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Phytochemical screening and antioxidant properties of alcoholic extract and antibacterial activity of *Rosmarinus officinalis* L., Leaves

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Abstract

Antibiotic resistance is increasing due to the increased use of antibiotics. The emergence of new antibacterial drugs with fewer side effects than antibiotics is an issue. The medicinal plant rosemary is widely used in traditional medicine. In this study, an ethanol extract from the leaves of this plant was tested against the most common hospital-acquired infections. The following study aimed to ascertain the phytochemicals, antibacterial, and antioxidant activities of commercial rosemary flavonoids in leaf extract used on major hospital pathogens through disc diffusion and MIC tests. The *Rosmarinus officinalis* plant was used in this experimental study to evaluate its antimicrobial efficacy against pathogens by disc diffusion and MIC tests. The leaves of this plant were extracted in ethanol at concentrations of 200, 100, 50, and 25 mg/ml and evaluated for their antimicrobial effect against several pathogenic strains. In addition, the optical density in the spectrophotometer (620 nm) was used to calculate the minimum inhibitory concentration (MIC) test. In this investigation, Pseudomonas aeruginosa, Enterobacter faecalis, Staphylococcus aureus, and Escherichia coli were all sensitive to the effects of the ethanolic extract of rosemary leaves at a concentration of 200 mg/mL. The minimum inhibitory concentration (MIC) of *E. coli* and *P. aeruginosa* in this extract changed from 25 mg/ml to 100 mg/ml concentration, while the other types of bacteria required only 200 and 100 mg/ml. These findings imply that high concentrations of rosemary extract inhibited all species, including *E. coli*, *S. aureus*, *P. Aeruginosa*, and *E. faecalis*.

Keywords

Rosmarinus officinalis; antimicrobial activity; plant extracts; flavonoids

Introduction

Rosemary, biologically known as *Rosmarinus officinalis* L, is a perennial woody plant native to Asia and the Mediterranean region. It has fragrant, needle-like, evergreen leaves and white, blue, purple, or pink flowers. The leaves of an aromatic evergreen plant called rosemary resemble hemlock needles (1). The leaves are added to stuffing and roasted lamb, chicken, pork, turkey, and beef dishes as a spice and condiment. Rosemary plant is a good source of iron, calcium, glutathione, enzymes, phenolic compounds, vitamins B-6 and E and C, and antioxidant substances, in addition to having a pleasant flavor in culinary preparations (2).

Since ancient times, it has been praised for its therapeutic qualities. According to published studies, "rosemary crude ethanol extract (RO)" may

be an effective antioxidant, anti-inflammatory and anti-tumor agent. In recent years, plant extracts have been created and used as antioxidants in foods. In addition to their antioxidant activity, these extracts may have antimicrobial activity against Gram-positive and Gram-negative bacteria, as they include a wide variety of phenolic compounds, including abietane diterpenoids, carnosol, and ursolic acid (3, 4).

Natural agents are currently being encouraged to replace synthetic antioxidants due to the development of bacteria resistance to antibiotics and the adverse effects of synthetic antioxidants. So, rosemary has long been used as an example in traditional medicine and cosmetics (5, 6). In addition to its therapeutic use, R. officinalis essential oil is significant due to its potent antibacterial, cytotoxic, antioxidant, antichloristic, and chemo-preventive qualities (7, 8).

Although it is evident that rosemary extracts are bioactive, nothing is known about their antibacterial capabilities. Despite their antibacterial properties, rosemary extracts' strong taste has kept them from being utilized extensively in food (9).

the other hand, microorganisms can create bioactive substances with cytotoxic, antifungal, and antibacterial properties through their metabolic pathways. All drugs or chemicals are referred to as general antimicrobial agents, which can kill or inhibit the growth of bacteria. The specific toxicity of an antimicrobial agent varies. Some have similar effects on all cell types and act in a relatively non-selective manner. Chemotherapy for infectious diseases often benefits greatly from antimicrobials with selective toxicity. Antimicrobial drugs affect bacteria to varying degrees. They have been found to have many targets within the thin microbial cell, and when all of these targets are disrupted, a microbicidal effect occurs (10, 11).

Interest in phytochemicals as a new source of organic antioxidants, antimicrobials, and hospital infection treatments is on the rise in developed countries. Pathogens are often attacked by synthetic chemicals, but sadly, this indiscriminate use of commercial antibiotics has led to the development of antibiotic resistance in many microorganisms. In addition, these drugs may have negative side effects such as immunosuppression and allergic reactions (12, 13). Therefore, the use of plant extracts is safer for the environment and human health.

Pathogens of the Enterobacteriaceae family are Gram-negative and opportunistic and include *E. faecalis* and *E. coli*. *S. aureus* is also common in hospitals as a Gram -positive bacterium. It regards one of the biggest problems plaguing people who use medications or people whose lifestyles are somehow linked and associated with hospitals is hospital-associated infections (14, 15). One of the most prevalent microorganisms causing infections in hospitalized patients is *P. aeruginosa*. In this work, we investigated the response of *P. aeruginosa* and other bacterial strains to concentrated extracts of rosemary extract. Enterobacteriaceae have recently become an important pathogen of intranasal infections (16). To identify the phytochemical, antibacterial, and antioxidant activities of commercial rosemary flavonoids for use against Gram-negative bacteria *(P. aeruginosa, E. faecium, E. coli* and Gram-positive bacteria (*S. aureus*).

Materials and Methods

This study was conducted at the Laboratory of the Clinical & laboratories Science Department, College of Pharmacy, University of Mosul, in 2019.

Samples Collection:

Leaves of rosemary were collected and identified in cooperation with the Herbarium and Herbal Research Center of Mosul. The test organisms *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa*, and *Enterobacter faecalis* were obtained from the Biology Department- College of the Science/University of Mosul Iraq.

Preparation of Plant extracts:

The method of Golshani and Sharifzadeh (17) was adopted for the preparation of plant extracts with little modifications. Briefly, the fresh leaves of *R. officinalis* were collected and dried in a place not exposed to sunlight. Then, the dried leaves were ground into a powder, and 25 g of the plant powder was placed in 150 ml of ethanol absolute and stirred for 24 h at room temperature. Whatman No. 1 filter paper was used to filter the mixture.

Phytochemical screening:

The evaluation and the screening of rosemary extract were done using a conventional approach as used by (18, 19).

Alkaloid:

A total of 1 ml of Mayer's reagent was added to a test tube containing a small amount of sample (mercuric potassium iodide). Alkaloid was present because a yellow-cream precipitate was formed.

Terpenoids testing:

After adding two drops of chloroform and 1 ml of strong hydrochloric acid to a small sample and heating for two minutes, a reddish-brown color appeared, indicating the presence of terpenoids.

Phenol testing:

A total of 2 ml of ferric chloride solution (5%) was added to a small portion of the sample and allowed to incubate at room temperature for 5 minutes. The presence of phenol is indicated by the production of a black, blue hue.

Tannin testing:

A few drops of natural lead acetate solution (10%) were added to a small amount of the sample to show the presence of tannins by producing a yellow ppt precipitate.

Carbohydrates testing:

Molisch test: The sulfuric acid-treated extract was carefully added along the side of the test tube, where it formed a violent ring indicating the presence of carbohydrates (20).

Saponins testing: A little sample was obtained, and 2-3

drops of distilled water were added before shaking thoroughly. The formation of foam shows the presence of saponin.

Coumarin glycosides testing:

The presence of coumarin was detected by the appearance of a yellow precipitate after the introduction of a few drops of the extract into diluted HNO_3 (21).

Test for flavonoids:

$FeCl_3$ solution was added to the sample solution, and the onset of a tingling, greenish-black color signifies the presence of flavonoids.

Quinine test:

Quinine can be detected by adding ml of sodium hydroxide solution to a tiny sample and waiting two minutes. Quinine will then turn reddish-brown.

Protein testing:

A little sample was obtained and placed in a test tube. A drop of concentrated HNO_3 was then added. Reddish color formation revealed the presence of the protein.

Steroid testing through Salkowaski reaction:

A small sample was mixed with 1 ml of concentrated H_2SO_4 and 1 ml of chloroform, stirred thoroughly, and left for 5 minutes. When the lower layer appears red, it indicates the presence of steroids.

Extraction of Flavonoid:

The dried leaves of rosemary were ground to a powder, and 25 g of the plant powder was placed in 150 ml of 100% ethanol and stirred for 24h at room temperature. Whatman No. 1 filter paper was used to filter the mixture. With the remaining residue, the above procedure was performed again, and 150 ml of absolute ethanol and the two filtrates were properly combined. The filtrate was then mixed with 100 ml of 1% lead acetate solution for 4 hours to ensure complete precipitation, after which the filtrate was collected. The filtrate was mixed with 250 ml of acetone and 30 ml of hydrochloric acid. The entire mixed solution was stored at 4°C. The 100 ml liquid sample was completely evaporated each time using a rotary evaporator while operating under reduced pressure to produce a dark brown precipitate, which was then used in various scientific experiments (22).

Chemical screening of Flavonoids:

The presence of flavonoids and phenolic compounds was checked by adding 5 ml of plant extract to a few drops of 1% potassium hydroxide (KOH) and 1% ferric chloride ($FeCl_3$. $2H_2O$) solutions, respectively, at a time. The formation of the yellow and blue-green colors showed the presence of flavonoids and phenolic chemicals in the plant extracts, respectively (23).

Determining the net flavonoid content in the extracts

A drop of glacial acetic acid, 1.0 ml of aluminum trichloride in ethanol (20 mg/ml), and 1 ml of rosemary extract were mixed to detect the flavonoid content. The mixture was then diluted with ethanol to a volume of 25 ml to quantify the overall flavonoid concentration. After 40 minutes, the absorbance at 415 nm was measured using (a spectrophotometer). 1.0 ml of rosemary extract, one drop of glacial acetic acid, and 25 ml of ethanol were used to prepare the blank. Under the same conditions, the absorption of a standard (quercetin) solution (0.5 mg/ml) of ethanol was determined.

The following equation was used to determine the total amount of flavonoids present in the rosemary extract as quercetin equivalents (QE):

X=(A.mg)/(Aq.m) (Eq.1)

Where: X is the number of flavonoids (mg/mg rosemary extract in QE), A is the amount of Rosemary extract absorbed, Aq is the number of standard quercetin solutions absorbed, m solution is the number of pomegranate flowers (mg) in the extract, and mq in rosemary s is the amount of quercetin in the solution (mg).

Effect of extract on DPPH radical scavenging

The ability of plant extracts to scavenge free radicals was assessed using the following method. Plant extracts were added to a known volume of test tubes (50-150 L), completed with a known volume (1.0 mL) by D.W., and then (1.0 mL) of DPPH solution (0.2 mM of ethanol) was added to each test tube. After that, the tubes were left at room temperature for 30 minutes. The same procedure was used to make a control but without the use of plant extracts. As a positive control, an ascorbic acid solution (0.03% w/v) was used. The absorbance (A) of this solution was determined at 517 nm using a Jenway 6300 spectrophotometer. The following formula was used to determine the percentage inhibition of DPPH radicals in this product. The percentage I am equal to [(Ac-As)/Ac] (24).

Identification of the compounds:

FTIR spectrophotometer was used to evaluate the plant extract. Japan's SIMADZU, Model No. 8400S.

Bacterial Culture:

The rosemary flavonoid extracts were then stored in a refrigerator, covered with aluminum foil to protect them from the effects of light. Flavonoid extracts were administered and dissolved in 5% dimethyl sulfoxide at doses of 25, 50, 100, and 200 mg/ml (DMSO). The inhibitory activity was then evaluated using the disc diffusion technique and MIC test.

Evaluation of the rosemary flavonoid extract's inhibitory activity:

Mueller-Hinton (MH) agar is used to maintain bacterial cultures. It was made according to the instructions provided by the manufacturer. The surfaces of agar plates previously infected with a standard number (1.5105) of microorganisms were covered with sterile filter paper sheets of uniform size (6 mm) that had been soaked with various concentrations, which were 25, 50, 100, and 200 mg/ml of RO alcohol extract. The incubation period of the plates was of one day at room temperature of 37°C. At the end of the incubation, the plates were checked for the presence of an inhibition zone (IZ) around the plate that would prevent bacterial growth.

MIC test:

The antimicrobial activity of the extracts was observed after six hours, and this was determined by the minimum inhibitory concentration of each target bacteria individually. Tubes containing nutrient broth enriched with different amounts (10-500 L) of extracts were filled with 100 L of 10^6 cells/ml. After 24 h at 37°C, sample readings were compared to negative controls by measuring the optical density (620 nm) in a spectrophotometer, and the results were recorded (media without bacteria), with samples without turbidity classified as MIC. Each test was performed three times, and the mean value was then displayed (25).

Antibiotic resistance:

Antibiotic plates were placed on inoculation plates at 37°C for 24 h. Following this, bacterial strains suspended in nutrient broth were inoculated onto the surface of each nutrient agar plate. Chloramphenicol (C), ceftriaxone (CRO), ciprofloxacin (CIP), and amoxicillin (AM) were utilized in this study.

Bacterial cultures and preparation of rosemary extracts:

The leaves were powdered after sun-drying. The leaves weighing up to 50 g were added to a sterilized Erlenmeyer flask. The next stage was the addition of 250 ml of 100% ethanol to dissolve the components of the herbs. The alcohol and herbal powder were placed in a conical flask and shaken for 48 hours until the solvent started to act at a temperature of 40°C. The solvent was then removed using the rotary method. Finally, the rosemary extract was stored in sterilized refrigerator-safe plates and covered with aluminum foil to protect it from light. The extracts were provided in 5% dimethyl sulfoxide at strengths of 25, 50, 100, and 200 mg/ml. Inhibitory activity was then evaluated using disc diffusion techniques and MIC testing.

Results

As shown in Table 1 and Fig. 1, the chemical analysis of rosemary leaf extracts revealed the presence of different concentrations of active substances and other unidentified components.

Table 1. Results of phytochemical analysis Results of phytochemical screening of *R. officinalis* leaf extracts.

Phytoconstituents test	Observations	Result
Alkaloids	formation of yellow	+
Terpenoids	Formation reddish colour	+
Phenols	Observed black-bluish precipitate	+
Tannins	Observed yellow precipitate	+
Saponins	No Formation foam	-
Coumarine glycoside	Observed yellow precipitate	+
Flavonoids	Formation greenish blue	+
Quinine	Formation reddish brown	+
Steroids	Formation red colour	+
Protein	No Formation of reddish colour	-
Carbohydrate	No Formation purple ring	-

(+) = positive reaction; (-) = negative reaction.

Flavonoid Content

In Table 2, the total flavonoids of rosemary ethanolic extract are given as quercetin equivalents (quercetin/g extract). The results showed that the total flavonoid content of the ethanolic extract was 8.34 mg QUE/g dry weight.

Table 2. Total solid and flavonoid content of *R. officinalis* leaves

Alcoholic *R. officinalis* leaves	Total solids (%)	Total flavonoids (mg/g)
	36	8.34

Fig. 1. Results of phytochemical Screening *R. officinalis* leaves extracts

Antioxidant Study:

DPPH radical scavenging activity

The radical scavenging potential of different concentrations of rosemary leaf extract varied from 75% concentration at 62.5 ppm to 76% at 125 pm and 79% at 187 ppm, depending on the concentration. Table 3 summarizes the results of scavenging DPPH activity by flavonoids of rosemary leaves ethanolic extract and compares them with the standard antioxidant ascorbic acid, as shown in (Fig. 2).

Table 3. Scavenging activity of ethanolic extract of rosemary leaves against DPPH.

Treatment	Conc. (ppm)	Scavenging activity (%)
Alcoholic rosemary leaves	62.5	75
	125	76
	187	79.3
Ascorbic acid	23	81.96

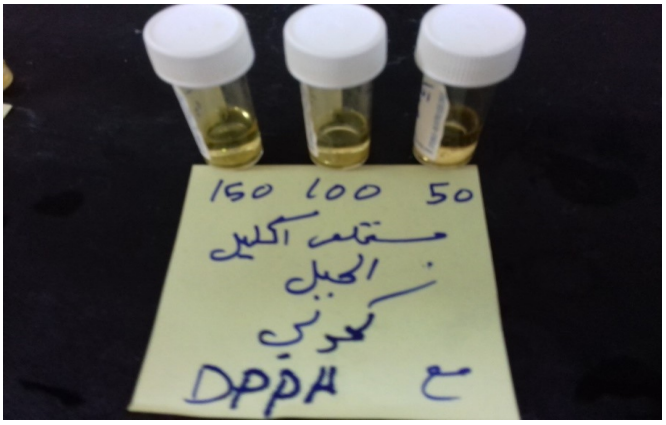

Fig. 2. Discoloration of DPPH under the influence of *R. officinalis* leaves extract at different concentrations.

Fourier Transform Infrared Spectroscopy (FTIR) Assay

The solutions were analyzed individually using an FT-IR spectrophotometer (SHIMADZU, Japan, model 8400S). According to the FT-IR analysis (Fig. 3), the molecule includes multifunctional groups such as phenolic -CH2-, -C=C-, C=O, etc. (Table 4).

Table 4. The functional groups of the purified flavonoid compound from the IR-spectrum

Wave no.2	Band shape	Band	Functional group
3329	Brand	OH	Alcohol
2927	sharp	-CH2-	Aliphatic stretch
1446-1517	sharp	-C=C-	Stretching of olefinic
1684	sharp	C=O	Stretching C =O of -CHO Group
1270	sharp	C-O	C-O Stretching

According to the data, quinoline alkaloids, phytol, eucalyptol, camphor, and verbenone were the most prevalent substances. Other substances, such as borneol flavor, borneol acetate flavor, fragrance, heliotrope, geraniol, and lidocaine, were found in lower concentrations.

Activities of referred antibiotics:

The bactericidal activities of the suggested antibiotics were also conducted on four types of bacteria that were taken into consideration in this study. (Table 5) (Fig. 4)

Table 5. Antimicrobial activities of the suggested antibiotics against tested microorganisms represented as resistant (R), susceptible (S), and intermediate (I).

Bacterial strains	Antibiotics			
	C*	CRO*	CIP*	AM*
Staphylococcus aureus	R	R	R	R
Escherichia coli	S	R	R	S
Pseudomonas aeruginosa	I	S	S	I
Enterobacter faecalis	I	R	S	S

*Chloramphenicol (C), Ceftriaxone (CRO) Ciprofloxacin (CIP), Amikacin (AM)

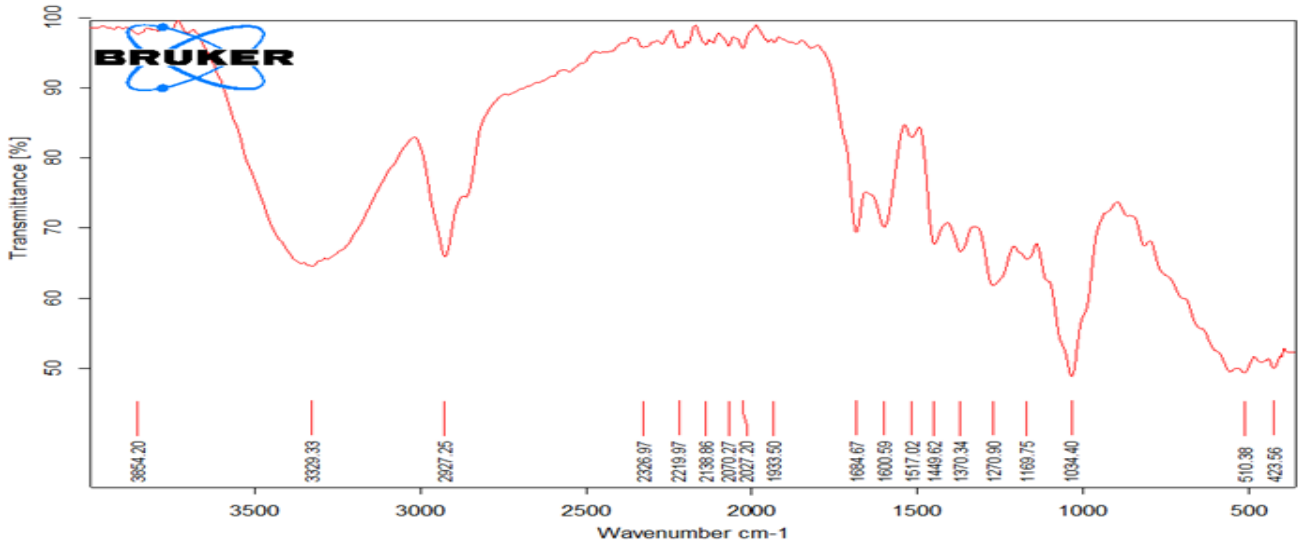

Fig. 3. The result of FTIR Spectroscopy

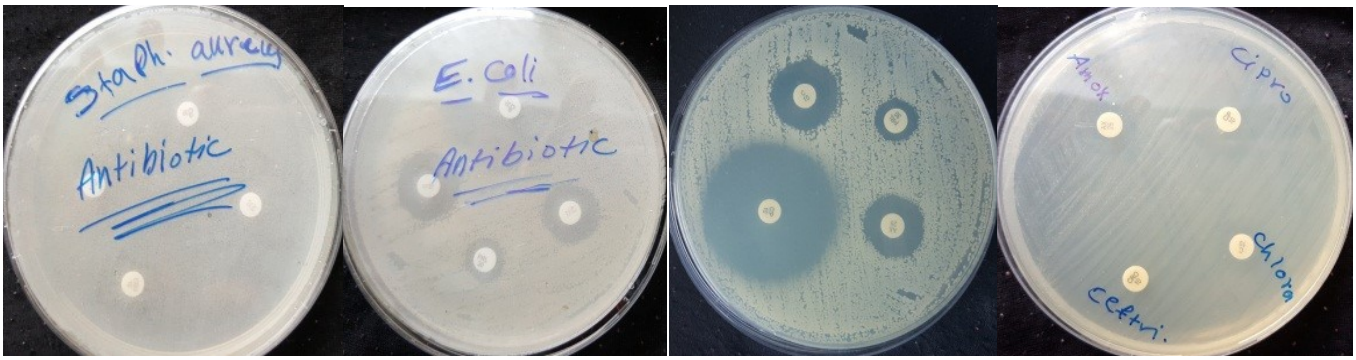

Fig. 4. Inhibition zones (mm) of antibiotics against 1- E. faecalis, 2- P. aeruginosa, 3- E. coli, 4- S. aureus, respectively.

Antibacterial activity of rosemary extracts on various microorganisms:

In this investigation, the presence of IZ in millimeters was used to assess the antimicrobial activity of phenolic components of rosemary leaf extracts against the tested types. (Table 6) (Fig. 5)

Table 6. Antimicrobial activities of RO alcoholic extract against tested microorganisms represented as inhibition zone diameter (mm)

Concentration of Extracts mg/ml	*E.coli*	*P. aeruginosa*	*S.aureus*	*E. faecalis*
200	19mm	21mm	10mm	10mm
100	13mm	15	8	8
50	10	11	-	-
25	8	8	-	-

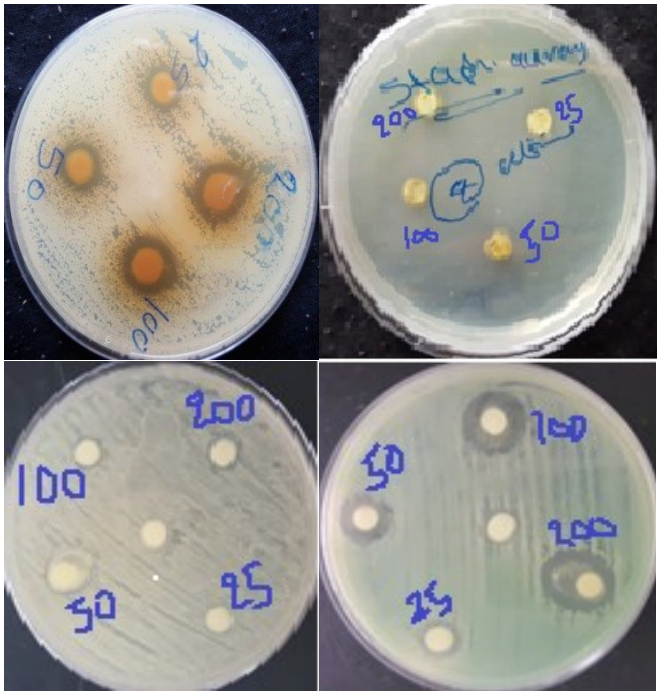

Fig. 5. Inhibition zones (mm) of rosemary ethanolic extract against : 1- *S. aureus*, 2- *E. coli*, 3- *P. aeruginosa* and *E. faecalis*, respectively.

Minimum inhibitory concentration (MIC)

As shown in Table 7, the ethanolic extract of rosemary leaves showed MICs ranging from 0.125 to 0.5 mg/ml against the indicated bacteria.

Table 7. MIC values of alcoholic rosemary extract determined with agar diffusion method.

Bacterial species	MIC
Pseudomonas aeruginosa	0.25
Escherichia coli	0.125
Enterobacter faecalis	0.5
Staphylococcus aureus	0.5

Discussion

The main causes of food quality deterioration and shelf life reduction are bacterial infection and lipid oxidation. Therefore, controlling bacterial cross-contamination and delaying lipid oxidation is of critical importance to food processors (26). Although the rosemary plant is a rich source of phenolic compounds with strong antioxidant and antimicrobial properties, little is known about its antimicrobial effects. Furthermore, there is some evidence that minor elements have an important role in antimicrobial activity, perhaps through synergistic effects with other elements. In this investigation, the flavonoids found to be most effective against bacterial species were rosmarinic acid and carnosic acid (27). Their unique antioxidant function may be related to their antibacterial effect. It was found that ethanolic plant extracts showed broad-spectrum activity against Gram-positive and Gram-negative bacteria (28).

The results of the study showed that phenolic flavonoids and substituted benzene hydrocarbons were the more readily available components of rosemary extracts. GC-MS analysis of rosemary led to the identification of more than 20 compounds by comparing the recorded mass spectra with those in a computer library, although the most active substances were eucalyptol, camphor, lobster, lobster acetate, and verbenone (29). The quinoline alkaloids found in rosemary extracts are of high medicinal value; in addition to their recognized antimicrobial properties, they are frequently used in the food and soft drink industries. Phytol had the largest percentage, and it was used as a mouthwash and preservative. Stearic acid, geraniol, and geraniene were the substances with the lowest percentages (0.64%) at (0.71%), respectively (30, 31). These findings are consistent with a related study that found that rosemary extract included higher levels of camphor, lobster, lobster acetate, and eucalyptol. These variations may be due to seasonal variations, plant conditions, and extraction techniques.

E. coli was sensitive to some drugs but resistant to ciprofloxacin and ceftriaxone, while *S. aureus* showed resistance to all prescribed antibiotics. On the other hand, *P. aeruginosa* showed some susceptibility to other antibiotics. These findings support the notion that *E. coli* strains resistant to some antibiotics become vulnerable to alcoholic extracts of *R. officinalis* plants (32).

The flavonoids in the rosemary extract were active against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*, and *E. faecalis*) pathogenic microorganisms at concentrations 200, 100, 50, and 25 mg/ml ranging from 8 to 20 mm (Table 6). The zone of inhibition increased with the increasing concentration of methanolic extract. Furthermore, the results showed that rosemary extract affected *P. aeruginosa*, contrary to the previous investigations (33, 34). In addition, *P. aeruginosa* and *E. coli* were more sensitive to the concentration of 200 mg/ml. The results of the study showed that there were significant differences in the sensitivity of the tested bacteria to ethanol extracts. In other words, Gram-negative bacteria such as *E. coli* and *P. aeruginosa* showed more sensitivity than Gram-positive bacteria such as *S. aureus*.

Their growth was stopped in 25 mg/ml of extract. These findings are inconsistent with those who claim that Gram-positive bacteria are the most sensitive compared to other bacterial species. According to those who found that the changes in antimicrobial activity could be caused by changes in the bacterial cell wall composition, the extract and its components were more active against the bacterial species (35). In addition, the presence of an inhibition zone does not always indicate that a compound is inactive. For example, non-polar substances may not disperse into the culture medium.

The MIC of rosemary extract is considered to be one of the lowest levels in inhibiting the growth of bacterial cells. The data suggest that higher extract concentrations are needed to inhibit microorganisms. *E. coli* can be inhibited with 25 mg/ml of extract, but *S. aureus* requires 100 mg/ml. The use of plants to treat diseases, including infectious diseases, has been widely used, and data in the literature, as well as our findings, reveal the great potential of phototherapy, although they have not been fully investigated (36, 37). Therefore, more research is needed to find new compounds. Once extracted, they should be tested for toxicity in vivo bioassays before being used in new therapeutic approaches, as has been demonstrated for different plant extracts.

The development of antibiotic resistance has been of particular concern and is currently one of the main therapeutic issues. Since rosemary extracts have been shown to have antibacterial effects against various bacteria, they are recommended for treating bacteria-causing infections. Further research into medicinal plants is recommended as potential alternatives to chemical drugs, which, although beneficial, have negative side effects (38).

The European Food Safety Authority (EFSA) has examined the safety of rosemary extracts. It has been concluded that estimates of excessive intake of rosemary alcohol and rosemary acid are 0.09 (elderly) to 0.81 (adolescents) mg/kg per day. Rosemary extracts are now added to food and beverages in the EU in amounts up to 400 mg/kg (as the sum of carnosic acid and carnosol).

Conclusion

In conclusion, it can be said that *Rosmarinus officinalis* L. leaf extract has inhibitory effects on different types of bacteria and can be used in the pharmaceutical industry to create new synthetic drugs to treat infectious diseases brought about by food pathogens of these bacterial species, such as *E. coli*, *P. aeruginosa*, etc., which may cause food spoilage and subsequently human diseases.

These findings imply that plant extracts belonging to *R. officinalis* might be employed as natural antibacterial agents in medical settings. Further study will be needed to determine the active component in each extract and to investigate its phytopharmaceutical properties.

Overall, the prevalence of drug-resistant strains has grown over the past several decades due to the widespread use of antibiotics and other antimicrobial treatments, especially in the treatment of infections. Due to the harmful effects and high levels of toxicity of most manufactured remedies, there has become a great urgent need to create alternative materials such as herbal extracts or their active ingredients in treatments.

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Authors' contributions

TA carried out the Conception and design, Analysis and interpretation of the data, and Drafting of the article, while EA performed the critical revision of the article for important intellectual content and provision of study materials. FO conceived the study and participated in its design and coordination. All authors contributed to the study design, performed the statistical analysis, and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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