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Original article

Effect of Marjoram Extract Containing Monoterpene Hydrocarbons, Oxygenated Monoterpene, and Phenolic Compounds on Some Gram-Positive and Gram-Negative Bacteria

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Marjoram, Natural Plant, Extraction, Bioactive Substances, Antimicrobial Activity.

Abstract
In the present study, an attempt has been made to study the chemical composition and antimicrobial activity of water and methanol extracts of Origanum Majorana against four types of bacteria, two-gram negative (E. Coil, S. typhi) and two-gram positive (B.sub, s.aur) using agar well diffusion method. Flavonoids, tannins, anthraquinones, terpenes, coumarins, alkaloids, and saponins were the primary bioactive substances identified in Origanum majorana. The antibacterial activity revealed that the methanol extract of Origanum majorana only inhibited Bacillus subtilis and Staphylococcus aureus. However, some bacteria did not exhibit extract resistance. According to the current study, Origanum majorana extract may be a promising option for the hunt for a natural antibacterial agent. The scientific knowledge gained from this investigation will help determine the antibacterial values and explore additional pharmacological features.

Introduction

Natural plants play a significant role in preserving Earth's biodiversity and environmental balance. They have piqued human curiosity since antiquity. Since plants have long been used to cure illnesses and provide medicines, studies have concentrated on their therapeutic applications. This interest persisted for over a thousand years, significantly contributing to the development of knowledge about plant properties and their diverse applications [1]. With the development of science and technology in the contemporary era, laboratory tests have been used in scientific investigations to examine the effects of medicinal herbs. Numerous pharmacological medications have plant origins, including morphine (produced from the opium poppy) and aspirin (extracted from willow bark).

Nowadays, medicinal plants continue to perform a significant role in alternative and traditional medicine, with different species being utilized to treat and prevent illness. According to the World Health Organization, 80% of people worldwide primarily use traditional medicine, and a significant portion of these treatments include plant extracts or their active ingredients [2]. Flavonoids, tannins, alkaloids, and essential oils are among the active ingredients found in medicinal plants that aid in producing a range of therapeutic benefits. These substances can be used to treat a variety of illnesses because they may have antibacterial, anti-inflammatory, and antioxidant properties [3]. Certain therapeutic plant compounds can interact with bacterial cell membranes, disrupting them and stopping the bacteria's ability to reproduce. One important mechanism in the fight against bacteria is this interaction [2].

The kingdom Prokaryotae includes bacteria, which are unicellular creatures. Their cellular structure is comparatively basic, lacking a proper nucleus and membrane-bound organelles. Being among the first living things on the planet, bacteria are found everywhere and can flourish in a variety of settings, including soil, water, air, and even the bodies of living things. They range in size from 0.1 to 5 micrometers and are distinguished by the absence of a genuine nucleus [4].

Numerous characteristics, including shape, gram staining (gram-positive), and oxygen requirements, can be used to categorize bacteria into different categories [5]. Many bacteria have become resistant to chemical antibiotics. However, some medicinal plants have demonstrated strong effects against these resistant bacteria. For example, parsley (*Petroselinum crispum*) contains antimicrobial compounds that combat antibiotic-resistant bacteria, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) [5]. Some medicinal plants may not exhibit bactericidal activity but can inhibit bacterial growth and reproduction. For instance, turmeric (*Curcuma longa*) contains curcumin, which disrupts intracellular bacterial processes and limits proliferation [3].

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Origanum majorana, commonly known as marjoram, is an aromatic, herbaceous perennial plant belonging to the family Lamiaceae. This self-supporting photoautotroph is a member of the genus *Origanum* [6].

Figure 1. Majorana Plant

Origanum majorana exhibits a Mediterranean distribution, with notable occurrences in Morocco, Algeria, Egypt, Spain, Serbia, Italy, and Portugal [6].
"Marjoram typically grows between 30 and 60 cm in height. Its stem is solid, ribbed, and covered with fine brownish-red hairs at the top. The leaves are tongue-shaped, with a darker upper surface. Marjoram produces small, pink flowers clustered in a spindle-shaped inflorescence [7]. The plant is characterized by a distinctive sweet scent, reminiscent of pine and citrus [7]. Seeds can be sown in spring after the last frost, allowing approximately 30 cm between plants. Alternatively, cuttings can be taken from established plants and planted in moist soil [8].
The most important components in the marjoram plant include sabinene, sabinene hydrate, carvacrol, and linalool. It also contains compounds with anti-inflammatory properties, making it beneficial in treating various inflammatory conditions. Studies confirming these anti-inflammatory effects highlight marjoram's utility in reducing inflammation. Furthermore, the active compounds in marjoram help improve cardiovascular health and strengthen blood vessels, thus aiding in the prevention of heart diseases [9]. Marjoram has been used as a flavoring and herbal spice since ancient times. Steam distillation of its leaves and flower heads yields a volatile oil, commercially known as oil of sweet marjoram. This oil is widely used in flavoring food and also in perfumery.
According to Ayurvedic and Yunani medical practices, this plant offers therapeutic benefits for numerous health issues. Its properties, described as pungent, bitter, and 'hot,' contribute to its use in promoting digestion, combating parasitic infections, and detoxifying the body. It is also employed to manage heart and blood-related problems, fevers, leucoderma, and inflammation. [10]. A plant infusion is utilized as a galactagogue, emmenagogue, stimulant, sudorific, and beneficial for paralysis, hysteria, and asthma [11]. Flowers of Origanum majorana have an impact on arterial hypertension [12].
Majorana flowers combined with leaves have been used in Libya to treat premenstrual syndrome, heliananthic colic, coughing, flatulence, menstrual pain, and appetite loss [13]. Numerous investigations have shown Origanum majorana's antibacterial properties. The plant works well as a natural treatment for germs because of its strong antibacterial qualities [11]. The phytochemicals thymol, tannins, arbutin, orientin, vitexin, limonene, terpinene, sabinin, triterpenes, and alkaloids are abundant in Origanum majorana L [12]. The study's objectives were to examine and assess the antibacterial efficacy of novel extracts derived from the majorana plant against a range of harmful bacteria, as well as to research active ingredients and raise public awareness.

Method

Collection of Plant material

A plant specimen, collected in the spring of 2024 from Libya's Al-Asabea region, underwent identification and authentication at the University of Tripoli's Faculty of Science, Department of Botany. Upon arrival at the laboratory, the sample was thoroughly rinsed with distilled water to eliminate contaminants. The complete plant was subsequently air-dried for a fortnight in a shaded, low-humidity environment, avoiding direct sunlight. After drying, it was pulverized using an automated grinder and stored in sterile, airtight glass receptacles for future analysis.

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Figure 2: Collection of Plant material

Chemicals and their sources
Analytical-grade organic solvents (methanol and chloroform) were obtained from Merck (Germany). Antibiotic paper discs (tetracycline and vancomycin) were sourced from Oxoid (England)

Organisms Test
Four bacterial strains were selected to assess the plant's biological activity: Gram-positive *Bacillus subtilis* (B.sub) and Gram-negative *Escherichia coli* (E. coli).

Table1. Four types of bacteria were selected to measure the biological activity of plant.

Gram (+ve)	Gram (-ve)
Bacillus subtilis (B.sub)	Salmonella typhimurium (Sal)
Staphylococcus aureus (S.aur)	Escherichia coli (E.coli)

Extraction Test
Dried plant material was pulverized using a clean electric blender and sieved to obtain a uniform powder. This powder was stored in a dark, airtight glass container (wrapped in aluminum foil) before extraction. The powdered material was then subjected to the following extraction procedures.

Aqueous extraction
Two extraction methods were employed using water as a solvent. For cold water extraction, 20 grams of milled plant material were soaked in 100 ml of distilled water for 6 hours at room temperature (25°C) with continuous agitation. The resulting extract was then filtered through Whatman No. 1 filter paper and stored at 4°C.
For hot water extraction, 20 grams of powdered plant material were boiled in distilled water for 10-15 minutes with constant stirring. After cooling, the extract was filtered using Whatman No. 1 filter paper and stored at 4°C.
For organic solvent extraction, 20 grams of milled, dry plant material were extracted three times with 200 ml of methanol for 24 hours each. The combined extracts were filtered, and the solvent was removed using a rotary evaporator at 40°C under reduced pressure. The resulting crude extract was then filtered through Whatman filter paper and stored at 4°C until use.

Phytochemical Screening
The presence of alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and terpenes was determined through phytochemical screening using standard qualitative methods [14]. The contents of the phytochemicals extracted from marjoram are rated from (+ ve) for light turbidity to (+++ ve) for thick turbidity, as shown in Table 1.

Chemical testing (Color reactions)
The components were identified by conducting chemical tests on the alcoholic and aqueous extracts of powdered specimens using conventional protocols as outlined by Sofowara. Coumarins were found at 366 nm UV light, and after 10% KOH was sprayed, the blue fluorescence spots became stronger [14].

Saponins Test
Saponins are naturally occurring amphiphilic glycosides made up of a hydrophobic aglycone (sapogenin) and a hydrophilic sugar moiety (glycone). They are known for their ability to produce foam in aqueous solutions and decrease surface tension. Approximately 2 grams of plant powder were boiled in 20 milliliters of water and then filtered. 5 ml of the filtered liquid was mixed with 2.5 ml of water and shaken to create a lasting foam. Three drops of olive oil were added to the stable froth, and the mixture was vigorously shaken

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again. The formation of an emulsion, a mixture of immiscible liquids, indicated the presence of Saponins [15] [16].

Anthraquinone Test
Anthraquinone was obtained by shaking 0.5g of extract with 10ml of benzene, filtering the mixture, and then adding 10% ammonia solution. Anthraquinones are indicated by the ammoniacal phase developing a pink, red, or violet color.

Alkaloids Test
Alkaloids are a class of naturally occurring organic compounds found predominantly in plants, characterized by the presence of nitrogen. They are recognized for their wide range of biological activities and are extensively researched for their potential therapeutic applications. Qualitative alkaloid tests are selected to identify the presence of their basic nitrogen-containing structure, typically through precipitation reactions with acidic reagents. A 2.5-gram sample of powdered plant material was subjected to an ethanol extraction (25ml). The ethanol was then removed by evaporation, and the resulting dry residue was treated with 5ml of 2N hydrochloric acid, followed by cooling. The mixture was filtered, and the filtered solution was separated into two equal volumes. One half was then treated with Mayer's reagent and the other half with Wagner's reagent. After that, the samples were examined for precipitation or turbidity [44]. 60ml of water was used to dissolve [HgCl2:1.358g], and then 5g/10ml of KI solution was added. (In 100ml of water, KI (2g) and I2 (1.27g) were dissolved) [16,17].

Tannins Test
Polyphenolic substances found in various plants, known as tannins, are distinguished by their ability to bind and precipitate proteins and alkaloids. These substances are broadly classified into hydrolysable tannins, derived from gallic or ellagic acid, and condensed tannins, which are polymers of flavonoids. Qualitative tests for tannins involve their reaction with specific chemical reagents. In this procedure, 0.5g of dried, powdered samples were boiled in 10 ml of water and filtered. The resulting filtrate was then treated with a few drops of 0.1% ferric chloride. The observation of a brownish-green or blue-black color change served as a positive indicator for the presence of tannins, with the color's intensity correlating to the tannin concentration.

Phenols *Test*
Phenols are a class of aromatic compounds defined by the presence of a hydroxyl group (- OH) directly bonded to an aromatic ring. They display acidic characteristics due to the stabilization of the phenoxide ion through resonance. Many plants contain phenols, which are known for their anti-inflammatory, antibacterial, and antioxidant qualities. Phenols are tested for using both qualitative and quantitative techniques that identify their unique chemical reactivity [16].

Flavonoids Test
Flavonoids are a diverse group of polyphenolic compounds found abundantly in plants, serving as antioxidants, antimicrobial agents, and plant pigments. They are defined by a C6-C3-C6 carbon backbone and are classified into subclasses, including flavones, flavonols, flavanones, isoflavones, and anthocyanidins. The detection of flavonoids utilizes both qualitative and quantitative methods, which exploit their distinct chemical properties. In this test, 5 milliliters of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract, followed by the addition of concentrated sulfuric acid. The observation of a transient yellow coloration in each extract indicated the presence of flavonoids [16].

Terpenoids Test
Terpenoids, commonly referred to as isoprenoids, are a broad and varied class of organic compounds that occur naturally and are produced from isoprene units. They are categorized into many groups, such as diterpenoids, sesquiterpenoids, and monoterpenoids. Terpenoids can be found in biological or chemical materials using a variety of qualitative and quantitative techniques, For example, five milliliters of each plant's aqueous extract were combined with two milliliters of chloroform, and three milliliters of concentrated H_2SO_4 were carefully added to create two layers. The presence of terpenoids is positively indicated by a reddish-brown hue at the contact. [16].

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Evaluation of Extracts' Antimicrobial Properties - Hole-plate diffusion methods

The "hole-plate diffusion methods" [18]. were used to assess the antimicrobial activity of the crude extracts. Each test organism was maintained on a nutrient agar slant and recovered for testing by growing in nutrient broth (No. 2, Biolab, Difco) for 24 hours at 37°C.

Cultures were regularly adjusted to a suspension of 1×106 to 2×106 CFU/ml before to streaking using a pre-made calibration curve that represented viable cell count (X ×106) versus OD 660nm (Y). Using a UV/VIS spectrophotometer, the optical density (OD) of each culture was determined at 660 nm. Fresh sterile nutrient agar was used to dilute each culture 1:10 to prepare agar plates [19]. Using a sterile Eppendorf micropipette with disposable tips, 150µl aliquots of the extract were added to wells in Petri plates (12 cm in diameter) that contained 30 ml of nutrient agar. Holes (8 mm in diameter) were aseptically punched into the agar using a hollow punch. In order to improve the diffusion of the extract into the agar, the plates were maintained at 4°C for one hour. After that, the plates were incubated for eighteen hours at 37°C. Tetracycline and vancomycin were performed as positive controls, while 70% methanol or sterile water was performed as negative controls. At the end of the incubation time, the diameters of the inhibitory zones were measured in millimeters, and the mean of the three experiments was recorded.

Result and discussion

The active phytochemical components of marjoram (Table 2) were identified through screening. The findings showed that the plant has a considerable concentration of anthraquinones, terpenes, alkaloids, flavonoids, and saponins. These substances were present in different amounts in each sample. Nevertheless, the plant was noticeably lacking in certain elements, like tannins and coumarins.

Table 2: Marjoram's qualitative screening results for common phytochemicals.

Phytochemicals	Methanol extract
Alkaloid	+++ve
Flavonoid	+++ve
Tannin	-ve
Terpene	++ve
Coumarin	-ve
Saponin	+ve
Anthraquinone	+ve

+++ve, High, ++ve, Medium, +ve, low, -ve, None

Antimicrobial activity

Aqueous extract

The results of the antimicrobial and phytochemical screening of the aqueous Marjoram extract (Table 2) showed that the plant contained high levels of Flavonoids and alkaloids, moderate levels of Terpenes, saponins, and anthraquinones and that Tannins and coumarins were consistently absent.

According to Table 3, the water extracts' antibacterial activity against both G+ve and G-ve bacteria was weak to extremely weak.

Table 3. Cold aqueous plant extract's in vitro antibacterial efficacy against narrow-spectrum sensitive bacteria

Aqueous extract of Marjoram	Local name	Inhibition Zone (mm)			
		Gram positive		Gram negative	
		S. aur	B.sub	E. coli	S. typhi
	Mardagosha	18	19	15	10

8mm means no observed inhibition, MP extracts are of 200mg/ml concentration, S. aur: Staphylococcus aureus B. sub: Bacillus subtilis E. coli: Escherichia coli S. typhi: Salmonella typhi

The results given in Table (4) showed that the plant's hot water extract exhibited almost the same level of activity against every tested bacterial strain as its cold-water extract.

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Table 4: Hot aqueous plant extracts' in vitro antibacterial efficacy against the arrow spectrum sensitive bacteria

Hot aqueous extract of Marjoram	Local name	Inhibition Zone (mm)*			
		Gram-positive		Gram negative	
		S. aur	B.sub	E. coli	S. typhi
	Mardagosha	18	18	20	18

8mm means no observed inhibition 20 18; MP extract are of 200mg/ml concentration
B. sub: Bacillus subtilis; S. typhi: Salmonella typhi; E. coli: Escherichia coli; S. aur: Staphylococcus aureus

The results obtained (Table 5) indicate that the methanolic extract of marjoram has a slight antibacterial action (DIZ = 18-24 mm on G -ve species and 26-32 mm on G +ve species). The remaining methanolic extract under test indicated exceptional antibacterial activity against both bacterial and Gram-positive organisms. Provided that it produces a DIZ range of 14–18 cm on G+ve bacteria, marjoram extract appears to have relatively superior action when compared to the reference antibiotic Vancomycin (at 30 µg/disc) (Table 5).

Table 5. Antimicrobial activity of marjoram methanolic extract against certain sensitive microorganisms in vitro.

Methanolic extract	Local name	Diameter of Inhibition Zone (mm)*			
		Gram-positive		Gram-negative	
		S. aur	B. sub	E. coil	S. typhi
Marjoram	Mardagosha	32	26	18	24
Vancomycin (30µg)		10	15	24	21
Tetracycline (30µg)		26	24	27	25

8mm means no observed inhibition, MP extracts are of 200mg/ml concentration while diluted methanol (as negative control) gave negative results on all test bacteria. S. aur: Staphylococcus aureus. S. typhi: Salmonella typhi. B. sub: Bacillus subtilis E. coli: Escherichia coli

The weak to moderate antibacterial activity exhibited by the aqueous plant extracts is attributed to the presence of alkaloids, which are known to inhibit microbial processes such as adhesion, enzymatic activity, and cell envelope transport [20]. Research indicates that methanol is more effective than water, ethanol, or hexane when extracting antimicrobial substances from medicinal plants [21]. Furthermore, compared to water extract, the phenolic content of Hieracium pilosella alcoholic extracts was higher [22]. The presence or absence of inhibitory zones was utilized as a qualitative indicator of antibiotic activity, and methanol was determined to be the preferred extraction solvent for this investigation (Tables 5).

Table 6. The MIC (mg/ml) values of methanolic plant extracts on susceptible bacterial Species

Plant extract of marjoram	Local name	B. sub	S. aur	E. coli	S. typhi
	Mardagosha	3.125	3.125	50	50

S. aur: Staphylococcus aureus. B. sub: Bacillus subtilis, E. coli: Escherichia coli, S. typhi: S. Salmonella typhi

Table 6 displays the methanolic extract of marjoram's minimum inhibitory concentration (MIC) values for its impact on four distinct bacterial species: S. aureus (S. aur), Bacillus subtilis (B. sub), Escherichia coli, or E. coli and Typhi, or Salmonella. Gram-negative bacteria have been producing extended-spectrum beta-lactamase enzymes, which have made infections more challenging to treat because they are resistant to a variety of medications [3]. Ofcinalis Rosmarinus [14], Origanum majorana [2], and Trigonella foenum-graecum have been used previously to treat a variety of diseases, including rheumatic cholecystitis, diarrhea, hypertension, and urinary tract infections [2]. Multiple studies have assessed their antibacterial properties, showing that they exhibit diverse levels of activity [2, 5]. Due to the high ESBL prevalence in Libya [11, 23], the antibacterial activity of local plants was investigated.

In this result, we determined that the minimum inhibitory concentration (MIC) of methanolic marjoram extract against *Bacillus subtilis* and *Staphylococcus aureus* was 3.125 mg/ml, revealing significant antibacterial activity against Gram-positive bacteria. The minimum inhibitory concentration (MIC) of the marjoram methanolic extract was determined to be 50 mg/ml for both *Escherichia coli* and *Salmonella typhi*, suggesting lower efficacy against Gram-negative bacteria.

Our results indicate that *Bacillus subtilis* and *Staphylococcus aureus*, being Gram-positive, are more readily affected by plant extracts because of their less intricate cell wall compared to Gram-negative bacteria. In contrast to Gram-positive bacteria, Gram-negative bacteria like Escherichia coli and Salmonella typhi have a more complex cell wall with an additional outer membrane consisting of lipids and proteins, resulting in increased resistance to plant extracts. As seen in other plant-based antimicrobial research, our current

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study supports the conclusion that essential oils are more effective against Gram-negative and Gram-positive bacteria than aqueous or alcoholic extracts.

Conclusion

The traditional Libyan plant under investigation has shown a dual function and is currently investigated as a potential for more, in-depth research on the toxicity and molecular makeup of its active components. Naturally, this could aid in the development of drug design research projects to create naturally occurring lead compounds that are antibacterial and/or antioxidant.

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المستخلص
في الدراسة الحالية تم دراسة التركيب الكيميائي والنشاط المضاد للميكروبات لمستخلصات الماء والميثانول من المردقوش ضد أربعة أنواع من البكتيريا، اثنان سلبيان للجرام واثنان موجبان للجرام باستخدام طريقة انتشار الآبار في الأجار. المواد النشطة البيولوجية الأولية الفعالة التي تم تحديدها من خلال الدراسة في نبات المردقوش هي الفلافونويدات والتانينات والأنثراكينونات والتربينات والكومارين والقلويدات كشف النشاط المضاد للبكتيريا أن مستخلص الميثانول من المردقوش يثبط Bacillus subtilis و Staphylococcus aureus. فقط .ومع ذلك، لم تظهر بعض البكتيريا مقاومة للمستخلص. وفقا للدراسة الحالية، قد يكون مستخلص المردقوش خيارا واعدا للبحث عن عامل طبيعي مضاد للبكتيريا. ستساعد المعرفة العلمية المكتسبة من هذا البحث في تحديد قيم مضادات البكتيريا واستكشاف ميزات دوائية إضافية .

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