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Antimicrobial effect of aqueous and ethanolic extracts *Teucrium polium L*. on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

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ABSTRACT

Teucrium polium L. (Labiatae) has long been recognized in folk medicine in the treatment of many pathophysiological implications, such as gastrointestinal disorders, inflammations, diabetes and rheumatism. In this study antimicrobial activity of two crude extracts obtained from *Teucrium polium L*. was tested against bacterial species. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using a microdilution analysis method. The antimicrobial effects of extracts were evaluated on *Streptococcus pyogenes* PTCC 1447, *Pseudomonas aeruginosa* PTCC 1310 and *Staphylococcus epidermidis* PTCC 1435 by "using the method of Collins" and "disk agar diffusion method". The results showed that aqueous and ethanolic extracts were quite effective in 2000 µg/ml concentration on *Streptococcus pyogenes* and *Staphylococcus epidermidis* and were prevented from growth them on medium, while both extracts have no certain antimicrobial effect on *Pseudomonas aeruginosa.* In "disk agar diffusion method", 10, 20, 30 and 40 mg/ml aqueous and alcoholic extracts concentrations, was inhibition effect on *Streptococcus pyogenes* and *Staphylococcus epidermidis*, and 30 and 40 mg/ml aqueous and ethanolic extracts concentrations, has inhibition effect on *Pseudomonas aeruginosa* prevent them growing, but at 10 and 20 mg/ml concentrations, no inhibitory effect on *Pseudomonas aeruginosa* was observed. The results indicate that ethanolic and

aqueous extracts of *Teucrium polium L.* have the greatest effect on gram-positive bacterium *Streptococcus pyogenes* ($p<0.05$). Results showed, aqueous and ethanolic extracts of *Teucrium polium L.*, have been strong antimicrobial activity against many food pathogen bacteria.

1. Introduction

Finding healing power in plant is an ancient idea. Numerous studies have been carried out in different parts of the globe to extract plant products for screening antimicrobial activity (Barbour *et al*., 2004; Cowan 1999) Herbal medicines are one of the important cultural and traditional part of the people. Today, most of the world population depends on herbal medicines for their health care needs (Mahmoud *et al*., 2011; Manandhar 1995).

Antibiotics provide the main basis for the therapy of microbial (bacterial and fungal) infections. Since the discovery of these antibiotics and their use as chemotherapeutic agents there was a belief in the medical fraternity that this would lead to the eventual eradication of infectious diseases. However, overuse of antibiotics has become the major factor in the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms (Khan *et al*., 2009). The genus Teucrium (germander) belongs to the family Lamiaceae, within the subfamily Ajugoideae. In the flora of Europe the genus Teucrium has been divided into six sections with 49 species. The section Stachyobotrys is represented by the species *Teucrium arduini L*., the section Scorodonia by *Teucrium scordium L*., the section Chamaedrys by *Teucrium chamaedrys L.* and the section polium by *Teucrium polium L*. and *Teucrium montanum L*. These are mostly perennial herbs, shrubs or sub shrubs, while *Teucrium botrys* is an herbaceous annual plant. The species of this genus are widespread in all continents of the world but a very large number of species are present in the Mediterranean (Ricci *et al*., 2005). *Streptococcus pyogenes*, and *Staphylococcus epidermidis*, organisms that cause respiratory and cutaneous infections, and members of the Pseudomonas families, organisms that cause diarrhea, urinary infection, and sepsis, are now resistant to virtually all of the older antibiotics. The aim of this study was evaluated of antimicrobial effects of aqueous and ethanolic extracts *Teucrium polium L*. against some of the important food pathogens bacteria.

2. Materials and methods

2.1. Preparation plant

Teucrium polium L. was collected from Birjand (southeast of Khourasan state, Iran). Taxonomic identification was performed by the Faculty of Science Herbarium, Ferdowsi University of Mashhad, Iran.

2.2. Preparation of aqueous and ethanolic extracts from Teucrium polium L.

The amount 100 gram of *Teucrium polium L*. powder was Added to 500 ml ethanol 96 degree or distilled water. The ethanolic extract mixture was preserved at laboratory temperature for 24 hours and was stirred every few hours with a glass rod. The aqueous mixture was boiled for 20 minutes with low flame until the cream colored liquid was obtained. The collecting supernatant was centrifuged by 6000 rpm for 5 min. The resulting extract (supernatant) volume has reached to the original with ethanol or distilled water, and then samples were stored into the dark container at refrigerator temperature after filtering by 0.45 µ Whatman filter paper (Ahmad and beg., 2001).

2.3. Source of microorganisms

Three bacterial species, Gram-positive (*Streptococcus pyogenes* PTCC 1447, *Staphylococcus epidermidis* PTCC 1435), Gram-negative (*Pseudomonas aeruginosa* PTCC 1310) the microorganisms used in this study are significant. *Pseudomonas aeruginosa* is opportunistic pathogens. *Pseudomonas aeruginosa* are frequently associated with infection of the urinary tract. Also, *Pseudomonas aeruginosa* is commonly found in hospital environment and can

easily infect immunosuppressed patients. *Staphylococcus epidermidis* can infect skin and wounds causing acne, boils, and pimples.

2.4. Preparation of microbial suspension

Some of the bacterial suspension was poured in sterile tubes containing Ringer solution and its turbidity, was measured by spectrophotometer at 530 nm wavelength. It was diluted by Ringer solution until the solution turbidity equalizes with 0.5 McFarland standard solutions. Suspension should have contains 1.5×10^8 CFU / ml (Valero and Salmeron., 2003).

2.5. Evaluation of antimicrobial activity

0.2 gram of aqueous and ethanol extract, were added to 5 ml of sterile distilled water. Then it was stirred with vortex system to assist being steady. Then 1 ml of this solution was added to sterile plates. The final concentration of the extract was 2000 µg/ml (Babayi *et al*., 2004). In the next step, Mueller Hinton agar (Merck-Germany) medium were sterile and used for bacteria, were added to the plates, and placed at room temperature, so the medium was prepared. One loop of each standard strain culture media was cultured inoculums on these medium. The plates were incubated for 48 hours at 37 °C. The culture with extract and without bacteria was used as control (Babayi *et al*., 2004). The disk agar diffusion method, at first a loop of each standard strain culture media was cultured on the plates, and then paper discs (from Whatman filter with 6 mm diameter) plates were saturated with 100 µl of the test compound allowed to dry and was introduced on the upper layer of the seeded agar plate with 10, 20, 30 and 40 mg/ml extract concentrations, were prepared in distilled water and was treated with *Teucrium polium L*. extract and placed in culture medium by Sterile loop. Then it was fixed on the media with a light little pressure. After incubation time, the diameter of free zone was measured exactly by using a ruler in millimeters. All experiments were performed with 3 replicates (Bauer *et al*., 1996).

2.6. Determination of minimum inhibitory concentration (MIC)

MIC was determined according to agar dilution method (Benger *et al*., 2004). Various concentrations of extract were prepared in 10 cm experimental tubes containing Mueller Hinton broth for bacteria. Each tube contains 9 ml of Mueller Hinton for bacteria were sterilized by autoclaving. On cooling, 1 ml of each extract (watery & ethanoli) concentration were added to each tube, to make the final concentrations of (2, 4, 8, 16, 32 and 64mg/ml). The mixture of Mueller Hinton and extract was poured into plates aseptically in a laminar flow cabinet. On solidification of the agar medium, 2 µl of adjusted spore suspension were added to each plate by micropipette and incubated 37 °C for bacteria (Yazdani *et al*., 2009). The Mueller Hinton without any herbal extract served as control. The MIC was regarded as the lowest concentration of the extract that did not show any visible growth after 12 days of incubation (compared with control).

2.7. Minimum bactericidal concentration (MBC)

MBC was determined according to agar dilution method (Espinel-Ingroff *et al*., 2002) with slight modifications. The MBC were determined by incorporating various concentrations of extracts (2, 4, 8, 16, 32, 64, 128 and 256 mg/ml) in Mueller Hinton broth for bacteria.The tubes which showed no visible growth after 2 days incubation were subculture on extract free Mueller Hinton plates and incubated at 37°C for 2days (Yazdani *et al*., 2009). The MBC was regarded as the lowest concentration of the extract that prevented the growth of any bacteria colony on the solid medium.

2.8. Statistical analysis

All the assays were carried out in triplicates. The experimental results were expressed as mean ± standard deviation. The data were analyzed using one way analysis of variance (ANOVA) using SPSS version 17.

3. Results

The results of the antimicrobial effects of aqueous and ethanolic extracts, by "using the method of Collins *et al.* (1995)" were show on in Tables 1 and 2. The results of the antimicrobial effects of aqueous and ethanolic *Teucrium polium L*. extracts, by "the agar diffusion method" are presented in Tables 3 and 4. Minimum Inhibitory Concentration (MIC**)** results of the aqueous and ethanolic extracts of *Teucrium polium L* are given in Table 5 and 6.

Minimum Bacterial Concentration (MBC) results of the aqueous and ethanolic extracts of *Teucrium polium L* are given in Table 7 and 8.

Table 1

Antimicrobial effects of 2000µg/ml ethanolic *Teucrium polium L*. extract concentrations, on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (using the method of Collins *et al*. (1995).

Microorganism	***Teucrium polium L.***
P. aeruginosa	-
S. pyogenes	++
S. epidermidis	+

(-) in Table showed the growth of bacteria on culture and the lack of antibacterial activity of ethanolic *Teucrium polium L*. extract.

(+) in Table showed no bacterial growth on culture and antibacterial activity of ethanolic *Teucrium polium L*. extract.

(++) in Table showed no bacterial growth on culture and strong antibacterial activity of ethanolic *Teucrium polium L*. extract.

Table 2

Antimicrobial effects of 2000µg/ml aqueous *Teucrium polium L*. extract concentrations, on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (using the method of Collins *et al*. (1995).

Microorganism	***Teucrium polium L.***
P. aeruginosa	-
S. pyogenes	+
S. epidermidis	+

(-) in Table showed the growth of bacteria on culture and the lack of antibacterial activity of aqueous *Teucrium polium L*. extract.

(+) in Table showed no bacterial growth on culture and antibacterial activity of aqueous *Teucrium polium L*. extract.

Table 3

Average diameter (mm) of microbial free zone area of aqueous *Teucrium polium L*. leaves extract, on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (disk agar diffusion method).

Microorganism			***P. aeruginosa***	
Teucrium polium L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	-	-	6.30±0/50	6.90 ±0/50
			S. pyogenes	
Teucrium polium L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	12.30±0/57	14.30±0/50	16 ±0/28	19.10±0/28
			S. epidermidis	
Teucrium polium L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	11.20±0/57	12.40±0/57	14.10±0/50	16.90±0/28

(-) in Table showed no inhibitory effects was shown.

Table 4

Average diameter (mm) of microbial free zone area of ethanolic *Teucrium polium L*. leaves extract, on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (disk agar diffusion method).

Microorganism			***P. aeruginosa***	
Teucrium polium L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	-	-	6.70 ±0/57	8.80±0/28
			S. pyogenes	
Teucrium polium L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	14.90±0/76	16.90±0/50	18.80 ±0/50	22.90±0/57
			S. epidermidis	
Teucrium polium L. concentration	10	20	30	40
Average diameter (mm) of microbial free zone area	13.10±0/28	14.90±0/50	16.90 ±0/76	18.60±0/57

(-) in Table showed no inhibitory effects was shown.

Table 5

Minimum Inhibitory Concentration (MIC) of *Teucrium polium L*. extract (aqueous) on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis.*

	Concentration (mg/ml)						
Microorganism species	**Control**	**2**	**4**	**8**	**16**	**32**	**64**
P. aeruginosa	-	-	-	-	-	-	+
S. pyogenes	-	-	-	-	+	+	+
S. epidermidis	-	-	-	-	-	+	+

+: Positive inhibition

- : Negative inhibition

Table 6

Minimum Inhibitory Concentration (MIC) of *Teucrium polium L*. extract (ethanolic) on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis.*

	Concentration (mg/ml)						
Microorganism species	**Control**	**2**	**4**	**8**	**16**	**32**	**64**
P. aeruginosa	-	-	-	-	-	+	+
S. pyogenes	-	-	-	+	+	+	+
S. epidermidis	-	-	-	-	+	+	+

+: Positive inhibition

- : Negative inhibition

Table 7

Minimum Bacterial Concentration (MBC) of *Teucrium polium L*. extract (aqueous) on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis.*

	Concentration (mg/ml)								
Microorganism species	**Control**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
P. aeruginosa	-	-	-	-	-	-	-	-	+
S. pyogenes	-	-	-	-	-	-	+	+	+
S.epidermidis	-	-	-	-	-	-	-	+	+

+: Positive inhibition

- : Negative inhibition

Table 8
Minimum Bacterial Concentration (MBC) of *Teucrium polium L*. extract (ethanolic) on *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis.*

	Concentration (mg/ml)								
Microorganism species	**Control**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
P. aeruginosa	-	-	-	-	-	-	-	+	+
S. pyogenes	-	-	-	-	-	+	+	+	+
S.epidermidis	-	-	-	-	-	-	+	+	+

+: Positive inhibition
- : Negative inhibition

4. Discussion

The antimicrobial compounds from plants may inhibit bacterial growth by different mechanisms than those presently used. Antimicrobials therefore, may have a significant clinical value in treatment of resistant microbial strains. In particular, the antimicrobial activities of plant oils and extracts have formed the basis of many applications including raw and processed food preservation, pharmaceuticals, alternative medicine, and natural therapies (Sarac & Ugur, 2007).

The results showed 2000 µg/ml concentration of both aqueous and alcoholic extracts, were quite effective on reduce of growth *Streptococcus pyogenes* and *Staphylococcus epidermidis* and were had prevent growth over the medium. However, 2000 µg/ ml concentration aqueous and alcoholic extracts, have no significant antibacterial effect on *Pseudomonas aeruginosa* and it is not able to prevent the growth of bacteria on culture. Based on the results ethanolic extract of *Teucrium polium L*. in this study have significant antimicrobial activity on the studied microorganisms.

The results show that *Teucrium polium L*. ethanolic and aqueous extracted at all concentrations (10, 20, 30 and 40 mg/ml) had the inhibitory effect on *Streptococcus pyogenes* and *Staphylococcus epidermidis* .The results show that *Teucrium polium L*. extracted at concentrations (30 and 40 mg/ml) had the inhibitory effect on *Pseudomonas aeruginosa*, However, 10 and 20 mg/ml concentration extracts, have no significant antimicrobial effect on *Pseudomonas aeruginosa* and it is not able to prevent the growth of bacteria on culture. Gram-positive bacteria are more sensitive than gram-negative bacteria to *Teucrium polium L* extract, due to differences in cell structure of gram-negative and gram-positive bacteria, because gram-positive bacteria have more mucopeptide in their cell wall composition while gram-negative bacteria have only a thin layer of mucopeptide and most of their cell structure is lipoprotein and lipo-polysaccharides. Thus, gram-negative bacteria are more resistant (Tassou and Nychas., 1995; Ghalem *et al*., 2008). These points were consistent with the results obtained in this study.

(Alizadeh Behbahani *et al*., 2012) report that, ethanol extract compared to the aqueous extract was more effective and have a greater deterrent. The reason of these phenomena may be extracting more effective materials extracted by ethanol from *Avicennia marina*. These points were consistent with the results obtained in this study. (Kizil and Sogut., 2003) expressed in their study where they used the disc diffusion method that *Teucrium spicata* var. spicata essential oil had a strong antibacterial impact on *Staphylococcus aureus*, *Escherichia coli* K 12, *Escherichia coli* pMK 3, *Escherichia coli* pBR 322 and *Escherichia coli* pUC 9.

In a study where antibacterial activity of *Teucrium spicata* var. spicata essential oil was examined through the method of disc diffusion, it was indicated that the essential *Staphylococcus aureus* ATCC 25923 was effective on *Bacillus cereus* ATCC 11778 and *Escherichia coli* ATCC 29998 and created the largest inhibition zone against *Bacillus cereus* ATCC 11778(Akin., 2010). In another study aiming at studying the effects of the essential oil of *Teucrium polium* plant obtained by hydro distillation on various bacteria and yeasts carried out by its absorption into empty antibiotic discs at different amounts through the method of disc diffusion, it was found that the 2µl and 4µl applications of the essential oil had no effect on *E. coli* DM, which is one of the bacteria of the study, it had also no effect on *Staphylococcus auresu* Cowan 1 in the application of 2µl while its application of 4µl created an inhibition zone (Pauli and Knobloch 1987). The results shows that MIC of aqueous extract of *Teucrium polium L*. for *Streptococcus pyogenes* was 16mg/ml, for *Pseudomonas aeruginosa* was 64 mg/ml and for the *S. epidermidis* was 32 mg/ml. The results shows that MIC of ethanolic extract of *Teucrium polium L*. for *Streptococcus pyogenes* was 8 mg/ml, for *Pseudomonas aeruginosa* was 32 mg/ml and for the *Staphylococcus epidermidis* was 16 mg/ml. The results indicate that ethanolic extract of *Teucrium polium L*. mostly had been effective on *Streptococcus pyogenes*

and has the least impact on *Pseudomonas aeruginosa*. The result shows that MBC of aqueous extract of *Teucrium polium L.* for *Streptococcus pyogenes* was 64 mg/ml, for *Pseudomonas aeruginosa* was 256 mg/ml and *Staphylococcus epidermidis* was 128 mg/ml. The result shows that MBC of ethanolic extract of *Teucrium polium L.* for *Streptococcus pyogenes* was 32 mg/ml, for *Pseudomonas aeruginosa* was 128 mg/ml and *Staphylococcus epidermidis* was 64 mg/ml. The obtained results were similar to the results in other studies, the ethanolic extract of *Teucrium polium L.* inhibited the growth of several bacteria with different Minimal Inhibitory Concentration (MIC) This extracts inhibited the growth of *Staphylococcus aureus*, *Salmonella typhi* with a MIC of 40 mg/mL, this concentration was 10 mg/mL *Bordetella bronchiseptica* ,and *Bacillus anthracis* (Darabpour *et al*., 2010). These points were consistent with the results obtained in this study.

5. Conclusion

In conclusion, it can suggest that *Teucrium polium L.* extract *"in vitro"*, have considerable antimicrobial ability over the studied strains. In addition, more studies are needed *"in Situ"* be done, to identifying the effective dose of the extract on the microorganisms, and finally introduce the extract as a natural and novel antimicrobial compound.

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