



Phytochemical Characterization of Polyphenolic Extracts from *Thymus capitatus* and Evaluation of Their Antimicrobial Efficacy

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التوصيف الكيميائي النباتي للمستخلصات البوليفينولية لنبات الزعتر الرأسي (*Thymus capitatus*)
وتقييم كفاءتها المضادة للميكروبات

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Abstract:

Thymus capitatus is renowned for its rich phytochemical profile and diverse therapeutic properties. This study aimed to characterize the polyphenolic composition of methanolic and ethanolic extracts of *T. capitatus* and evaluate their biological efficacy against a spectrum of pathogenic microorganisms. The phytochemical screening was conducted using High-Performance Liquid Chromatography (HPLC) to identify and quantify bioactive metabolites. The antimicrobial potential was assessed against Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli*, using Ampicillin and Amphotericin B as standard reference drugs. HPLC analysis identified a robust profile of bioactive compounds, notably rosmarinic acid, protocatechuic acid, p-hydroxybenzoic acid, catechin, caffeic acid, apigenin, kaempferol, ferulic acid, and rutin. Both extracts exhibited potent broad-spectrum antimicrobial activity. The presence of these phenolic and flavonoid constituents was strongly correlated with the observed inhibitory effects on both bacterial growths, demonstrating competitive efficacy relative to commercial antibiotics. These findings highlight *T. capitatus* as a significant natural source of antimicrobial agents. The results validate its traditional use and justify further in vivo toxicological and pharmacological investigations to establish its potential as a precursor for novel natural therapeutic formulations.

Keywords: *Thymus capitatus*; Polyphenols; Antimicrobial activity; HPLC analysis; Bioactive compounds.

المخلص

تُعرف نبتة الزعتر الرأسي (*Thymus capitatus*) بغناها بالمركبات الكيميائية النباتية وخصائصها العلاجية المتنوعة. هدفت هذه الدراسة إلى تحديد التركيب البوليفينولي للمستخلصات الميثانولية والإيثانولية لهذه النبتة، وتقييم كفاءتها البيولوجية ضد مجموعة من الكائنات الحية الدقيقة المسببة للأمراض. أُجري

الفحص الكيميائي النباتي باستخدام تقنية الكروماتوغرافيا السائلة عالية الأداء (HPLC) لتحديد وقياس الأيضات النشطة بيولوجياً. كما تم تقييم القدرة المضادة للميكروبات ضد بكتيريا موجبة الجرام (المكورات العنقودية الذهبية (*Staphylococcus aureus*) - وبكتيريا سالبة الجرام (الإشريكية القولونية - (*Escherichia coli*)) مع استخدام الأمبيسيلين والأمفوتيريسين (ب) كأدوية مرجعية قياسية. كشف تحليل الـ HPLC عن ملف قوي من المركبات النشطة بيولوجياً، لا سيما حمض الروزمارينيك، وحمض البروتوكاتيكويك، وحمض هيدروكسي بنزويك، والكاتيكين، وحمض الكافيك، والأبيجينين، والكامفيرول، وحمض الفيروليك، والروتين. أظهر كلا المستخلصين نشاطاً قوياً واسع المدى كمضاد للميكروبات. وارتبط وجود هذه المكونات الفينولية والفلافونويدية ارتباطاً وثيقاً بالتأثيرات المثبطة الملحوظة على نمو البكتيريا، مما أظهر كفاءة تنافسية مقارنة بالمضادات الحيوية التجارية. تسلط هذه النتائج الضوء على نبتة الزعتر الراسي كمصدر طبيعي هام للعوامل المضادة للميكروبات، وتؤكد صحة استخدامها التقليدي، كما تبرر إجراء المزيد من الدراسات الدوائية والسمية في المختبر وعلى الكائنات الحية (*in vivo*) لتحديد إمكانية استخدامها كركيزة لتركيبات علاجية طبيعية مبتكرة.

الكلمات المفتاحية: الزعتر الراسي؛ البوليفينول؛ النشاط المضاد للميكروبات؛ تحليل HPLC؛ المركبات النشطة بيولوجياً.

Introduction

The Lamiaceae family, particularly the genus *Thymus*, represents a cornerstone of ethnomedicine and pharmacognosy. For centuries, various species within this family have been utilized to manage a myriad of infectious diseases, gaining significant global commercial and clinical importance due to their robust and diverse biological activities (Abdel-Hamid et al., 2021; Nordine, 2022). *Thymus capitatus*, a perennial aromatic herb indigenous to the Mediterranean basin and widely distributed across North Africa, derives its name from the Greek word "Thyme," meaning "to fumigate"—a testament to its potent and characteristic fragrance (Barros et al., 2022, Salem & Lakwani, 2024; Soof et al., 2025).

The genus *Thymus* encompasses approximately 215 species, many of which serve dual roles as culinary herbs and therapeutic agents. *T. capitatus* is particularly esteemed for its multi-target pharmacological properties, serving as an antispasmodic, carminative, cough remedy, and expectorant (Salem et al., 2025). Historically, it has been a vital intervention for intestinal infections, exhibiting broad-spectrum efficacy against unicellular and multicellular fungi, as well as a variety of Gram-positive and Gram-negative pathogenic bacteria (Salem & Moammer, 2024).

While extensive literature has characterized the volatile essential oils of *T. capitatus*, the non-volatile polyphenolic fraction remains comparatively under-explored. Emerging evidence suggests that rosmarinic acid is the primary bioactive constituent within the *Thymus* genus, often occurring in synergy with flavone derivatives such as apigenin and luteolin (Alshawish et al., 2025). Consequently, the present study seeks to address this knowledge gap by investigating the polyphenolic profile and biological potential of various solvent extracts of *T. capitatus* (Khalil et al., 2025).

The therapeutic versatility of *T. capitatus* is largely attributed to its complex matrix of secondary metabolites. Among these, apigenin stands out as a significant flavone with well-documented antimicrobial, antiviral, antifungal, and antiparasitic properties (Salem et al., 2025). Furthermore, gallic acid and its derivatives are highly valued in the pharmaceutical and food sectors, primarily due to their exceptional capacity for scavenging reactive oxygen species (ROS) and their potency as antimicrobial agents (Salem, 2024; Salem and Salem., 2025).

Phenolic acids constitute another critical class of compounds within these extracts. Protocatechuic acid (PCA) demonstrates remarkable broad-spectrum antibacterial activity,

effectively inhibiting clinically relevant pathogens such as *Escherichia coli* and *Staphylococcus aureus* (Salem et al., 2025). Additionally, hydroxycinnamic acids—including caffeic acid (CA), p-coumaric acid, and ferulic acid—are prevalent in thyme and are recognized for their protective roles (Khalil et al., 2025). Notably, caffeic acid is regarded as one of the most promising polyphenolic candidates for antimicrobial therapy, particularly against both methicillin-sensitive and methicillin-resistant *S. aureus* strains (Salem, 2024).

Finally, rosmarinic acid (RA)—an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid—serves as a definitive biomarker for the *Thymus* genus. Its antibacterial efficacy has been validated across numerous studies, substantially enhancing the pharmacological profile of the *T. capitatus* polyphenolic fraction (Alshawish et al., 2025; Salem et al., 2025).

In light of these attributes, the current study was designed to provide a comprehensive characterization of *T. capitatus* extracts. The primary objectives involve determining the phytochemical profile via High-Performance Liquid Chromatography (HPLC) and evaluating the plant's nutritional composition. Furthermore, the study assesses the comparative antimicrobial efficacy of methanolic and ethanolic extracts against diverse pathogens, establishing a correlation between specific bioactive metabolites and biological potency. This research aims to substantiate the suitability of thyme extracts as viable natural alternatives for pharmaceutical and nutraceutical applications (Salem, 2025; Soof et al., 2025).

Materials and Methods

1. Chemicals and Instrumentation

All chemicals and analytical standards used were of high analytical grade from Sigma-Aldrich. Chromatographic analysis was performed using the Agilent Technologies 1100 HPLC system, equipped with an autosampler and a Diode-Array Detector (DAD). Compound separation was carried out using an Eclipse XDB-C18 column (150*4.6 mm; 5 μ m) with a C18 guard column (Phenomenex, Torrance, CA). For sample preparation and elemental analysis, a IKA® RV 10B rotary evaporator, microwave digestion system, Inductively Coupled Plasma (ICP) analyzer, and UV-Vis spectrophotometer (Spectronic, Milton Roy) were used.

2. Plant Material and Species Identification

T. capitatus and its fresh aerial parts were obtained. The aerial parts (leaves, flowering tops, and inflorescences) of *T. capitatus* were collected in July 2024 from the Al-Heisha region in southern Derna, Libya (32° 33' 42" N, 22° 31' 08" E). The botanical identity of this plant was officially verified, and the plant material was air-dried indoors before being ground into a uniform fine powder. The identification of the plant species was conducted by Dr. Anwagy ALMASAWRIY, an expert in botany and plant taxonomy.



Figure 1. The aerial parts of the thyme plant as well as the extraction of the oil by the researcher.

3. Preparation of Alcoholic Extracts

The extraction process was conducted using the cold maceration technique to safeguard thermolabile bioactive constituents from thermal degradation. Briefly, 100 g of finely pulverized *T. capitatus* powder was immersed in 1 L of absolute ethanol or methanol (analytical grade). The extraction was maintained at a controlled temperature of 25°C for 72 hours with periodic mechanical agitation. Following filtration, the solvent was eliminated under reduced pressure utilizing a rotary evaporator at 40°C. The resulting crude dry extracts were preserved at 4°C until further analytical procedures (Bilen et al.,2020).

4. Qualitative Phytochemical Screening and Proximate Analysis

The extracts underwent preliminary phytochemical screening to identify key secondary metabolites, including alkaloids, flavonoids, and tannins, utilizing validated biochemical assays (Salem et al., 2025). Furthermore, proximate analysis was performed on both the raw powder and the extracts to determine total protein (via the Kjeldahl method), crude fat (via Soxhlet extraction), total dietary fiber, moisture, and ash content, adhering to the standardized protocols of the AOAC .

5. HPLC-DAD Analysis of Phenolic Compounds

For the precise identification and quantification of phenolic constituents, an optimized alkaline hydrolysis procedure was implemented. A 1 g sample was hydrolyzed using 20 mL of 2M NaOH under a nitrogen atmosphere for 4 hours at room temperature to prevent oxidative loss. The mixture was subsequently acidified to pH 2.0 with 6M HCl and subjected to centrifugation at 5000 rpm for 10 minutes. The resulting supernatant was partitioned twice with a diethyl ether and ethyl acetate mixture (1:1, v/v). The organic phase was concentrated at 45°C, and the residue was reconstituted in 2 mL of HPLC-grade methanol for chromatographic injection (CLSI,2018).

6. Microwave-Assisted Digestion and Mineral Profiling

Elemental determination was executed via Inductively Coupled Plasma (ICP) spectroscopy following a rigorous microwave-assisted acid digestion. A 0.2 g aliquot of the dried plant material was digested with concentrated nitric acid (HNO₃) in a closed-vessel microwave system. The digestion parameters were optimized to ensure complete mineralization of the organic matrix, resulting in a clear, pale-yellow solution suitable for multi-elemental analysis.

Table 1: Optimized Microwave Digestion Program Parameters.

Step	Time (min)	Pressure (Psi)
1	2	120
2	15	150
3	25	180

7. Post-Digestion Protocol and ICP-OES Quantitative Analysis

Upon completion of the microwave-assisted digestion program, the vessels were allowed to reach ambient temperature. In strict adherence to laboratory safety protocols, the vessels were depressurized and opened within a high-efficiency fume hood while utilizing appropriate personal protective equipment (PPE), including chemical-resistant gloves, safety goggles, and laboratory coats (Fraser, 1997; Kim, 2006).

The resulting digestates, characterized by their clarity and transparency, were quantitatively transferred and diluted to a final volume of 50 mL using deionized distilled water. The concentrations of essential minerals, specifically sodium (Na), potassium (K), magnesium

(Mg), selenium (Se), and zinc (Zn), were determined via Inductively Coupled Plasma (ICP) spectrometry (Salem et al., 2025).

8. Polyphenol Chromatographic Analysis

The identification and quantification of polyphenolic compounds were executed using an Agilent Technologies 1100 HPLC system. The apparatus was equipped with a quaternary pump, an autosampler, and a Diode-Array Detector (DAD). Chromatographic separation was achieved using an Eclipse XDB-C18 analytical column (150 × 4.6 mm; 5 μm particle size) coupled with a C18 guard column to ensure column longevity and analytical precision.

8.1. Chromatographic Conditions The mobile phase employed a binary solvent system consisting of Acetonitrile (Solvent A) and 2% aqueous acetic acid (Solvent B). The flow rate was precisely maintained at 0.8 mL/min, with a total run time of 70 minutes. Prior to analysis, all samples were filtered through a 0.45-μm Acrodisc syringe filter to ensure clarity and prevent particulate interference. An injection volume of 50 μL was utilized for each analytical run. The elution was conducted according to a meticulously optimized gradient program, as delineated in Table 2.

Table 2: HPLC Gradient Elution Program.

Time (min)	Solvent A (%)	Solvent B (%)	Elution Phase
0 – 30	0 - 15	85 - 100	Linear Gradient
30 – 50	15 - 50	50 - 85	Linear Gradient
50 – 55	50 - 100	0 - 50	Cleaning/Wash
55 – 60	0 - 100	0 - 100	Re-equilibration
60 – 70	0	100	Isocratic Hold

8.2. Detection and Peak Identification the DAD was monitored at dual wavelengths: 280 nm for detecting benzoic acid derivatives and 320 nm for cinnamic acid derivatives. The identification of polyphenolic compounds was carried out by comparing their retention times (Rt) and UV-Vis spectral characteristics with authentic commercial standards analyzed under the same conditions (Iqbal et al., 2015).

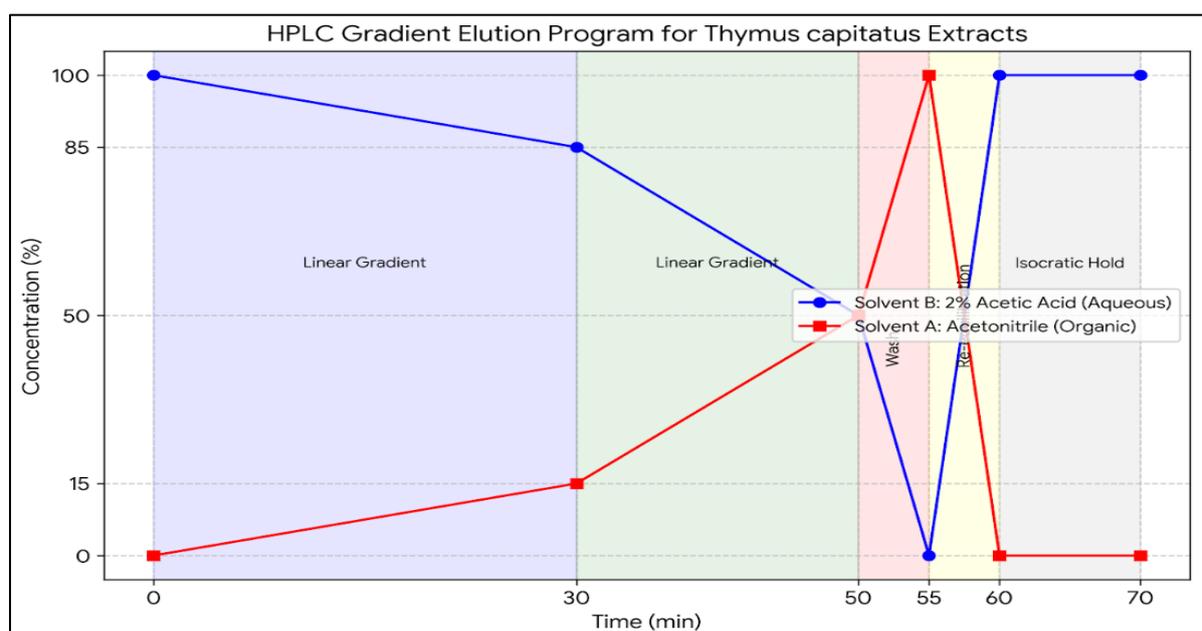


Figure 2: HPLC profile of separation using Gradient Elution.

The separation initiated with a high-aqueous phase (100% Solvent B) to isolate highly polar phenolic acids. A linear increase of the organic modifier (Solvent A: Acetonitrile) was applied up to 55 minutes to elute flavonoids and less polar cinnamic acid derivatives. This was followed by a rapid re-equilibration phase (55–60 min) to restore the column to its initial condition, ensuring reproducibility for subsequent analytical injections.

9. Microbiological Testing

9.1. Microorganisms and Inoculum Preparation The antimicrobial efficacy of *T. capitatus* extracts was evaluated against a diverse group of microorganisms, including the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. Microbial suspension preparations were standardized by adding 100 µL of a live culture into 10 mL of growth medium. The turbidity was adjusted to achieve a final density of approximately 10^8 CFU/mL, in accordance with the 0.5 McFarland standard (Salem et al., 2025; Salem, 2024).

9.2. Modified Kirby-Bauer Disk Diffusion Method Antimicrobial susceptibility was determined using the modified Kirby-Bauer disk diffusion technique (Salem et al., 2025). Aliquots of 100 µL of each standardized suspension were uniformly spread across appropriate agar media. Blank paper disks (Schleicher & Schuell, 8.0 mm diameter) were impregnated with 10 µL of the test extracts and placed onto the inoculated plates. The compounds diffused radially from the disks into the medium, creating a concentration gradient. The plates were then incubated under optimized conditions as detailed in Table 3.

Table 3: Optimized Incubation Parameters for Tested Microorganisms.

Microorganism Type	Representative Strains	Temperature (°C)	Duration (h)
Gram-Positive Bacteria	<i>S. aureus</i>	35 – 37	24 – 48
Gram-Negative Bacteria	<i>E. coli</i>	35 – 37	24 – 48

9.3. Measurement of Inhibition Zones and Controls

- **Positive Control:** Standard Ampicillin antibiotic disks (10 µg) were used to confirm the sensitivity of the bacterial strains.
- **Negative Control:** Disks impregnated with 10 µL of distilled water, chloroform, or DMSO were used to ensure that the solvents did not contribute to the observed inhibitory effects.

Following incubation, the susceptibility of the microorganisms was quantified by measuring the diameter of the Zones of Inhibition (ZOI) in millimeters (mm) using a digital vernier caliper. All measurements and interpretations were conducted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Soliman et al., 2021). The disk diffusion method was selected due to its recognized reliability, accuracy, and efficiency compared to traditional broth dilution techniques (Salem, 2024; Alshawish et al., 2025).

Results and Discussion

1. Phytochemical Screening

The preliminary phytochemical investigation of *T. capitatus* (Table 4) revealed a diverse and rich profile of secondary metabolites. The Liebermann-Burchard reaction confirmed the presence of terpenoids and a notably high concentration of steroids (+++). Furthermore, the

extracts demonstrated significant levels of polyphenols and flavonoids (++), which are widely recognized as the primary drivers of the plant's bioactive and therapeutic potential (Alshawish et al., 2025). Other metabolites, including alkaloids, saponins, and coumarins, were also detected, indicating a complex and chemically synergistic matrix that contributes to the plant's overall biological efficacy (Salem & Lakwani, 2024).

Table 4. Preliminary Phytochemical Screening of *T. capitatus* Extracts

Phytochemical Constituent	Result
Terpenoids	+
Steroids	+++
Alkaloids	+
Saponins	+
Flavonoids	++
Coumarins	++

Key: (+++) Strongly positive; (++) Moderately positive; (+) Positive; (-) Negative.

2. Analysis of Overall Composition and Minerals

Nutritional and mineral analysis (Table 2) exhibited significant variations between the raw plant powder and the alcoholic extracts. The methanolic extract demonstrated superior recovery of total protein (28.0%) and total fat (39.3%) compared to both the ethanolic extract and the raw powder. Regarding mineral content (via ICP-OES), the profile revealed a notably high potassium content (K: 146.91 mg/g) relative to sodium (Na: 30.261 mg/g). This elevated K/Na ratio is clinically significant, providing a scientific basis for the traditional use of thyme in managing hypertension and enhancing cardiovascular health (Salem, 2025; Soliman et al., 2021). Additionally, the presence of zinc (Zn: 4.12 mg/g) and selenium (Se: 0.70 mg/g) highlights the potential of this species as a source of essential antioxidant co-factors (Salem et al., 2025).

Table 2. Proximate Chemical Composition of *T. capitatus* Powder and Alcoholic Extracts

Sample	Total Protein (%) ± SE	Total Fat (%) ± SE	Total Fiber (%) ± SE	Moisture Content (%) ± SE	Total Ash (%) ± SE
Dried Leaf Powder	14.6 ± 0.56	3.9 ± 0.15	4.3 ± 0.16	2.47 ± 0.07	17.18 ± 0.49
Ethanolic Extract	13.74 ± 0.39	15.12 ± 1.13	15.9 ± 1.18	4.17 ± 0.31	11.49 ± 0.21
Methanolic Extract	28.0 ± 0.50	39.3 ± 0.71	10.45 ± 0.10	2.76 ± 0.037	7.54 ± 0.074

Table 3. Mineral Content Analysis via ICP-OES

Element	Symbol	Concentration (mg/g)
Sodium	Na	30.261
Potassium	K	146.91
Magnesium	Mg	41.467
Selenium	Se	0.7002
Zinc	Zn	4.1211

3. Identification of Polyphenols via HPLC-DAD

The chromatographic profiling (HPLC-DAD) identified twenty bioactive compounds (Table 4). The constituents present in the highest concentrations were Caffeic acid (2378.2 µg/g), followed by Protocatechuic acid (1340.62 µg/g) and Ferulic acid (551.72 µg/g). Other pharmacologically significant compounds, such as rosmarinic acid, apigenin, and kaempferol, were identified at robust levels. These molecules are documented for their ability to disrupt microbial membranes and scavenge free radicals, establishing the chemical foundation for the observed antimicrobial activity.

Table 4. Quantitative HPLC-DAD Analysis of Polyphenolic Compounds

Compound	Conc. (µg/g)	Compound	Conc. (µg/g)
Gallic acid	5.12	Sinapic acid	56.87
Protocatechuic acid	1340.62	p-Coumaric acid	23.77
p-Hydroxybenzoic acid	231.22	Rutin	181.62
Gentisic acid	0.00	Rosmarinic acid	146.53
Catechin	208.10	Apigenin-7-glucoside	0.00
Chlorogenic acid	22.33	Cinnamic acid	21.07
Caffeic acid	2378.2	Quercetin	36.05
Syringic acid	88.91	Apigenin	97.35
Vanillic acid	10.31	Kaempferol	176.82
Ferulic acid	551.72	Chrysin	0.00

4. Antibacterial Efficacy

The antimicrobial potential was evaluated against key pathogenic microorganisms (Table 5). Both alcoholic extracts demonstrated potent, broad-spectrum inhibitory activity. The methanolic extract exhibited superior efficacy against *S. aureus* (22.67 mm), comparable to standard antibiotic discs. Significant activity was also recorded against *E. coli* (20.67 mm). The relative resistance observed in Gram-negative bacteria is typically attributed to the structural complexity of their outer membrane, which functions as a selective barrier against hydrophobic and bioactive compounds

Table 5. Antimicrobial Activity (Zones of Inhibition in mm)

Sample	<i>S. aureus</i>	<i>E. coli</i>
Standard Disk	27 ± 0.0	25 ± 0.0
Control (DMSO)	0.0 ± 0.0	0.0 ± 0.0
Ethanollic Extract	20.67± 1.15	18.67± 1.15
Methanolic Extract	22.67± 3.06	20.67± 1.15
Thyme Powder	15.67± 1.15	15.67± 1.15

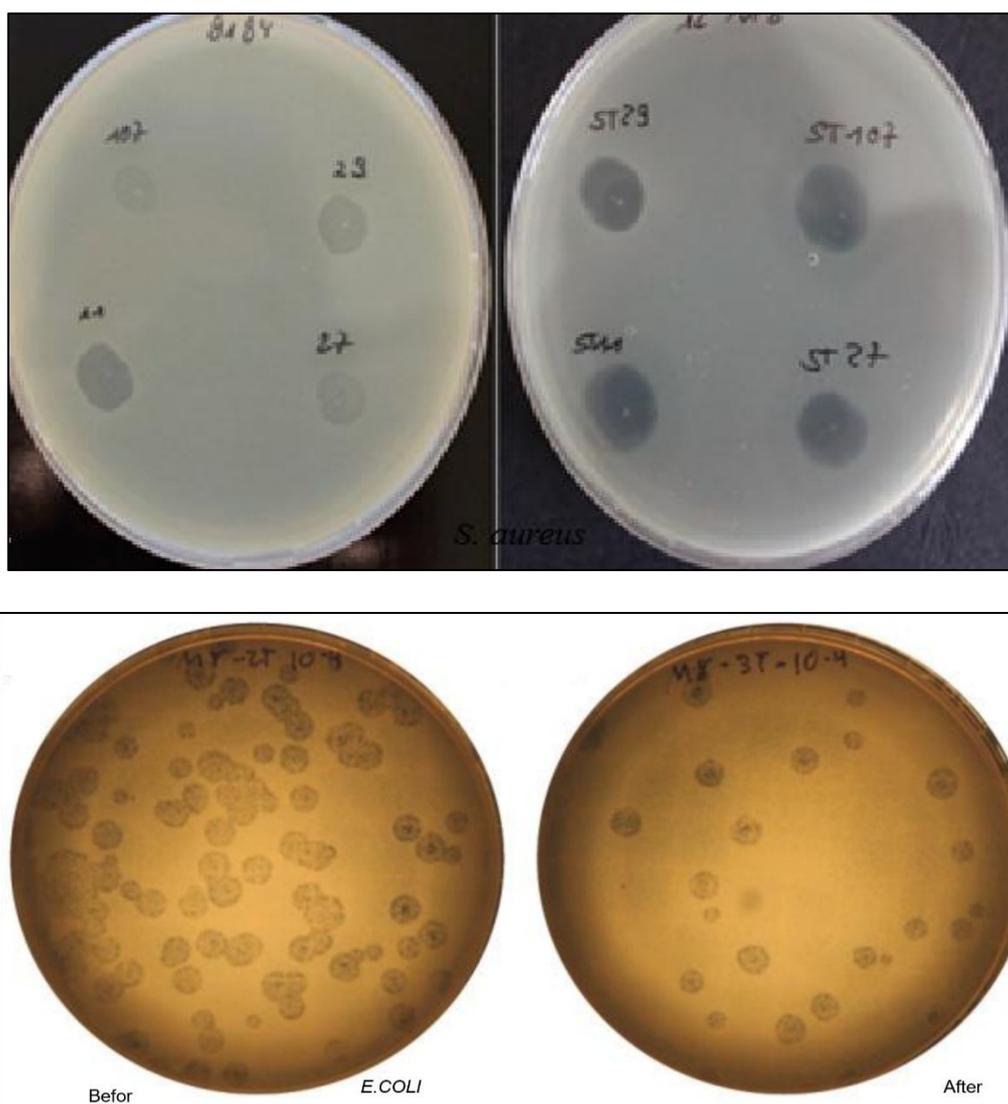


Figure 3. The effect of *T. capitatus* on bacterial growth.

The enhanced performance of the methanolic extract in recovering total proteins and fats is likely due to the higher polarity of methanol, which facilitates better penetration of plant cell walls and increases the solubility of nitrogenous and lipophilic substances (Soliman et al., 2021). Furthermore, the high K/Na ratio promotes sodium excretion and attenuates blood vessel wall tension, thereby assisting in blood pressure regulation (Salem, 2025). Mechanistically, the high concentrations of caffeic and ferulic acids exert their antibacterial effects by compromising the integrity of the microbial plasma membrane, leading to the leakage of intracellular constituents and subsequent cellular death (Thakur et al., 2021; Salem et al., 2025).

Conclusion

The research confirms that methanol is a more effective solvent for extracting nitrogenous compounds and fat-soluble substances from *T. capitatus*. The potent antimicrobial activity is directly associated with the high concentrations of caffeic acid and protocatechuic acid. Furthermore, the mineral profile validates the therapeutic value of this plant for hypertensive patients, providing a scientific basis for its use in health supplements and medicinal formulations.

Recommendations

1. **Food Applications:** Use *T. capitatus* extracts (especially methanolic) as natural alternatives to chemical preservatives in the food industry.
2. **Pharmaceutical Potential:** Further pharmacological studies should focus on isolating pure caffeic and protocatechuic acids to develop novel natural antibiotics.
3. **Clinical Studies:** Transition from *in vitro* testing to *in vivo* animal models to confirm efficacy and assess potential toxicity.
4. **Cosmeceuticals:** Utilize the high flavonoid content in the production of antiseptic creams and solutions for external skin infections.
5. **Biodiversity Conservation:** Implement plans to protect *Thymus* species in the Al-Haisha area (Green Mountain) from overgrazing and deforestation.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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