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Original Article

In Vitro Antibacterial Activity of Hydroalcoholic and Aqueous Extracts of *Echinophora platyloba* against Some Pathogenic Bacteria Related to the Oral Cavity

Running title: *Echinophora platyloba* extracts have Antibacterial Activity

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ABSTRACT

Bacteria's antibiotic resistance has increased due to the use of antibiotics as a treatment strategy for pathogenic bacteria. New substances with relatively stronger antimicrobial properties are being discovered in research. Medicinal plants have become an interesting research topic due to their effectiveness against pathogenic bacteria. This *in vitro* study aimed to evaluate the antimicrobial properties of *Echinophora platyloba* DC, hydroalcoholic, and aqueous extracts against some important pathogenic bacteria related to the oral cavity. For this purpose, samples of *E. platyloba* were collected from western Iran, and their extract was extracted. Subsequently, the antibacterial activity of various concentrations of the extracts was evaluated *in vitro*. The results showed that hydroalcoholic and aqueous extracts in all concentrations (100, 50, 25, and 12.5%) could significantly inhibit the growth of all bacterial pathogens with a probability level of 1% compared to the negative control (DMSO 100%). Furthermore, at 100% concentration, they were capable of suppressing the growth of bacterial pathogens (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, and *Streptococcus sanguis*) with a significant difference (p-value < 0.01) compared to the Gentamicin antibiotic. The MIC ranged from 3.12% to 50% in the present study. Therefore, the current research findings indicate that the aqueous and hydrocolic extracts of *E. platyloba* have a high potential to inhibit human pathogenic bacteria related to the oral cavity. This study's results will pave the way for developing novel functional pharmaceutical and food products.

Keywords: Antimicrobial, Medical plants, Human pathogenic Bacteria, MIC

INTRODUCTION

Over time, humans have had to deal with various diseases and attempts to antagonize them through different methods [1, 2]. Multidrug-resistant bacteria pose a significant global health threat. The emergence of antibiotic-resistant bacteria is causing the availability of antibiotics to become less effective in treating many of the infections we face today [3, 4]. The scientific research community focuses on discovering new antibacterial agents to combat antibiotic-resistant bacteria [5]. Natural compounds from plants that are powerful potential therapeutic tools have emerged in this field [6-8]. Antimicrobial treatment with botanical drugs is currently used by 65 to 80% of people in developing countries [9].

In response to environmental factors, including biotic and abiotic stress and interspecific interactions, plants produce an invaluable resource of secondary metabolites [10]. Humans have used plant secondary metabolites for medicine since ancient times. Plant secondary metabolites are a natural reservoir for research into discovering new antimicrobial compounds due to their vast chemical diversity and long history of traditional use. Recently, many antibacterial agents have been identified due to the rapid advancement in technology and the use of newer, more efficient methodologies [8, 11, 12]. Alkaloids, saponin, flavonoids, thiosulfates, glucosinolates, phenolic compounds, and organic acids are the most common secondary metabolites in plants that have antimicrobial

effects on various pathogenic microorganisms. The antimicrobial efficacy of plant secondary metabolites is primarily contingent upon their chemical structures, essential constituents, and effective dose [13, 14].

The *Echinophora* genus belongs to the *Apiaceae* family, and in Iran, it is known as Khosharizeh, Khosharuzeh, or Tighe Turagh. *Echinophora* is an aromatic plant with a pleasurable flavor, with numerous uses for food and medicinal purposes [15, 16]. There are multiple species within the genus. In Iran, four species include *E. platyloba* DC, *E. cinerea* (Boiss) Hedge & Lamond, *E. orientalis* Hedge & Lamond, and *E. sibthorpiana* Guss [16]. The *Echinodora* genus' essential oil compounds, specifically its phenolic and flavonoid compounds, have been shown to have antimicrobial, antifungal, and antioxidant properties. These compounds can suppress the growth of two main groups of bacteria (Gram-negative and Gram-positive) [16]. These compounds have a wide range of antimicrobial activity, highlighting their potential as natural antibiotic alternatives [17]. Antimicrobial effects of *Echinophora* related to the essential oil of this species on the many pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Shigella dysenteriae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* have been reported [17-21]. It is necessary to conduct more research to identify certain compounds, comprehend their functions, and explore potential synergies with present antibiotics [17, 22].

Even though molecular modeling and synthetic chemistry methods have recently become popular in drug discovery, natural product-derived compounds are still a valuable source of medicines for humans [23]. This study examined the antimicrobial effects of *E. platyloba* hydroalcoholic and aqueous extracts on oral pathogenic bacteria *in vitro*, given its effective antibacterial properties, low costs, and fewer complications of herbal drugs.

MATERIALS AND METHODS

Sample Collection

In 2021, *Echinophora platyloba* DC, as a traditional medicinal plant, was collected from Sar Firuzabad District of Kermanshah County, Kermanshah province, Iran (34°6'39.81"N, 47°5'5157"E). The plant was identified and appropriately authenticated at Agriculture College in Razi University, Kermanshah, Iran.

Plant Extracts Preparation

To prepare the plant extract, the collected samples were first washed under running tap water and air-dried in the shade at room temperature. The plant parts were ground up to fine powder using a home grinder. Ten grams of dried plant powder were dissolved separately into 100 mL of hydroalcoholic solvent (50 mL of ethanol + 50 mL of distilled water) and aqueous solvent (distilled water 100 mL) in an Erlenmeyer flask on a shaker for 24 hours. Then, the solution was filtered through the Whatman filter paper. This work lasted three times, each time for 24 hours, and finally, the filtered solution of all three stages was added to each other. The solution was placed in a rotary evaporator for one hour at 45°C for concentrated. Finally, the crude extract solutions were placed in an oven at 45°C until the extract was arid [24]. Until the antibacterial experiment was conducted, the dry crude extract was kept in a dark, sealed container at 4°C. Additionally, by weighing the dry crude extract and the dry weight of plant material, the extraction yield was calculated using the following formula:

$$\text{Extraction Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant material}} \times 100 \qquad (1)$$

To dilute the plant extracts from the initial stock solution (100%), 1 mL was added to the first tube, and then sequential two-fold serial dilutions of the extracts were performed. The resultant concentrations in the tubes were 100, 50, 25, 12.5%. The stock solutions were prepared using dimethyl sulfoxide (DMSO) as a solvent [25].

Bacterial Pathogens and Culture Conditions

The antibacterial properties of plant extract were tested against six pathogenic bacterial strains *Streptococcus salivarius* PTCC 1448, *Streptococcus sanguis* PTCC 1449, *Streptococcus mutans* PTCC 1683, *Staphylococcus epidermidis* PTCC 1436, *Staphylococcus aureus* PTCC 1431, *Pseudomonas aeruginosa* PTCC 1620. All bacterial strains were purchased from the Iranian Research Organization for Science and Technology (IROST, Tehran, Iran). The bacterial strains were grown and maintained on a nutrient agar medium (NA). To preserve them, the strains were stored in nutrient broth (NB) supplemented with 15% glycerol at -20°C [26, 27]. The pathogenic bacteria were pre-cultured in Mueller Hinton broth medium (Merck Co.) in a rotary shaker at 37°C for 24 h. Afterward, the formed colonies were picked up using a sterile inoculating loop and transferred into a

sterile saline solution. Subsequently, each strain suspension was adjusted at a 1.5×10^8 CFU/mL concentration using 0.5 McFarland standard [28].

Antibacterial Assay

Well Diffusion Method

The antibacterial activity of hydroalcoholic and aqueous extracts of *E. platyloba* was tested using an agar well diffusion method [25, 28]. For this purpose, 1 mL of pathogenic bacterial suspension (0.5 McFarland) was cultured on the Mueller Hinton agar medium (Merck Co.). Then, using a sterile cork borer, wells with a diameter of 7 mm were made, and with a sampler, 100 µL of different concentrations of the prepared extracts (100, 50, 25, and 12.5%) were poured into the wells. Subsequently, petri plates were incubated at 37∘C for 24 h. After incubation, antibacterial activity was measured by measuring the zone of inhibition with a ruler (in millimeters), and the mean values of three repeated tests were recorded. This study used a Gentamicin antibiotic disc with a concentration of 1.25 µg/ml as a positive control and 100% dimethyl sulfoxide DMSO (solvent) as a negative control. The list of treatments is given in Table 1.

Table 1 The list of treatments in this study to measure the antibacterial activity of aqueous and hydroalcoholic extracts of *Echinophora platyloba*.

Treatments code*	Bacterial species	Extract type	Extract Concentration (%)
PTCC 1448	*Streptococcus salivarius* PTCC 1448	Hydroalcoholic	100, 50, 25, 12.5
		Aqueous	100, 50, 25, 12.5
PTCC 1449	*Streptococcus sanguis* PTCC 1449	Hydroalcoholic	100, 50, 25, 12.5
		Aqueous	100, 50, 25, 12.5
PTCC 1683	*Streptococcus mutans* PTCC 1683	Hydroalcoholic	100, 50, 25, 12.5
		Aqueous	100, 50, 25, 12.5
PTCC 1436	*Staphylococcus epidermidis* PTCC 1436	Hydroalcoholic	100, 50, 25, 12.5
		Aqueous	100, 50, 25, 12.5
PTCC 1431	*Staphylococcus aureus* PTCC 1431	Hydroalcoholic	100, 50, 25, 12.5
		Aqueous	100, 50, 25, 12.5
PTCC 1620	*Pseudomonas aeruginosa* PTCC 1620	Hydroalcoholic	100, 50, 25, 12.5
		Aqueous	100, 50, 25, 12.5

*C+: Inhibition of bacterial growth with Gentamicin (1.25 µg/mL) as a positive control; C-**:** Inhibition of bacterial growth with DMSO 100% as a negative control.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was determined using the broth microdilution method in a 96-well plate using different concentrations of the *E. platyloba* extracts [24]. At first, 100 µL of Mueller Hinton Broth medium (Merck Co.) were poured into 96 wells of a microplate. After that, 100 µL of the achieved successive two-fold serial dilutions of the extracts concentrations were combined. Subsequently, 100 µL of pathogenic bacteria inoculum (0.5 McFarland) were added to the wells containing the Mueller Hinton Broth and the plant extracts. At the end, the microplates were incubated at 37°C for 24 hours. After incubation, the microplates were read by an ELISA reader at 630 nm. In this study, 100 µL of plant extract and pathogenic bacteria suspension for negative and positive control were added to the control wells, respectively. All treatments were performed in triplicate. The MIC was considered the lowest extract concentration that completely inhibits bacterial growth.

Statistical Analysis

All the tests were performed in a completely random design, and three repetitions were considered for each treatment. Results were subjected to analysis of variance (ANOVA), and mean comparison was performed using Duncan's multiple range test procedure in SAS statistical software version 9.3. Differences between means were considered significant at P-value < 0.01.

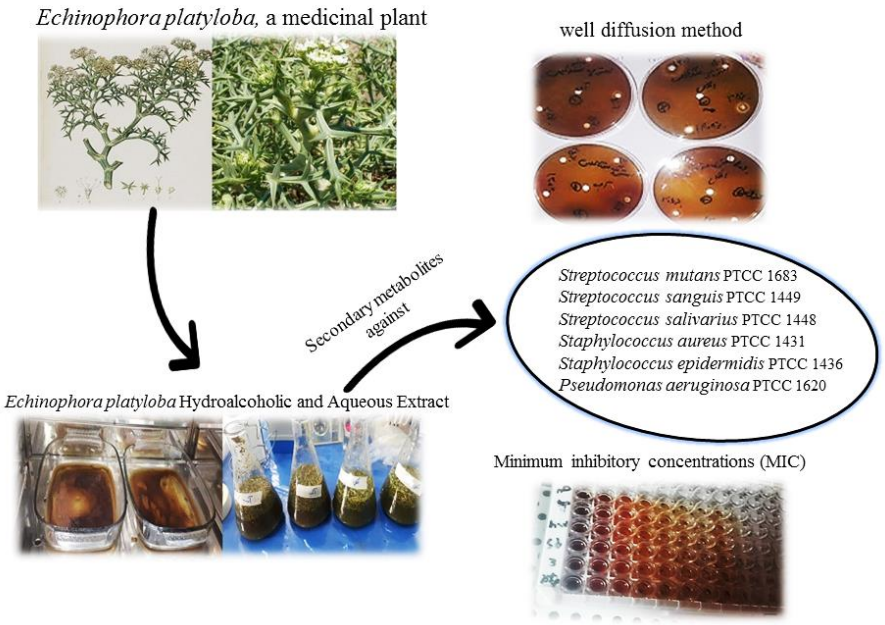

Fig. 1 The *Echinophora platyloba* plant, hydroalcoholic and aqueous extracts, and antibacterial tests were performed in this study.

RESULTS

Extraction yield

The plant extract of the collected samples was extracted using the maceration method. The extraction yield of both hydroalcoholic and aqueous extracts was 10%. Additionally, the concentration of the initial extract (100%) of the hydroalcoholic extract was 890 µg/mL, and the aqueous extract was 870 µg/mL.

Antibacterial activity

The well diffusion method examined the antibacterial properties of the *E. platyloba* plant's aqueous and hydroalcoholic extracts against some oral pathogenic bacteria. The research showed that hydroalcoholic and aqueous extracts in all concentrations (100, 50, 25, and 12.5%) could significantly inhibit the growth of bacterial pathogens with a probability level of 1% compared to the negative control with varying intensities (Figures 2 and 3).

As shown in Figure 2. In all concentrations, the hydroalcoholic extracts of the *E. platyloba* plant could significantly inhibit the growth of bacterial pathogens compared to the negative control (DMSO 100%). Furthermore, at 100% concentration, they were capable of suppressing the growth of bacterial pathogens (*Staphylococcus epidermidis* PTCC 1436, *Staphylococcus aureus* PTCC 1431, *Streptococcus salivarius* PTCC 1448, *Streptococcus mutans* PTCC 1683, and *Streptococcus sanguis* PTCC 1449) with a significant difference (P-value < 0.01) compared to the positive control (Gentamicin antibiotic). In contrast, there was no significant difference compared to the positive control (Gentamicin antibiotic) against *Pseudomonas aeruginosa* PTCC 1620. It should also be noted that the hydroalcoholic extract at a concentration of 50% successfully suppressed the growth of two pathogens, *Staphylococcus epidermidis* PTCC 1436 and *Staphylococcus aureus* PTCC 1431, with a significant difference in comparison to the positive control (Figure 2).

In addition to inhibiting all bacterial pathogens with a significant difference in comparison to the negative control, the aqueous extract also inhibited the growth of *Staphylococcus epidermidis* PTCC 1436 and *Staphylococcus aureus* PTCC 1431 at a concentration of 100%, with a significant difference (P-value < 0.01) in comparison to the positive control (Figure 3). The results of ANOVA analysis are given in Table 2. This indicates that the P-value is statistically very highly significant at the probability level of 0.0001. As shown in Figure 4. Aqueous and hydroalcoholic extracts had a high correlation with each other in inhibiting the growth of oral pathogenic bacteria (Pearson correlation coefficient of 0.9462 and P-value < 0.01).

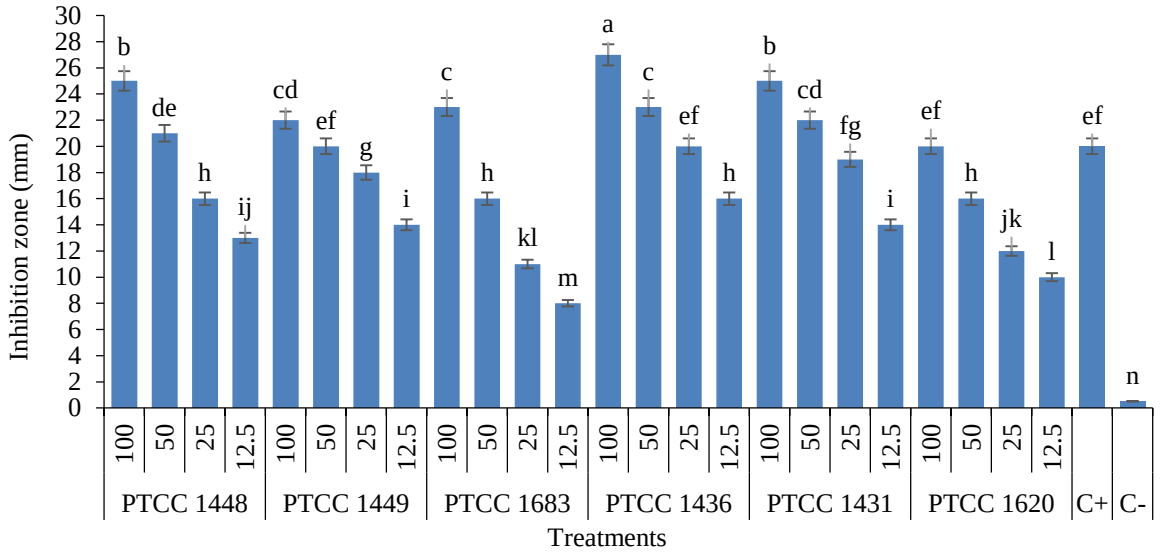

Fig. 2 Antibacterial activity of *Echinophora platyloba* hydroalcoholic extract against some oral pathogenic bacteria used in this study. The means were compared at the level of 1% probability using Duncan's multiple range test. The difference between means with common letters is not statistically significant.

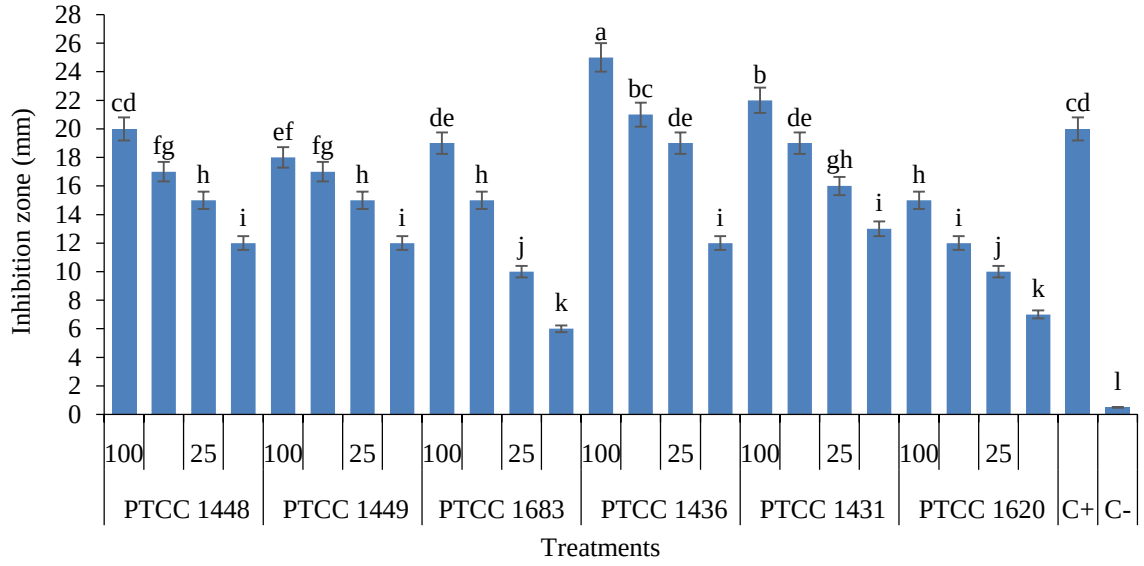

Fig. 3 Antibacterial activity of *Echinophora platyloba* aqueous extract against some oral pathogenic bacteria used in this study. The means were compared at the level of 1% probability using Duncan's multiple range test. The difference between means with common letters is not statistically significant.

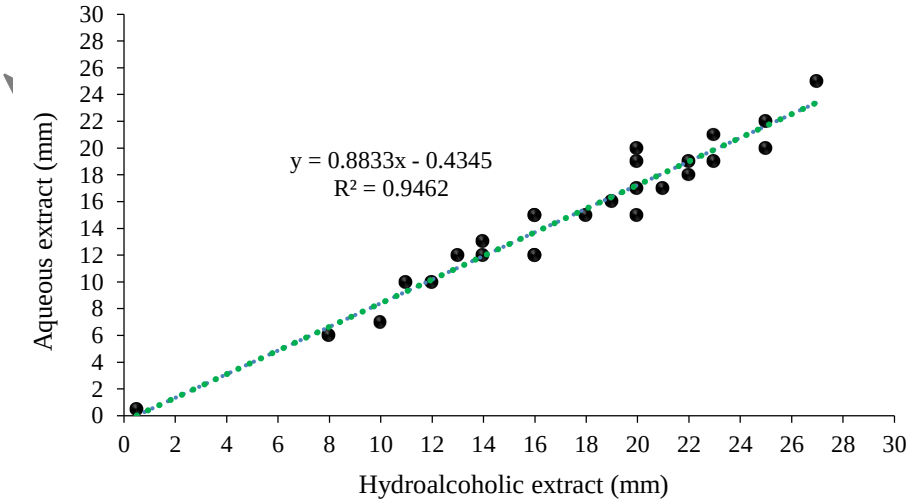

Fig. 4 Correlation between antibacterial activity of aqueous and hydroalcoholic extracts against some oral pathogenic bacteria used in this study, *in vitro*.

Table 2 Results of two-way ANOVA analysis were obtained using SAS statistical software (ver 9.3).

A. Hydroalcoholic extract						
Variation source	DF	Sum of Squares	Mean Squares	F-Value	*P*-Value	R-Square
Treatments	25	1814.88	78.907609	184.76	<0.0001****	0.988831
Error	52	20.5	0.427083			
Total	77	1835.38				
B. Aqueous extract						
Variation source	DF	Sum of Squares	Mean Squares	F-Value	*P*-Value	R-Square
Treatments	25	2238.02884	89.521154	179	<0.0001****	0.988516
Error	52	26	0.5			
Total	77	2264.028846				

******=** Statistically very highly significant

MIC Determination

The minimal inhibitory concentration (MIC) was determined by selecting the lowest plant extract concentration that completely inhibited pathogenic bacteria growth. The present study had a range of 3.12% to 50%. The results of the MIC determination for the hydroalcoholic and aqueous extract against the bacterial pathogens used in this study are in Table 3. The result of this study indicates that Hydroalcoholic extract shows better antibacterial activities against oral bacterial pathogens used in this study.

Table 3 The minimal inhibitory concentration (MIC) value of aqueous and hydroalcoholic extracts of *Echinophora platyloba* against the oral pathogenic bacteria used in this study.

Bacterial species	Extract type	MIC (%)
Streptococcus mutans PTCC 1683	Hydroalcoholic	6.25
	Aqueous	25
Streptococcus sanguis PTCC 1449	Hydroalcoholic	6.25
	Aqueous	25
Streptococcus salivarius PTCC 1448	Hydroalcoholic	3.12
	Aqueous	12.5
Staphylococcus aureus PTCC 1431	Hydroalcoholic	3.12
	Aqueous	6.25
Staphylococcus epidermidis PTCC 1436	Hydroalcoholic	3.12
	Aqueous	3.12
Pseudomonas aeruginosa PTCC 1620	Hydroalcoholic	12.5
	Aqueous	50

DISCUSSION

The use of antibiotics as a treatment strategy for pathogenic bacteria has resulted in an increase in bacteria's resistance to these drugs. New substances with relatively stronger antimicrobial properties are being discovered in research. The effectiveness of plant-derived essential oils against pathogenic bacteria makes them an interesting research topic [29]. Antimicrobial effects on various organisms can be obtained from plant-derived Essential Oils, and they are less harmful than common antibiotics [30, 31]. *Echinophora*, a medicinal plant, was considered one of the best antibacterial agents [17]. According to these activities, it has the potential to be both an antimicrobial agent and a food preservation agent. Most studies were conducted in Iran due to *E. platyloba* DC and *E. cinerea* being native plant species there. Therefore, our present research aimed to investigate the antibacterial efficiency of aqueous and hydroalcoholic extracts of the *E. platyloba* plant, which is natively grown in the region and can be used as a product for many medical and food purposes.

This study's findings indicate that *E. platyloba* DC, as a medicinal plant, effectively inhibited the growth of pathogenic bacteria related to the oral cavity. Scientific evidence has confirmed the antimicrobial properties of *E. platyloba* [16-18]. In a study by Entezari et al. 2009, results showed that methanolic extract of *E. platyloba* could inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Furthermore, another study investigated the antibacterial effect of E. platyloba essential oil against *Staphylococcus aureus*. The findings indicated that *E. platyloba* has a delicious taste and has a potent antimicrobial capacity against *Staphylococcus*

aureus [32]. In another study, it was found that the ethanolic extract of *E. platyloba* had a significant inhibitory effect against three Gram-negative (*Escherichia coli, Shigella flexneri, Acinetobacter baumannii*) and two Gram-positive bacteria (*Staphylococcus aureus, Enterococcus faecalis*) [33]. In a study by Fayyaz et al., 2015, the results showed that the *E. platyloba* essential oil has antimicrobial activity against *Escherichia coli* O157, *Staphylococcus aureus*, *Bacillus subtilis*, *Listeria monocytogenes*, and *Aspergillus niger* [34]. Finally, the review article by Pilavar et al., 2024, provides records of the antimicrobial properties of *E. platyloba* DC, which demonstrate the significant potential of this medicinal plant in controlling various human pathogens.

Antimicrobial activity is generally explained by two mechanisms: chemical interference with the synthesis or function of fundamental bacteria components (suppression of protein biosynthesis, suppression of nucleic acid synthesis, degradation of bacterial cell wall, and inactivation of extracellular enzymes) and/or circumvention of antibacterial resistance mechanisms [35]. Developing inherent resistance to multiple antimicrobial agents can be achieved by bacteria through selective pressures or the acquisition of resistance machinery from neighboring microbes [36, 37]. Even though many countries have accepted synthetic antimicrobial agents, the use of medicinal plants remains a hot topic among researchers [38]. Medicinal plants hold great promise in discovering novel bioactive compounds that can combat resistant microorganisms [17, 35]. A wide range of chemical compounds can be found in medicinal plants. They can restore the clinical application of former antibiotics by enhanced their potency, which will inhibit the development of resistance [39]. These compounds affect the microbial cell through multiple strategies [35]. Past studies have indicated that *Echinophora* genus is the source of phenolic compounds and flavonoids, which can be used as long-lasting antioxidants and antibacterial agents [17, 40]. The antibacterial properties of sterols, alkaloids, triterpenes, polyphenols, flavonoids, and saponins have been reported [41-43]. Therefore, in the present research, it is believed that the plant extract of *E. platyloba* has shown antibacterial potential through the production of secondary metabolites using the mentioned mechanisms. In future research, by identifying the composition and chemical structure of these metabolites and extracting them, the use of these compounds can be directly evaluated on pathogenic microorganisms.

Secondary metabolites are the main constituents of plant-derived bioactive compounds that have therapeutic value and are used for medicinal purposes. The chemical structure, topography, and climate of the country of origin significantly impact their antimicrobial activities. Actually, the antimicrobial activity of medicinal plant species is affected by the type of plant species, quality, and quantity of secondary metabolites [35, 43]. Hence, detecting, identifying, and characterizing medicinal plants with antimicrobial potential can lead us to obtain efficient bioactive compounds for controlling microbial diseases. According to the results of the present research, which indicated the high potential of *E. platyloba* hydroalcoholic and aqueous extracts in inhibiting the growth of pathogenic bacteria, in the future, by conducting more in vitro and in vivo experiments, we can promise to obtain a herbal medicine with high antibacterial potential to suppress bacterial pathogens.

CONCLUSION

The use of herbal antimicrobials is of interest due to the proven risks of synthetic preservatives and the growing rate of antibiotic resistance worldwide. *Echinophora*, as a medicinal plant, contains bioactive compounds that affect the normal function of microbial cells. As previously mentioned, *E. platyloba* and its essential oils possess significant antibacterial properties. Therefore, the current research findings indicate that the aqueous and hydrocolic extracts of *E. platyloba* have a high potential to inhibit human pathogenic bacteria related to the oral cavity. Although additional in vivo and clinical studies will still be necessary, the results of this study will lead to the development of novel functional pharmaceutical and food products.

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