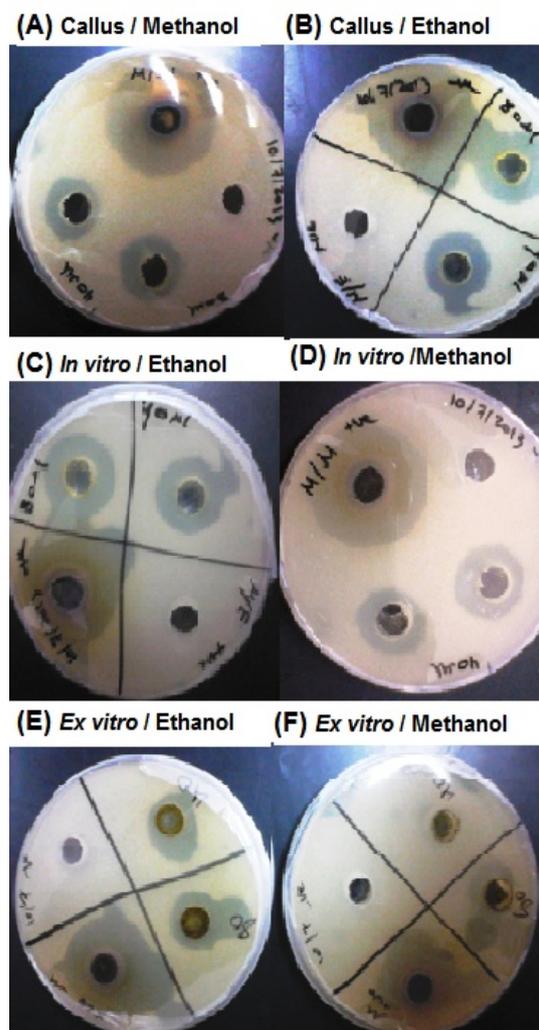


Figure (8): Agar well-diffusion assay of *R. graveolens* extracts against *M. latus*: (A) activity of callus extracted with methanol, (B) activity of callus extracted with ethanol, (C) activity of *in vitro* extracts obtained with ethanol, (D) activity of *in vitro* extracts obtained with methanol, (E) activity of In Vivo extracts obtained with ethanol, and (F) activity of In Vivo extracts obtained with methanol. The inhibition zone was detected around the well.

6.4. *Escherichia coli* (*E.coli*)

Is a rod-shaped, facultatively anaerobic, gram-negative coliform bacteria of *Escherichia* frequently found in warm-blooded species' lower intestines (De Marco, 2025). Along with other facultative anaerobes, most *E. coli* strains comprise around 0.1% of the gut's typical microbiota. For the most part, these bacteria are benign or even helpful to us. Certain strains of *Escherichia coli* benefit their hosts by producing vitamin K2 or preventing the growth of harmful pathogenic bacteria in the gut. These positive interactions between humans and *E. coli* demonstrate a mutualistic biological relationship, where both parties gain advantages from one another (Ng et al., 2025). Fecal matter contains *E. coli* that is released into the environment. Under aerobic circumstances, the bacteria thrive enormously in new fecal matter for three days, after which their numbers gradually decrease. Specific serotypes are harmful and can cause

their hosts to suffer from severe food poisoning. The main way that pathogenic forms of the bacteria spread illness is by fecal-oral transmission. Sometimes, food contamination events that lead to product recalls are caused by this transmission mode (Nnah et al., 2025). Because cells have a short half-life outside the body, they can be used as indicator organisms to check environmental samples for fecal contamination. Figure (8) displays an agar well-diffusion experiment to assess the antibacterial activity of *Ruta graveolens* extracts against *Escherichia coli* (*E. coli*). In this test, several *R. graveolens* extracts are applied to wells made on an agar plate infected with the bacterium. Since the extracts prevent *E. coli* from growing, inhibition zones surrounding the wells suggest antimicrobial action.

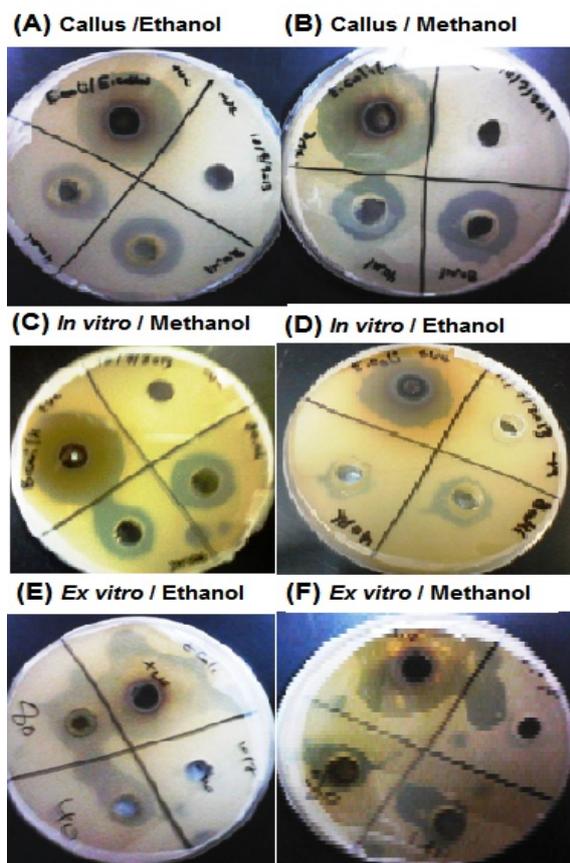


Figure (8): Agar diffusion assay of *R. graveolens* extracts against *E. coli*: (A) activity of callus extracted with ethanol, (B) activity of callus extracted with methanol, (C) activity of *in vitro* extracts with methanol, (D) activity of *in vitro* extracts with ethanol, (E) activity of *In Vivo* extracts with ethanol, and (F) activity of *In Vivo* extracts with methanol. The inhibition zone was detected around the well.

6.5. *Salmonella Enterica Serovar Typhimurium* (*S. Typhimurium*)

It is an intracellular facultative bacterial pathogen that has been used as a model organism for research on host-pathogen interactions for many years. Its capacity to create *Salmonella*-induced filaments (SIFs), a network of membrane tubules in infected host cells, contributes to its appeal from a cell biology standpoint. Despite the attention given to its intracellular lifestyle, *S. Typhimurium* is the world's most common cause of diarrhea and foodborne illnesses (Cao et al., 2025).

To evaluate the antibacterial activity of *Ruta graveolens* callus extracts against *Salmonella typhimurium* (*S. typhimurium*), Figure (9) shows an agar well-diffusion experiment. In this test, callus extracts prepared with either methanol (A) or ethanol (B) are added to wells on an agar plate inoculated with the bacteria. The appearance of inhibition zones surrounding the wells suggests that the extracts have an antibiotic action, as they limit the development of *S. typhimurium*. Because methanol and ethanol can extract bioactive chemicals differently, affecting their efficacy against the bacteria, the size of the inhibitory zones may vary depending on the solvent utilized. The potential of *R. graveolens* callus extracts as antimicrobial agents against *S. typhimurium* is ascertained with the aid of this test. According to previous studies on rue's alkaloid-rich extracts (Al-Ajlouni et al., 2023). In summary, the antibacterial action against Gram-positive bacteria, such as *Staphylococcus aureus*, aligns with the findings of the current study. However, the limited effectiveness against Gram-negative organisms, such as *Escherichia coli*, suggests that either concentration-dependent effects or structural barriers, like outer membrane proteins, may be involved (Oliva et al., 2019). The identified antibacterial activities, particularly against Gram-positive bacteria (*S. aureus* and *Bacillus cereus*), are consistent with the phytochemicals found in rue, including furanocoumarins and alkaloids (Oliva et al., 2019).

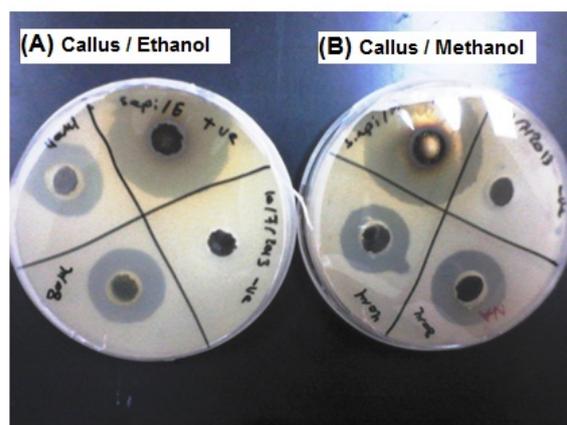


Figure (9): Agar well-diffusion experiment of *R. graveolens* extracts against *S. typhimurium*: (A) activity of methanol-extracted callus and (B) activity of ethanol-extracted callus. The inhibition zone was detected around the well.

7. Conclusion

This study explored the micropropagation of Rue (*Ruta graveolens* L.) and the antimicrobial effects of its extracts on five bacterial species. The MS medium included various BAP, Kinetin, and GA3 concentrations, with the most effective results for microshoot growth recorded at 0.30 mg/L BAP, 0.10 mg/L Kinetin, and 1.0 mg/L GA3. The highest root and shoot development occurred with 2,4-D at 0.4 mg/L. Both *in vitro* and *in vivo* extracts exhibited antimicrobial activity against all tested bacterial species, suggesting that *R. graveolens* may be a natural antibacterial treatment in pharmaceuticals.

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Conflict of interests

The authors declare no conflict of interests.

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