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Original Article

Phenolic Compounds, Antioxidants, and Antibacterial Activity of Some Native Medicinal Plants Against *Pseudomonas Aeruginosa*

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ABSTRACT

Background: Traditional medicines have a wide range of pharmacological properties. **Objectives:** This work was done to investigate the antimicrobial effect of *Thymus vulgaris*, *Matricaria chamomilla*, *Melissa officinalis*, and *Rhus coriaria* extracts against clinical isolates of *Pseudomonas aeruginosa.*

Methods: A total of 180 *P. aeruginosa* clinical isolates were examined. Using the disc diffusion method, MIC and MBC of the methanol and alcohol herbal extracts were measured. Furthermore, microtiter plate assay evaluated biofilm development in the *P. aeruginosa* isolates. The free radical scavenging activity of the plant extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined.

Results: Results showed that *P. aeruginosa* strains were resistant to more than three different classes of antibiotics. Our results demonstrated that the MBC value of ethanol and methanol extracts of thyme was 43.75 mg/mL, and 31.25 mg/mL, respectively. The highest MIC and MBC values were observed for the thyme and Lemon balm had the lowest MIC and MBC values. Our study showed that thyme extract was the most efficient plant extract against *P. aeruginosa*. The Mean±SD scavenging activity of thyme, chamomile, sumac, and lemon balm was 91.05±1.10, 89.55±0.70, 64.65±1.95, and 80.1±0.30%, respectively

Conclusion: These findings may help in reducing our dependence on antibiotics and handling infections of opportunistic pathogens more efficiently. Further studies are required to distinguish the most important phytochemical compounds and estimate their antibiofilm activities and their mechanisms of action.

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Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic rod opportunistic pathogen commonly found in soil and water as well as in plants and humans [1]. *P. aeruginosa* causes disease infrequently in normal hosts but is a major cause of infectious diseases in patients with underlying conditions, such as urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, and a variety of systemic infections, particularly in individuals with severe burns [2].

Improper use of antibiotics has led to the emergence of resistance to microbial isolates, which is increasing every day. Due to the spread of chemical-resistant strains, emergency efforts seems to be necessary to find new antimicrobial agents [3]. Plants and their compounds including essential oils and extracts have the potential to use as a medicine. The side effects of these compounds are less compared to chemical drugs. It has been found that most of the plant extracts have insecticidal, antifungal, antiparasitic, antibacterial, antiviral, antioxidant, and cytotoxic properties [4, 5]. Some plants have shown the ability to overcome antimicrobial resistance in some organisms and this has led researchers to investigate their mechanisms of action of desolation of active compounds. Therefore, these antimicrobial compounds produced by plants are used against pathogenic microorganisms [6].

Flavonoids are one of the main groups of natural phenolic compounds found in different organs, like leaves, flowers, bark, fruits, etc. The genus *Thymus vulgaris* has has different kinds of flavonoids, especially aglycones [7]. Nine different phenolic acids have been reported in the genus *T. vulgaris*, like caffeic and rosmarinic acids. These terpene phenols join amine and hydroxylamine groups of bacterial proteins in the membrane and alter their permeability resulting in the death of the bacteria [8]. In addition, thymol and carvacrol decrease the intracellular adenosine triphosphate (ATP) pool of Escherichia coli. *T. vulgaris*, in addition to antimicrobial and antioxidant effects, also has oral uses, which indicates that it has fewer side effects compared to other compounds [9].

Matricaria chamomilla is the most important medicinal plant of the chicory family and its flowers are widely used in the pharmaceutical, cosmetic, and food industries. *M. chamomilla* extract has antiseptic, sedative, antispasmodic, anti-allergic, and anti-flatulence properties [10]. In addition, its flowers have a moisturizing and softening effect due to their flavonoids, and for this reason, they are widely used in the health and cosmetic industries.

Thyme (*Thymus vulgaris*) has a strong and pleasant aroma and is currently used as a flavoring agent in various foods in Iran. The major constituents of *T. vulgaris* are phenolic compounds, such as thymol and carvacrol with antibacterial activities [11].

Sumac (*Rhus coriaria*) is a medicinal herb used as a drug in traditional medicine. *R. coriaria* extract represents antibacterial effects against Gram-positive and Gram-negative bacteria. Several studies have been conducted to identify biological properties of the mentioned species [12].

Melissa officinalis a popular traditional medicine used in many countries has various complex constituents, which have been reported to have anti-inflammatory, antioxidant, and cardioprotective effects. *M. officinalis* contain steroids, flavonoids, glycoside, saponins, tannins, and phenolic compounds [13].

The present study evaluated the antibacterial effects of *T. vulgaris*, *M. chamomilla*, *M. officinalis*, and *R. coriaria* on *P. aeruginosa*. We investigated antibiotic resistance for *P. aeruginosa* and tried to identify plant-derived natural products as antibacterial agents and extraction approaches for the identification of potential components. These natural antibiotic agents are promising candidates, which could provide novel strategies for combating pathogenic bacteria and the treatment of infectious diseases.

Materials and Methods

Plant materials

T. vulgaris, *M. chamomilla, M. officinalis*, and *R. coriaria* obtained from Sinafaravar pharmaceutical Co. (Isfahan, Iran) and were identified in the Agricultural and Natural Resources Research Center of Isfahan province. All parts of the plant were washed and dried in the shade. Then, the aerial parts, including the stems, leaves, and flowers of the plants were powdered by an electric mill.

Plants extracts

In this method, the first 50 g of plant powder was weighed and poured separately into a flask containing 250 mL of ethanol and methanol. The above solution was placed on a shaker at room temperature for 48 to 72 hours. The extract was filtered with Whatman 30 filter paper. The filtered extract was poured into glass plates and placed in an incubator at 30°C for 24 to 48 hours until the extract was completely dried. The obtained extracts were stored in the refrigerator at 4°C until use.

Microorganisms and growth conditions

Totally, 180 *P. aeruginosa* clinical isolates were examined. The clinical specimens were collected from Al-Zahra Hospital in Isfahan. The isolates were cultivated on blood agar, nutrient agar, CLED agar, and MacConkey agar. Colonies grown on different media were subjected to further morphological and biochemical identification. The isolated bacteria as well as the standard strain of *P. aeruginosa* (PTCC 1704) were characterized using Gram stain, morphological and cultural characteristics on nutrient agar, motility and carbohydrate fermentation tests, and catalase, hydrogen sulphide, and indole production [14, 15].

Antibiotic susceptibility test

In this study, 11 antibiotics, including ceftazidime (30 µ/disc), cefotaxime (30 µ/disc), cefixime (30 µ/disc), ciprofloxacin (5 µ/disc), carbenicillin (2 µ/disc), cloxacillin (15 µ/disc), clindamycin (2 µ/disc), co-trimoxazole (30 µ/disc), co-amoxiclav (30 µ/disc), nitrofurantoin (20 µ/disc) and vancomycin (30 µ/disc) were employed to detect the drug resistance of *Pseudomonas* isolates according to the guidelines of the Clinical and Laboratory Standard Institute [16] (Table 1).

Antibiotics

Antibacterial effect of extracts

Disc diffusion method

First, the bacterial suspension of *P. aeruginosa* was prepared (0.5 McFarland comparable to a bacterial suspension of 1.5×108 CFU/mL). Then, uniform culture was performed with 100 µL of prepared suspension on the surface of Mueller Hinton agar medium.

The extract-impregnated discs were then placed at a certain distance from the edge of the plate on the surface of the agar culture medium. The diameter of the non-growth halo was measured after three repetitions. A diameter of less than 8 mm growth was considered to be resistant, 8 to 9 mm was relatively resistant, more than 10 to 12 mm was considered to be relatively sensitive, and more than 12 mm was considered as sensitive [17].

Microdilution broth

Using microdilution broth, the minimum inhibitory concentration (MIC) of the methanolic and alcoholic extracts of the studied plants was determined. Therefore, 1 mL of 1.5×15 CFU/mL bacterial suspension was added to the 0.1-100 mg/mL methanolic extract and 0.1-100 mg/mL of alcohol extract. The optical density (OD) was measured at 680 nm wavelength using the ELISA reader (Update, Belgium). The samples were then placed at 37°C, and the OD was read at 12 and 24 hours. Finally, the minimum concentration of the extract, in which the OD decreased was calculated and considered as MIC.

In order to determine the MBC of the extract against each microorganism, 20 µL of each tube with no visible growth of the microorganism was inoculated on plates containing Brain Heart Infusion (BHI) agar. After storing for 16-24 hours at 35°C, the growth of microorganisms was evaluated. Each test was repeated three times.

Table 1. Different types of antibiotics

Name	Dose (µg/disc)
Ceftazidime (SF)	30
Cefotaxime (CTX)	30
Cefixime (CFM)	30
Ciprofloxacin (CP)	5
Carbenicillin (CB)	2
Cloxacillin (CX)	15
Clindamycin (CC)	2
Co-trimoxazole (SXT)	30
Co-amoxiclav (AMC)	30
Nitrofurantoin (NIF)	20
Vancomycin (V)	30

MBC was determined as the minimum concentration of the extract, where no bacterial growth was observed.

DPPH radical scavenging assay

The free radical scavenging activity of the plant extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined using the method proposed by Silva and Domingues [18]. A methanol solution of DPPH (9µM) was decolorized by plant extractives. DPPH produces a violet/purple color in methanol solution and fades to a yellow color in the presence of antioxidants. A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 mL of this solution was mixed with 1 mL of extracts in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The results were expressed in milligram equivalents of quercetin per milligram of dry weight. The calibration line was established using the following concentrations of quercetin: 0.001, 0.002, 0.005, 0.01, 0.02, and 0.04 mg/mL. On the last day, methanol was removed by a rotary evaporator.

Extraction and separation methods for phenolic compounds

The total polyphenol content (TPC) was determined by spectrophotometry, using gallic acid as standard, according to the method described by the International Organization for Standardization (ISO14502, 2005). Briefly, 0.200 g of each sample was weighed in an extraction tube, and 5 mL of 70% methanol at 70°C was added. The extract was mixed and heated at 70°C on a vortex for 10 min. After cooling at room temperature, the extract was centrifuged at 200 g for 10 min. The supernatant was decanted in a graduated tube. The extraction step was repeated twice. Both extracts were pooled and the volume was adjusted to 10 mL with cold 70% methanol. One milliliter of the extract was diluted with water to 100 mL. Then, 1.0 mL of the diluted extract was transferred in duplicate to separate tubes containing 5.0 mL of a 1/10 dilution of Folin-Ciocalteu's reagent in water. Then, 4.0 mL of a sodium carbonate solution (7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The TPC was expressed as gallic acid equivalents (GAE) /100 g material. The concentration of polyphenols in samples was derived from the standard curve of gallic acid ranging from 10 to 50 µg/mL.

Statistical analysis

The results are demonstrated as the Mean±SD of three separate experiments. All statistical analyses of the variances between the controls and tests were conducted using one-way ANOVA followed by a post hoc Tukey test. Only results at $P<0.05$ were regarded as significant.

Results

In this descriptive cross-sectional study, 180 *P. aeruginosa* samples were isolated from patients of Alzahra Hospital in Isfahan. The highest frequency of isolates was related to urine samples and the lowest was related to skin. The frequency of isolates were as follows: urine (63 isolates; 34%), trachea (41 isolates; 22%), sputum (30 isolates; 16%), blood (23 isolates; 12%), wound sources (12 isolates; 11%) and skin (11 isolates; 5%), respectively. In addition, 94% of the strains had beta-hemolytic activity, and only 6% had no hemolytic activity.

Antimicrobial susceptibility testing

All strains of *P. aeruginosa* isolated from various infections were exposed to the antibiotics tobramycin, ampicillin, gentamicin, cotrimoxazole, imipenem, ceftazidime, nitrofurantoin, amikacin, cefotaxime, carnosine, and carbenicillin. *P. aeruginosa* standard strain PTCC 1074 was resistant to ampicillin, amikacin, cefixime, and cephalexin antibiotics. Also, 83% of isolates were resistant to at least one or more antibiotics, while the total number of susceptible and semi-susceptible strains was 17%. Multidrug-resistant (MDR) strains were evaluated with a multiple authority index. MDR was observed in 68% of isolates and antibiotic resistance was higher in burns, lung, and intensive care units than in other departments. The lowest resistance was related to outpatients (35%). The antibiotic resistance pattern was obtained from agar diffusion method. Clinical strains of *P. aeruginosa* showed that the highest percentage of resistance was related to imipenem (84%), and the lowest percentage belonged to gentamicin (46%). Examination of this pattern of antibiotic resistance showed a high level of resistance of isolated *P. aeruginosa* strains for all antibiotics. Then, in determining antibiotic susceptibility and resistance, strains that were resistant to more than three different classes of antibiotics were selected as MDR strains (Table 2).

Table 2. Effects of different classes of antibiotics on *P. aeruginosa.*

Antimicrobial Agent	*P. aeruginosa*	
	Susceptible (s) %	Resistant (R) %
Tobramycin (TOB)	36	64
Ampicillin (AM)	26	74
Gentamicin (GEN)	54	46
Co-trimoxazole (SXT)	38	62
Imipenem (IPM)	16	84
Ceftazidime (CAZ)	40	60
Nitrofurantoin (NIF)	30	70
Amikacin (AK)	34	66
CefotaximeN (CTX)	38	62
Carbenicillin (CB)	24	76
Piperacillin/Tazobactam (PIT)	50	50
Cefalexin (CN)	36	64
Ciprofloxacin (CIP)	42	58

Antibacterial activity of different extracts

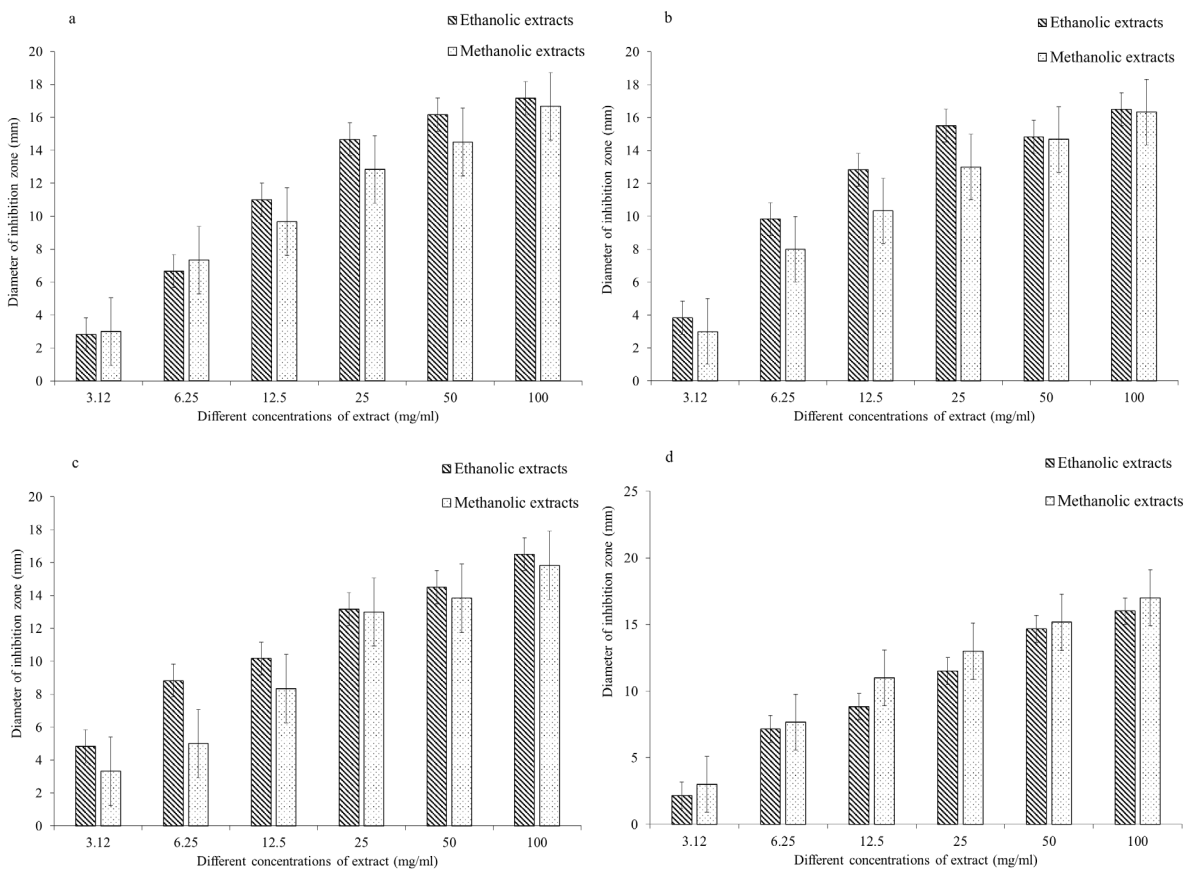

Figure 1. Inhibitory effect of ethanolic and methanolic plant extracts on *P. aeruginosa*
a) *T. vulgaris*; b) *M. chamomilla*; c) *R. coriaria*; d) *M. officinalis*.

Table 3. MIC and MBC values of ethanolic and methanolic extracts of *T. vulgaris*, *M. chamomilla*, *R. coriaria*, and *M. officinalis* on *P. aeruginosa* (mg/mL)

Plants	Mean±SD			
	MIC (mg/mL)		MBC (mg/mL)	
	Ethanolic Extracts	Methanolic Extracts	Ethanolic Extracts	Methanolic Extracts
T. vulgaris	19.21±1.12	14.18±0.08	43.75±0.25	31.25±1.12
M. chamomilla	19.48±1.16	14.54±0.65	42.64±1.12	30.38±0.45
R. coriaria	21.31±0.085	15.04±0.071	41.29±0.78	28.42±1.16
M. officinalis	21.81±0.21	15.62	40.02±0.56	27.32±0.44

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Disc diffusion method

Antibacterial activity of ethanol and methanolic extracts of *T. vulgaris, M. chamomilla*, *M. officinalis*, and *R. coriaria* was assessed by measuring the diameter of growth inhibition zone on *P. aeruginosa* and the results are presented in Figure 1. The antibacterial activities of extracts according to the zone of inhibition ranged between 2.4 and 17.1 mm.

The highest zone of growth inhibition was found for the ethanol extract of *T. vulgaris* (17.1 mm). The mean diameter of the growth inhibition zone of ethanolic extract was studied on *P. aeruginosa*. Ethanolic extract of *T. vulgaris* at concentrations of 100 and 50 mg/mL showed a growth inhibition zone of 17.1 and 16.1 mm, for methanolic extract, these values were 6.16 and 5.14 mm, respectively. This test showed that there was a significant relationship between the concentration and growth inhibition zone diameter. The minimum zone of inhibition was given by the ethanolic extract of *R. coriaria* at 3.12 mg/mL (2.4 mm).

The *in vitro* antibacterial activity of plants was evaluated against *P. aeruginosa* strains using the microdilution method to determine MIC and MBC. The MIC values obtained from plants exhibited antibacterial activity ranging between 14.18 and 21.81 mg/mL. Ethanol and ethanol extracts of *T. vulgaris* showed MIC values of 21.81 and 15.62 mg/mL, respectively. On the other hand, ethanol and methanol extracts of *T. vulgaris* showed MBC values of 43.75 and 31.25 mg/mL, respectively. Therefore, the highest MIC and MBC were observed for the *T. vulgaris* and *M. officinalis* had the lowest MIC and MBC (Table 3).

Total phenols and flavonoids

The total extractable phenols and flavonoids were different between the ethanolic and methanolic extracts of four different plants ($P<0.05$) (Figure 2). Total phenolic content was greatest in the ethanolic extracts of *M. officinalis* (50.50 mg GAE/ g DW), followed by *M. chamomilla*, *T. vulgaris*, and *R. coriaria* with contents of 42.30, 35.21, and 28.29 GAE/100 g DW, respectively (Figure 1b). Total flavonoid content in different plant extracts

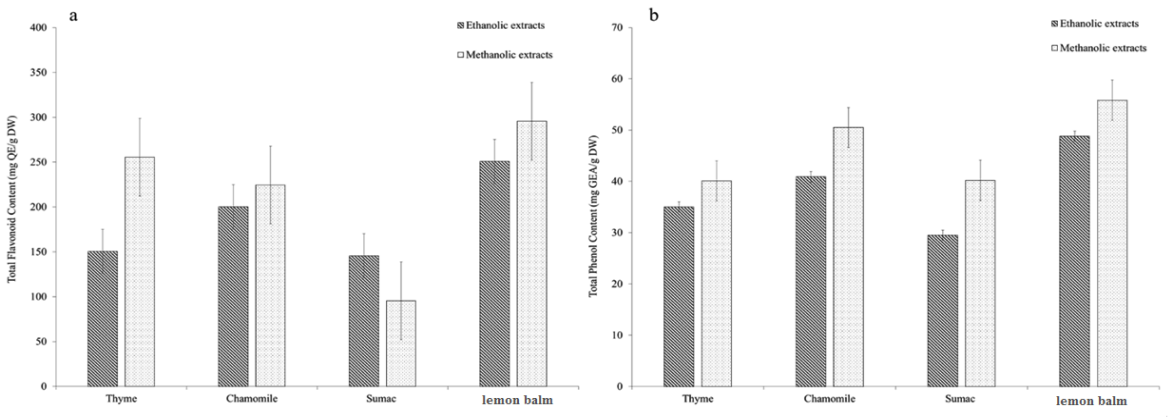

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Figure 2. Total extractable a) flavonoids and b) phenols of the *T. vulgaris* , *M. chamomilla*, *R. coriaria*, and *M. officinalis* extracts

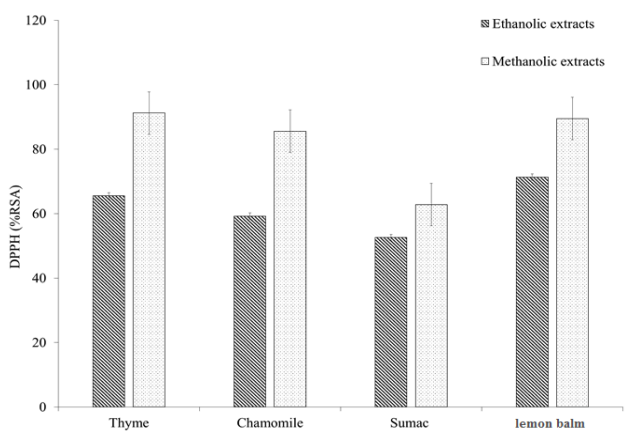

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Figure 3. Radical scavenging activity of extracts of *T. vulgaris*, *M. chamomilla*, *R. coriaria,* and *M. officinalis*.

ranged from 54.25 to 300.25 mg QE/g DW. The greatest and smallest values were recorded in the ethanol extracts of *M. chamomill* and *R. coriaria*, respectively (Figure 2).

DPPH radical scavenging assay

Figure 3 shows the free radical scavenging activity of the methanolic and ethanolic extracts of *T. vulgaris*, *M. chamomilla*, R. coriaria, and M. officinali, of whom thyme showed the highest activity. The Mean±SD scavenging activity of *T. vulgaris*, *M. chamomilla*, *R. coriaria* and *M. officinalis* was 91.05±1.10, 89.55±0.70, 64.65±1.95, and 80.1±0.30%, respectively.

Discussion

Due to the use of numerous plant species as a source of phytotherapeutic products, the study of their antimicrobial activity is interesting in recent years. *P. aeruginosa* is the most prevalent opportunistic pathogen, which can lead to nosocomial infections in patients who are immune-compromised [19]. Resistance to *P. aeruginosa* causes different problems and limits therapies. *P. aeruginosa* possesses numerous resistance mechanisms that overcome most conventional antibiotics [20]. The need for new therapeutics that can hinder biofilm formation and decrease the virulence of *P. aeruginosa* without more resistance is continuously growing. Plant-derived products have been a good option due to their effectiveness and considerably low side effects.

In this study, four different plants, including *T. vulgaris*, *M. chamomilla*, R. coriaria, and *M. officinalis*. were applied because of their multiple applications in medicine as antimicrobial agents. All tested plant extracts showed variable antibacterial activities against *P. aeruginosa* isolates. On the other hand, *T. vulgaris* was the most potent antibacterial agent. We can relate these effects to their rich constituents of bioactive compounds. The activities of these plants are mainly due to the presence of polyphenols, the most abundant of which is catechin, particularly epigallocatechin gallate (EGCG), which is known for its inhibitory effect on *P. aeruginosa* [21]. Apparently, the observed antibacterial activity of these plants is attributed to the high concentrations of phenolic compounds, such as oleuropein and hydroxytyrosol [22, 23]. Moreover, phenolic compounds can potentially, increase the permeability of cell membranes, leading to facilitating their rupture. The obtained results agreed with those of Alkuraishy1et al. who reported that chamomile alcoholic extract exhibited potent antimicrobial activity on all selected isolates and it can also inhibit and swarm motility in *P. aeruginosa* [24]. Another report by Liu et al. revealed that thyme oil inhibited numerous virulence factors of *P. aeruginosa* [25].

In plants, the main compounds with antioxidant activity are phenols, and as they have an aromatic ring, stabilize the unpaired electrons of their structure, thus facilitating the donation of hydrogen atoms and electrons from their hydroxyl groups. One of these mechanisms is the prevention of oxidative stress, keeping ROS under dangerous levels, and using them for efficient signaling [26]. Phenols as secondary metabolites of plants that can adjust the concentration of ROS, thus activating a network of biochemical events to increase tolerance [27, 28].

Our findings demonstrated that the difference between tested extracts might be due to their differences in the type and concentration of the active constituents, as well as the polyphenol contents. These results are consistent with other studies [29]. These compounds are considered to be bioactive and may be responsible for the activities of extracts [30]. Flavonoids, such as catechin

reduce virulence factors in *P. aeruginosa*. Subsequently, other functionally related flavones, such as baicalein and quercetin are also antibacterial compounds [31]. The excellent ability of the plant extracts to interfere with the initial stage of biofilm formation may be attributed to interference with forces that favor the deposition and adherence of bacteria to surfaces. In addition, since certain organic and inorganic molecules and other nutrients are important for cell growth and cell adhesion, the plant extracts may inhibit the availability of nutrients [32, 33]. The active plant extracts may hold promise for a reduction in the colonization of epithelial surfaces in the body, thereby preventing infections [34, 35].

Conclusion

T. vulgaris extract was the most efficient plant extract against antibiotic resistance. These findings may help in reducing our dependence on antibiotics and handling infections of opportunistic pathogens more efficiently. Further studies are required to distinguish the most important phytochemical compounds and estimate their antibiofilm activities and their mechanisms of action. In addition, *in vivo* studies are required to enable their application in the prevention and treatment of biofilm-related *P. aeruginosa* infections.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and Supervision: Nima Bahador and Alireza Khodavandi; Methodology: Mandana Ahmadi and Nima Bahador; Investigation, Writing–original draft, and Writing–review & editing: All authors; Data collection: Mandana Ahmadi; Data analysis: Nima Bahador and Alireza Khodavandi.

Conflict of interest

The authors declared no conflict of interest.

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