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Phytochemical Screening and Antibacterial Activity of the Aerial Parts of *Origanum majorana* L. (Lamiaceae)

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Abstract

This study explores the phytochemical composition and antibacterial activity of the methanolic extract obtained from the aerial parts of *Origanum majorana* L. (Lamiaceae), a plant commonly used in traditional medicine. Phytochemical screening highlighted the presence of several secondary metabolites, including polyphenols, flavonoids, tannins, coumarins, anthocyanins, and cardiotonic glycosides, while alkaloids, saponins, and anthraquinones were not detected. The antibacterial activity was evaluated using the agar diffusion method at decreasing extract concentrations. The extract showed a concentration-dependent inhibitory effect. A strong activity was observed against *Staphylococcus aureus* ATCC 25923 (inhibition zone > 28 mm) and methicillin-sensitive *S. aureus* (28.70 mm). *Pseudomonas aeruginosa* CIP showed high susceptibility at higher concentrations, whereas *P. aeruginosa* ATCC 27853 was resistant. Moderate inhibition was recorded for *Escherichia coli* MCR-1 (18.83–6 mm), *E. coli* ATCC 25922 (12.74–6 mm), and *Bacillus cereus* (16.53–4.75 mm). The results suggest that *Origanum majorana* exhibits notable antibacterial activity, particularly against Gram-positive bacteria, which could be related to its richness in bioactive compounds.

Keywords: *Origanum majorana* L.; Phytochemical Screening; Secondary Metabolites; Antibacterial Activity; Methanolic Extract

1. Introduction

Origanum majorana L. (Lamiaceae) is a well-known aromatic herb widely used in traditional healing practices. Its therapeutic relevance has been attributed to a wide range of reported biological effects, notably antimicrobial, antioxidant, and antispasmodic activities [1,2]. In traditional medicine, the leaves of this plant are commonly utilized to alleviate various health disorders, including respiratory conditions, metabolic diseases such as diabetes, and certain neurological impairments. Phytochemical investigations have shown that the aerial parts of *O. majorana* are a rich source of secondary metabolites. These include essential oils as well as phenolic constituents, particularly flavonoids and phenolic acids, which are widely recognized for their biological activity. Such compounds are thought to be largely responsible for the pharmacological properties associated with this species, highlighting its importance as a potential source of bioactive agents [3]. Accordingly, the present work focuses on the phytochemical characterization of the aerial parts of *O. majorana* and the evaluation of its antibacterial activity. The antimicrobial effect was investigated using the agar well diffusion assay, whereby a methanolic extract was tested against a selection of bacterial strains.

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2. Materials and methods

2.1. Plant material

The plant material consisted of the aerial parts of *Origanum majorana* L. (Lamiaceae) collected from the Ouled Driss region, Souk-Ahras province (Algeria). Following collection, the plant material was left to dry at ambient temperature in a shaded, well-ventilated environment to prevent degradation. Once dried, it was finely ground and passed through a sieve to obtain a uniform powder. The resulting aerial parts powder was stored in a sealed glass container, protected from light at room temperature until further use.

2.2. Preparation of the Extract

For extraction, 20 g of the powdered plant material were macerated with 200 mL of methanol in an Erlenmeyer flask. The mixture was continuously stirred and maintained at room temperature for 24 h. After extraction, the solution was filtered using Whatman No° 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator set at 40 °C until a green, dry extract was obtained. This extract was later reconstituted in methanol and stored in a tightly sealed amber vial at 4 °C until analysis.

2.3. Phytochemical Screening

Phytochemical screening enables the detection of bioactive constituents within plant material. It is a qualitative approach designed to identify the main classes of chemical compounds, including polyphenols, coumarins, alkaloids, among others. Prior to analysis, an extract is prepared from dried leaves. The identification of these secondary metabolites is then performed using this extract, through specific chemical reactions involving appropriate reagents selected according to the target compound class.

2.3.1. Detection of Polyphenols

- Extract 0.2 g of powdered plant material with 5 mL of an ethanol/water mixture (6:2, v/v).
- Place the mixture in a water bath for 5 minutes, then filter.
- Take 1 mL of the filtrate and add a few drops of 1% $FeCl_3$ solution.
- The formation of a greenish-black precipitate indicates the presence of phenolic compounds [4].

2.3.2. Detection of Tannins

Condensed Tannins

- **Stiasny Reaction**
  - In a test tube, add 3 mL of a 2% decoction, then add 1.5 mL of Stiasny reagent (10 mL of 40% formaldehyde + 5 mL of concentrated hydrochloric acid).
  - Heat in a water bath for 15 to 30 minutes at 90°C
  - The formation of a flocculent precipitate indicates the presence of catechin (condensed) tannins.
- **Bate-Smith Reaction**
  - Place 0.2 g of plant powder in a test tube, add 2 mL of hydrochloric butanol.
  - Place the tube in a water bath for 15 minutes at 90°C.
  - The appearance of a reddish-brown coloration, which intensifies by agitation, indicates the presence of condensed tannins [5].

Hydrolyzable Tannins

- Filter the solution after treatment with Stiasny's reagent.
- Neutralize the filtrate with sodium acetate.
- Then, 1 mL of 1% $FeCl_3$ solution was added.
- The formation of a black precipitate indicates the presence of gallic tannins not precipitated by Stiasny reagent [5].

2.3.3. Detection of Flavonoids: Cyanidin Reaction

In alcoholic solution and in the presence of nascent hydrogen, flavonoids produce characteristic color changes depending on their type.

- Extract 0.2 g of defatted powder with 80% ethanol in a test tube.
- The tube was incubated in a water bath at 65 °C for 10 minutes.

- The solution is filtered while hot.
- To 2 mL of the filtrate, 0.5 mL of concentrated hydrochloric acid and a few magnesium turnings are added. Observe the appearance of an orange-pink color with flavones, pink-violet with flavanones, cherry red with flavanols, and no coloration with chalcones [4].

2.3.4. Detection of Coumarins

- Macerate 1 g of powdered material in 10 mL of 80% methanol and bring to a boil for 10 minutes.
- Filter and dilute the filtrate to half its concentration.
- Divide the solution into two test tubes (previously checked to ensure absence of fluorescence).
- Add 0.5 mL of 10% ammonia solution to the first tube, while the second serves as a control.
- Observe fluorescence under UV light at 360 nm.

The appearance of blue, purple, or yellow fluorescence indicates the presence of coumarins [5].

2.3.5. Detection of Anthocyanins

- To 5 mL of aqueous extract, add 5 mL of sulfuric acid (H_2SO_4), followed by 5 mL of ammonium hydroxide (NH_4OH).

If the color intensifies under acidic conditions and then turns bluish-purple in alkaline medium, the presence of anthocyanins can be confirmed [5].

2.3.6. Detection of Alkaloids

Alkaloids have the ability to form colored precipitates in acidic aqueous media when treated with certain heavy metal salts and iodinated complex reagents, commonly referred to as general alkaloid reagents.

- To 1 g of powdered plant material, add 10 mL of 10% H_2SO_4.
- Allow the mixture to macerate for 2 minutes.
- Filter and divide the filtrate into three test tubes.
- Add a few drops of each of the following reagents to separate tubes:
  - Dragendorff's reagent (potassium iodobismuthate)
  - Mayer's reagent (potassium mercuric iodide)
  - Bouchardat's reagent (iodine–potassium iodide)

The presence of alkaloids is indicated by the formation of a precipitate: white with Mayer's reagent, brown with Bouchardat's reagent, and orange-red with Dragendorff's reagent [6].

2.3.7. Detection of Saponins

- 2 g of dried and powdered plant material were used to prepare a decoction with 100 mL of distilled water.
- The mixture was brought to a boil for 30 minutes. After cooling and filtration, the volume was adjusted to 100 mL.
- From this stock solution, 10 test tubes (1.3 cm internal diameter) were prepared containing 1, 2, ..., up to 10 mL, and the final volume in each tube was adjusted to 10 mL with distilled water.
- Each tube was vigorously shaken in a horizontal position for 15 seconds.
- After standing for 15 minutes in a vertical position, the height of the persistent foam was measured in centimeters.

The foam index was determined as follows

- If the foam height is less than 1 cm in all tubes, the foam index is lower than 100.
- If a foam height of 1 cm is observed in one of the tubes, the dilution of the plant material in that tube corresponds to the foam index [7].

2.3.8. Detection of Anthraquinone Derivatives

Bornträger's Reaction

- 0.1 g of powdered plant material was macerated in 5 mL of dichloromethane for 15 minutes.

- The mixture was occasionally shaken.
- The solution was filtered into a test tube, and 1 mL of diluted ammonia (1:1) was added to the filtrate.
- The mixture was shaken, and the color change was observed.

Shouteten Reaction

- 50 mg of powdered plant material was mixed with 15 mL of distilled water in an Erlenmeyer flask.
- The mixture was boiled for 15 minutes.
- After cooling, it was filtered.
- The filtrate was divided into two test tubes: one served as a control, while the other received an equal volume of 4% sodium borate solution.
- Both tubes were observed under UV light at 365 nm.
- The appearance of a yellowish-green fluorescence in the borate-treated tube indicates the presence of C-glycosides [8].

2.3.9. Detection of Cardiotonic Glycosides

- 1 g of powdered plant material was extracted with a hydroalcoholic mixture (ethanol/water, 50:50).
- The mixture was brought to a boil for 2 minutes.
- After filtration, lead subacetate (5 drops) was added for clarification, followed by filtration.
- The filtrate was transferred into a separatory funnel, and 15 mL of dichloromethane was added.
- The mixture was shaken and allowed to separate.
- The organic phase was collected and dried over anhydrous sodium sulfate.
- The extract was distributed into evaporating dishes and evaporated to dryness in a sand bath.
- All the following reactions were carried out on the obtained dry residue [6].

Liebermann Reaction

- To the dry residue, add a few drops of acetic anhydride followed by a few drops of concentrated sulfuric acid.
- The appearance of a reddish-brown color changing to olive green indicates the presence of a steroidal nucleus [6].

Keller–Kiliani Reaction

- To the dry residue (prepared as described above), add 2 mL of glacial acetic acid and one drop of 1% $FeCl_3$ solution.
- Carefully layer this mixture onto 2 mL of concentrated sulfuric acid in a test tube.
- The formation of a reddish-brown ring at the interface and a green coloration in the upper phase indicate a positive reaction [6].

Baljet Reaction

- To the dry residue (prepared as described above), add a few drops of 10% NaOH and a few drops of 1% picric acid.
- The appearance of an orange-red coloration indicates a positive reaction [6].

2.4. Evaluation of Antibacterial Activity

The antibacterial activity of the methanolic extract was evaluated using the agar diffusion method [9]. This technique is similar to the standard antibiogram method, in which the antibiotic is replaced with the extract at different concentrations.

2.4.1. Bacterial Strains

The antibacterial activity of the methanolic extract of *Origanum majorana* L. leaves was assessed against various bacterial strains (Table 1), including clinical and foodborne isolates and reference strains provided by the microbiology laboratory of Badji Mokhtar University, Annaba (Algeria).

Table 1 Bacterial strains used in this study

Bacterial strains	Resistance	Type
Escherichia coli ATCC 25922	/	Gram-negative bacilli
Escherichia coli	MCR-1	Gram-negative bacilli
Pseudomonas aeruginosa ATCC 27853	/	Gram-negative bacilli
Pseudomonas aeruginosa	CIP	Gram-negative bacilli
Staphylococcus aureus ATCC 25923	/	Gram-positive cocci
Staphylococcus aureus	MRSA	Gram-positive cocci
Staphylococcus aureus	MSSA	Gram-positive cocci
Bacillus cereus	/	Gram-positive bacilli

2.4.2. Reactivation of Bacterial Strains

Bacterial strains were reactivated by subculturing from the preservation medium onto a solid culture medium (nutrient agar), which was previously melted and poured into Petri dishes to a thickness of approximately 4 mm (the plates were allowed to cool and dry before inoculation). After incubation at 37 °C for 24 h, the plates were removed, and fresh pure cultures were used to prepare bacterial suspensions.

2.4.3. Preparation of Dilutions

From the concentrated extract (C), obtained by dissolving 100 mg of dry residue in 1 mL of DMSO, a series of successive dilutions was prepared to obtain different concentrations: C/2, C/4, C/8, C/16, and C/32. These dilutions were achieved by progressively diluting a volume of the previous solution with an equal volume of dimethyl sulfoxide (DMSO).

2.4.4. Preparation of Discs

Discs with a diameter of 6 mm were prepared using filter paper. They were placed in test tubes, sterilized in an autoclave at 121 °C for 15–20 min under steam pressure, and then stored at room temperature until use.

2.4.5. Preparation of Bacterial Suspensions

Fresh and pure 18-hour-old cultures were used to prepare bacterial suspensions in physiological saline. Briefly, 9 mL of sterile physiological saline was aseptically distributed into screw-capped tubes. Three to five colonies of each bacterial strain were collected using a sterile swab and suspended in tubes with gentle agitation to obtain homogeneous bacterial suspensions.

2.4.6. Preparation of Culture Media

Melted Mueller–Hinton agar (MH) was poured into Petri dishes to a thickness of approximately 4 mm. The pouring was performed under aseptic conditions near a Bunsen burner flame. After pouring, the plates were allowed to cool and solidify at room temperature for a few minutes before use.

2.4.7. Inoculation

- The dried plates were inoculated with sterile swabs dipped in bacterial suspensions under aseptic conditions.
- The swab was soaked in the suspension and pressed against the inner wall of the test tube to remove the excess liquid. Inoculation was performed by streaking the surface of the agar in parallel lines.
- This operation was repeated three times, rotating the plate by 60° each time to ensure the uniform distribution of the inoculum over the entire agar surface. A final sweep was performed around the edge of the plate to remove excess moisture.
- Under aseptic conditions and using sterile forceps, Whatman paper discs impregnated with the extract were placed on the agar surface at equal distances from each other to avoid overlapping inhibition zones. Gentle pressure was applied to ensure the proper adhesion. The discs were not moved once placed.
- The plates were left at room temperature for 30 minutes to allow diffusion of the extract.

2.4.8. Incubation

After the 30-minute diffusion period, all prepared Petri dishes were incubated at 37 °C for 24 hours.

2.4.9. Measurement of Antibacterial Activity

Antibacterial activity was indicated by the appearance of a clear inhibition zone around the discs containing the tested extract, reflecting the inhibition of microbial growth.

After 24 hours of incubation, the plates were examined, and the diameters of the inhibition zones were measured in millimeters (mm) using a caliper, without opening the Petri dishes. Bacterial strains were then classified according to the size of the inhibition zones.

2.4.10. Interpretation of Results

The larger the diameter of the inhibition zone, the more sensitive the bacterial strain is to the tested substance. Conversely, smaller inhibition zones indicate higher resistance. The diameter of the inhibition zones is proportional to the bacteriostatic activity of the extract against the tested strain.

According to the classification described by Konan et al. [10], inhibition zone diameters reflecting antibacterial activity can be grouped into four distinct categories, namely:

- **Resistant (−):** inhibition diameter equal to 6 mm or less than 8 mm
- **Low sensitivity (+):** inhibition diameter between 8 and 14 mm
- **Moderate sensitivity (++):** inhibition diameter between 14 and 20 mm
- **High sensitivity (+++):** inhibition diameter greater than 20 mm

3. Results and Discussion

3.1. Phytochemical Screening

3.1.1. Results

The phytochemical study of *Origanum majorana* L. revealed a rich and diverse composition of secondary metabolites. Qualitative tests showed a strong presence of polyphenols, tannins, flavonoids, coumarins, anthocyanins and cardiotonic glycosides. In contrast, other classes of compounds such as alkaloids, anthraquinones, and saponins were not detected.

The results of the qualitative phytochemical screening are illustrated in Figures 1–12 and summarized in Table 2.

Figure 1 Identification result of polyphenols by the $FeCl_3$ test

Figure 2 Identification results of condensed tannins by the Stiasny reaction

Figure 3 Identification results of tannins condensed by the Bate-Smith reaction

Figure 4 Identification results of hydrolyzable tannins

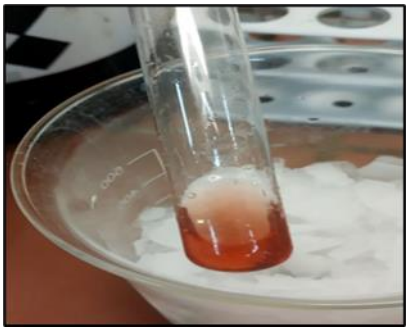


Figure 5 Identification results of flavonoids by the cyanidin reaction

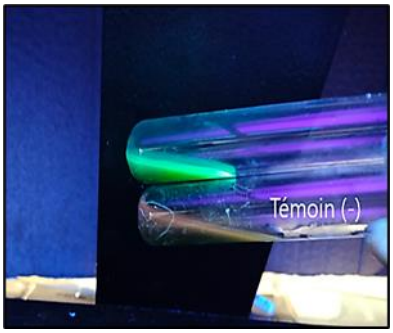


Figure 6 Identification results of coumarins by the fluorescence

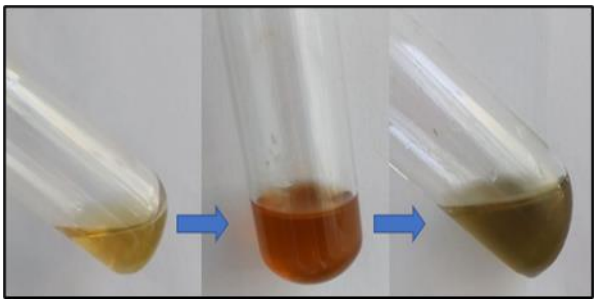


Figure 7 Identification result of anthocyanins

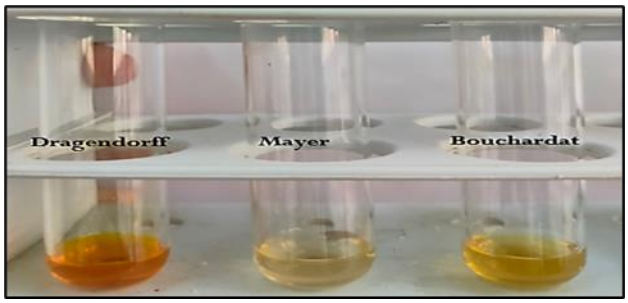


Figure 8 Identification results of alkaloids by the Dragendorff, Mayer and Bouchardat reaction

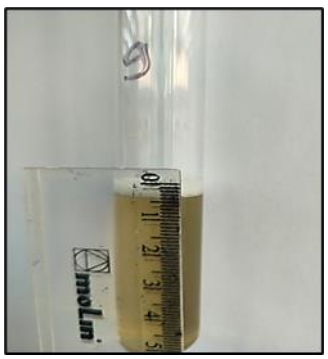


Figure 9 Identification results of saponins by the foam index

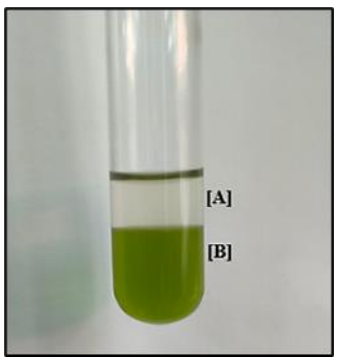


Figure 10 Identification result of anthraquinones by the Bornträger [A]: aqueous phase; [B]: organic phase

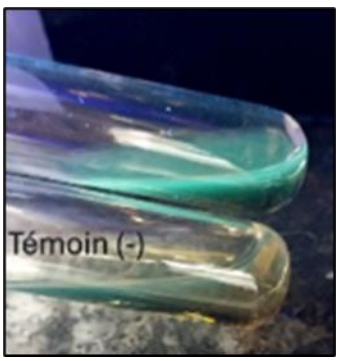
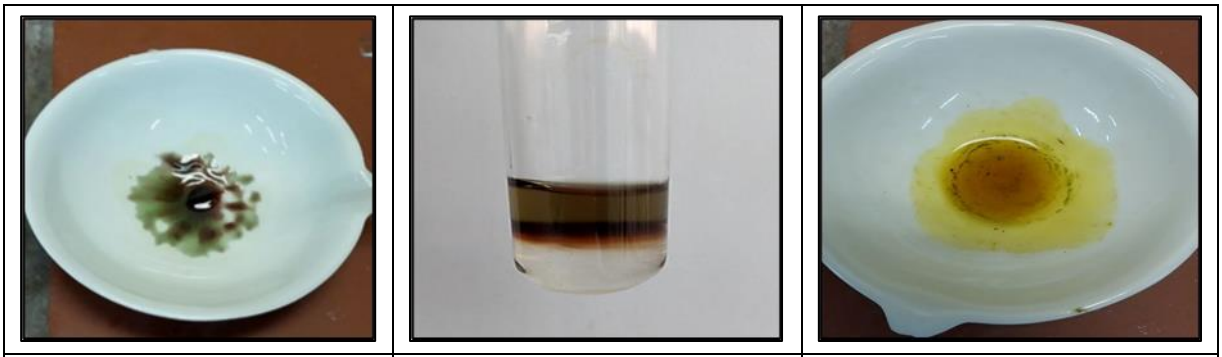


Figure 11 Identification result of anthraquinones by the Shouteten reaction



A	B	C

Figure 12 Identification results of cardiotonic glycosides by the reactions of :(A) Liebermann (B) keller-kiliani (C) Baljet

Table 2 Summary table of physicochemical screening

Phytochemical compounds investigated		**Result**	
Polyphenols		Positive	Greenish-black precipitate
Condensed tannins	Stiasny	Positive	Flocculent precipitate
	Bate-Smith	Positive	Red coloration
Hydrolyzable tannins		Positive	Bluish-black precipitate
Flavonoids		Positive	Red coloration
Coumarins		Positive	Blue-green fluorescence under UV lamp
Anthocyanins		Positive	Change in color in different pH levels
Saponins		Negative	Foam index < 1 cm
Alkaloids	Dragendorff	Negative	Absence of orange-red precipitate
	Mayer	Negative	Absence of white precipitate
	Bouchardat	Negative	Absence of brownish precipitate
Anthraquinone derivatives	Bornträger	Negative	Absence of pink coloration in aqueous phase
	Shouteten	Positive	Yellow-green fluorescence under UV light
Cardiotonic glycosides	Libermann	Positive	Reddish-brown coloration turning to olive green
	Keller kiliani	Positive	Reddish-brown ring; green coloration of the upper phase
	Baljet	Positive	Orange-red coloration

3.1.2. Discussion

The results obtained are generally consistent with the data reported in the literature, highlighting the richness of *Origanum majorana* in phenolic and flavonoid compounds (Ennaji et al., 2020; Bouyahya et al., 2021) [11,12]. The absence of anthraquinones and alkaloids observed in the present study is also in agreement with several previous reports (Vasudeva, 2015; Wang, 2021; Złotek, 2017) [13–15].

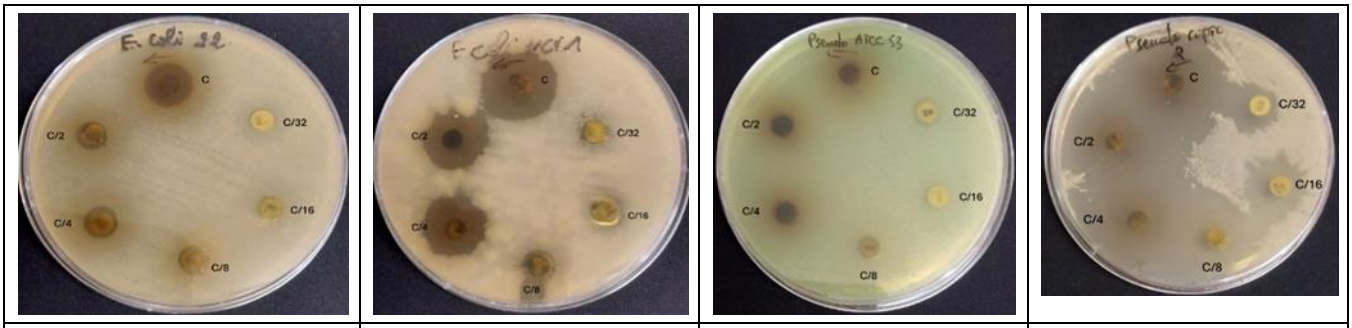
However, the absence of saponins contrasts with some findings in the literature. These discrepancies between studies may be attributed to several factors, including differences in extraction solvents, geographical origin of the plant material, and sensitivity of the detection methods employed.

The presence of phenolic compounds and flavonoids, which are known for their antimicrobial properties, may have contributed to the antibacterial activity observed in this study.

3.2. Antibacterial activity

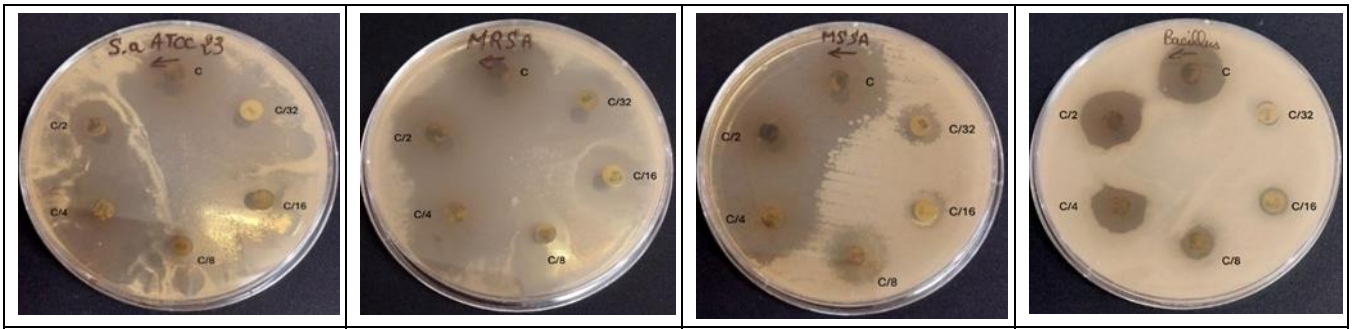
3.2.1. Results

The antibacterial activity of the methanolic extract of *Origanum majorana* L. against the tested bacterial strains is illustrated in Figures 13 and 14.



A B C D

Figure 13 Antibacterial activity of the methanolic extract of *Origanum majorana* L. against: (A) *Escherichia coli* ATCC 25922 (B) *Escherichia coli* MCR-1, (C) *Pseudomonas aeruginosa* ATCC 27853, and (D) *Pseudomonas aeruginosa* CIP



E F G H

Figure 14 Antibacterial activity of the methanolic extract of *Origanum majorana* L. against: (E) *Staphylococcus aureus* ATCC 25923, (F) *Staphylococcus aureus* MRSA, (G) *Staphylococcus aureus* MSSA, and (H) *Bacillus cereus*

Table 3 Inhibition zone diameters (mm) of bacterial strains exposed to the methanolic extract (C) and its serial dilutions

Strains	**Diameters of inhibition zones (mm)**					
	C	C/2	C/4	C/8	C/16	C/32
E. coli ATCC25922	12.74	7.34	7.30	6	6	6
E. coli MCR-1	18.83	15.00	14.95	6.70	6	6
P. aeruginosa ATCC 27853	6	6	6	6	6	6
P. aeruginosa CIP®	>28	>28	>28	>28	>28	15.05
S. aureus ATCC 25923	>28	>28	>28	>28	>28	>28
S. aureus MRSA	>28	>28	>28	>28	19.48	19.00
S. aureus MSSA	28.70	28.01	28.30	15.66	9.75	8.46
Bacillus cereus	16.53	15.75	14.40	7.72	5.48	4.75

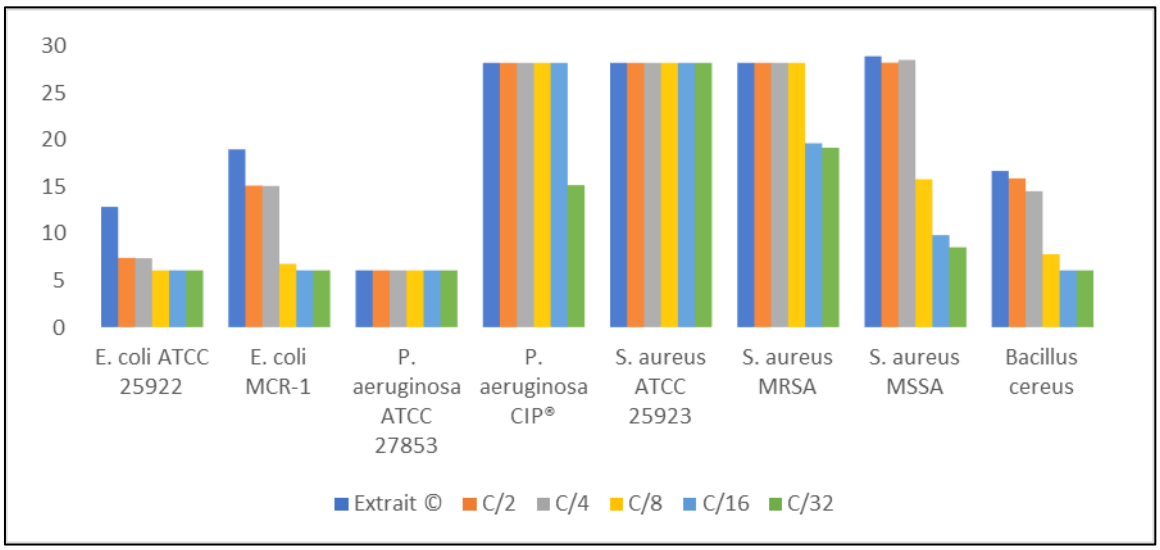


Figure 15 Grouped histogram representing the inhibition zone diameters of the different bacterial strains under the effect of the methanolic extract (C: concentrated) and its various dilutions

3.2.2. Discussion

The methanolic extract of *Origanum majorana* L. showed that most of the strains tested are sensitive to the extract with inhibition zone diameters varying depending on the bacterial species. A progressive decrease in inhibition zones with decreasing concentrations indicated a dose-dependent effect. *Staphylococcus aureus* strains, including multidrug-resistant forms (MRSA) were the most sensitive with large inhibition zone diameter. the richness of the extract in bioactive compounds, particularly phenolics and flavonoids, which are widely recognized for their antimicrobial properties could explain this antibacterial effect. Similar findings have been reported by Saleh et al. [16] and Ellali et al. [17]. A comparable sensitivity was also observed for *Bacillus cereus*, this higher sensitivity may be explained by the structural characteristics of Gram-positive bacteria, especially the absence of an outer membrane that allows easier penetration of active compounds. Benslama et al. [18] reported comparable results indicating the significant inhibition of *B. cereus* using *O. majorana* extracts. Gram-negative bacterial strains, particularly *Escherichia coli* and *Pseudomonas aeruginosa*, were less sensitive. However, the presence of an outer membrane may limit the penetration of active compounds and therefore could explain the lower susceptibility. The results obtained are in agreement with those of Leeja and Thoppil [19] who also reported that a crude methanolic extract showed moderate activity against *P. aeruginosa* at a concentration and strain dependent manner. According to Benslama and others, standard strains of *P. aeruginosa* recorded moderate activity comparable to sensitive strains.

4. Conclusion

This work contributes to the valorization of medicinal plant resources traditionally used in Algeria. It focused on the phytochemical investigation and evaluation of the antibacterial activity of the leaves of *Origanum majorana* L., belonging to the Lamiaceae family.

The phytochemical screening revealed the presence of several secondary metabolites, notably polyphenols, tannins, flavonoids, coumarins and cardiotonic glycosides, while alkaloids, saponins, and anthraquinone derivatives were absent.

The antibacterial activity of the methanolic extract obtained from *Origanum majorana* L., evaluated by the agar diffusion method, revealed a concentration-dependent effect against the tested bacterial strains. *Staphylococcus aureus* ATCC 25923 showed the highest susceptibility with inhibition zones exceeding 28 mm at all concentrations, while *S. aureus* MSSA also exhibited strong activity (28.70–8.46 mm). *Pseudomonas aeruginosa* CIP® was highly sensitive at higher concentrations (>28 mm), but showed reduced inhibition at C/32 (15.05 mm). In contrast, *P. aeruginosa* ATCC 27853 was completely resistant (6 mm at all concentrations). Moderate activity was observed against *Escherichia coli* MCR-1 (18.83–6 mm), E. coli ATCC 25922 (12.74–6 mm), and *Bacillus cereus* (16.53–4.75 mm).

In conclusion, these findings highlight the promising phytochemical and antibacterial potential of *Origanum majorana* L. This species may represent a valuable source of bioactive compounds of pharmaceutical interest and warrants further investigations to identify the molecules responsible for the observed activity.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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