An investigation on the effect of alcoholic and aqueous extracts of *Dorema aucheri* (Bilhar) on some pathogenic bacteria in vitro

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ABSTRACT

Dorema aucheri is a plant that grows in Iran. In Persian it is called (Bilhar). This experimental study was carried out at Ferdowsi University of Mashhad in 2014. After collection and preparation of aqueous and ethanolic extracts of *Dorema aucheri* (Bilhar), The antibacterial activity of ethanolic and aqueous extracts of Bilhar was evaluated against 7 laboratory strains of microorganisms, including 4 Gram positive (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus* and *Bacillus subtilis*) and 3 Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*). Its effects against human pathogen microorganism were determined using "Spreading of the Extract on Medium Surface" and "Disk Agar Diffusion Method", Minimal Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) were determined for this extract. Collected data were analyzed by SPSS software using one-way ANOVA. The zone of inhibition for the ethanolic extract varied from 8 mm for *P. aeruginosa* to 24 mm for *S. pyogenes* and from 7 mm for *P. aeruginosa* to 19 mm for *S.pyogenes* in the aqueous extract. The minimum inhibitory concentration (MIC) of the extracts ranged between 2 mg/ml and 64 mg/ml while the minimum lethal concentration (MLC) ranged between 4 mg/ml and 256 mg/ml. Among of tested strains, *P. aeruginosa* has maximum MIC and MBC. 30 and 40 mg/mL Concentrations of Redcurrant have significant antimicrobial effect on bacteria. Antibacterial effect of extracts was decreased with decrease of extract concentration in disk. According to result, ethanolic extract of *Dorema aucheri* have antimicrobial effect on growth of all of the strains exposed analyzes and antimicrobial effect of that was maximum on Gram-positive bacterum of *S. pyogenes*. *P. aeruginosa* showed the highest level of resistance against the aqueous and ethanolic Bilhar extracts. The present study demonstrated that the ethanol leaf extract of *Dorema aucheri* hold an excellent potential as an antibacterial agent.

Keywords: *Dorema aucheri, Pseudomonas aeruginosa*, *Bacillaceae*, Extract.

INTRODUCTION

Dorema aucheri (Bilhar) is from the piaceae family that exists in margins of Zagros mountains in Iran. It's normally growing in mountain especially in west of Iran (Kermanshah) and in Percian called "kandale kohi" and in Kermanshah it's known as "zo" normally cooked with Steam and served with butter as meal. Root known as medicin (traditionally) and using it in different ways (creame and smoke) [1]. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity [2]. The use of, and search for, drugs and dietary supplements taken from plants have accelerated in recent years. Pharmacologists, botanists, microbiologists, and natural-products chemists are combing the earth for phytochemicals and leads that could be developed for treatment of various diseases [3]. Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 75 percent show a positive relationship between their modern remedial use and the traditional use of the plants from which they are insulate [4].

Enterobacteriaceae is a large family of Gram-negative bacteria that includes, along with many harmless symbionts, many of the more familiar pathogens, such as Salmonella, Escherichia coli and Proteus [5]. *P. vulgaris* can cause urinary tract infections and hospital-acquired infections. *Proteus* is unique, however, because it is highly motile and does not form regular colonies [6]. *E. coli* causes septice mias and can infect the gall bladder, surgical wounds, meninges, skin lesions and the lungs especially in debilitate and immunodeficient patients [7]. Bacillaceae is a family of Gram-positive, heterotrophic, rod-shaped bacteria that may produce endospores. Bacillus is responsible for a minority of foodborne illnesses (2–5%), causing severe nausea, vomiting and diarrhea. Bacillus foodborne illnesses occur due to survival of the bacterial endospores when food is improperly cooked [8]. In this study, the antibacterial activity of *Dorema aucheri* ethanol and aqueous extracts were evaluated through disk agar diffusion method, extract on medium surface method and microdilution method against 7 laboratory strains of microorganisms, including 4 Gram positive (*S. aureus*, *S. pyogenes*, *B. cereus* and *B. subtilis*) and 3 Gram negative bacteria (*E. coli*, *P. aeruginosa* and *P. vulgaris*) in vitro.

MATERIALS AND METHODS

Collection of Sample

This experimental study was conducted at Industrial Microbiology Laboratory, Department of Food Science and Technology, Ferdowsi University of Mashhad in 2014. The *Dorema aucheri* were collected from countryside of Kermanshah (Kermanshah Province, Iran) this was taken for identification in the department of Institute of Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Razavi Khorasan Province, Iran. Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles [9].

Plant extraction

20 g of air-dried powder was mixed with distilled water. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000 g for 10 min. The supernatant was collected. This procedure was repeated twice more. After 6 h, the supernatant, collected at an interval of every 2 h, was pooled and concentrated to make the final volume one-fourth of the original volume. For solvent extraction, 20 g of airdried powder was mixed with 100 ml of organic solvent (ethanol) in a conical flask, plugged with cotton and then kept on a rotary shaker at 190 - 220 rpm for 24 h. After 24 h, it was filtered through 8 layers of muslin cloth and centrifuged at 5000 g for 10 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fourth of the original volume, and stored at 4^{o}C in air-tight bottles [10].

Test organisms

Seven bacteria were tested in this study include; *P. aeruginosa* ATTC 27853, P. *vulgaris* ATTC 8427, *E. coli* ATTC 25922, *B. cereus* ATTC 14579, *B. subtilis* ATTC 23857, *S. aureus* ATTC 25923 and *S. pyogenes* ATTC 19615 were obtained from the Microbiology Department of Mashhad University of Medical Sciences, Mashhad. The typed cultures of bacteria was sub-cultured on Nutrient agar (Oxoid) slants respectively and stored at 4^{o}C until required for study.

Preparation of medium

Mueller–Hinton Agar and Nutrient Agar were supplied by Department of Food Science and Technology, Ferdowsi University of Mashhad. Inocula of test organisms obtained from source were prepared by growing each pure isolate in Nutrient broth for 18 hours at 37^{o}C. The overnight broth culture was matched with Macfarland turbidity standard to give an approximate 1.5×10^{8} cfu/ml [11].

Suspension preparation

Fresh cultivated *P. aeruginosa* ATTC 27853, P. *vulgaris* ATTC 8427, *E. coli* ATTC 25922, *B. cereus* ATTC 14579, *B. subtilis* ATTC 23857, *S. aureus* ATTC 25923 and *S. pyogenes* ATTC 19615 colonies were suspended in 5 mL of 0.85% normal saline. Suspension was mixed for 15 seconds with a vortex. Then its concentration was adjusted to 1.5×10^{8} CFU/mL based on a standard 0.5 McFarland [12].

Paper Disc Technique

Sterile filter paper discs (6.00 mm diameter) were soaked with the test extracts and dried at 20^{o}C for 30 minutes. The prepared Nutrient agar plates were seeded with each of the test bacteria and the filter paper discs were placed on each plate. The plates were incubated at 37^{o}C for 24 hours [13].

Determining antimicrobial activities using the extract on medium surface method

The extract on medium surface method or pour-plate technique also will yield isolated

colonies and has been extensively used with bacteria. The original sample is diluted several times to reduce the microbial population sufficiently to obtain separate colonies upon plating. The small volumes of several diluted samples are added to sterile petri plates and mixed with liquid tryptic soy agar that has been cooled to about 48°C to 50°C. 0.2 gram of extract, were added to 5 ml of sterile distilled water. At the next step, the sterilized Mueller Hinton Agar (Merck-Germany) medium were added to the plates and placed at room temperature. One loop of each standard strain culture media was cultured inoculums on these medium. The plates were incubated for 24 hours at 37°C [14].

Minimum Inhibitory Concentration (MIC)

The broth micro-dilution method was used to determine MIC using Mueller Hinton broth supplemented with Tween 80 at a final concentration of 0.5% (v/v). Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged 264, 128, 64, 32, 16, 4 and 2 mg/ml. To each well, 10 µL of indicator solution and 10 µL of Mueller Hinton broth were added. Finally, 10 µL of bacterial suspension 1.5×10^8 CFU /ml (equivalent to 0.5 McFarland standards) was added to each well. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates and then they were placed in an incubator at 37^oC for 18-24 h. The color change was then assessed visually and the lowest concentration at which the color change occurred was taken as the MIC value. The average of 3 values was calculated providing the MIC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity [15].

Minimum Lethal Concentration (MLC)

The minimum lethal concentration (MLC) test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MLC is determined using a series of steps, undertaken after a Minimum Inhibitory Concentration (MIC) test has been completed [16].

Statistical analysis

The test results were processed by using one-way variance analysis (ANOVA). Differences at $p<0.05$ were considered to be significant. The employed software was SPSS $_{V18}$ (USA، Il، Chicago، SPSS Inc).

RESULTS

The results of the antimicrobial effects of ethanolic and aqueous extracts, by "extract on medium surface method" were show on in Tables 1.

Table 1. Antimicrobial effects of ethanolic and aqueous *Dorema aucheri* extracts concentrations on some pathogenic bacteria.

Extract	Microorganism	Result
Aqueous	*P. aeruginosa* ATTC 27853	R
Aqueous	*P. vulgaris* ATTC 8427	**S**
Aqueous	*E. coli* ATTC 25922	**R**
Aqueous	*B. cereus* ATTC 14579	**S**
Aqueous	*B. subtilis* ATTC 23857	**S**
Aqueous	*S. aureus* ATTC 25923	**S**
Aqueous	*S. pyogenes* ATTC 19615	**S**
Ethanolic	*P. aeruginosa* ATTC 27853	**R**
Ethanolic	*P. vulgaris* ATTC 8427	**S**
Ethanolic	*E. coli* ATTC 25922	**S**
Ethanolic	*B. cereus* ATTC 14579	**S**
Ethanolic	*B. subtilis* ATTC 23857	**S**
Ethanolic	*S. aureus* ATTC 25923	**S**
Ethanolic	*S. pyogenes* ATTC 19615	**S**

R: Resistant S: Sensitive

Table 2. Average diameter (mm) of microbial free zone area of aqueous and ethanolic *Dorema aucheri* extracts concentrations on some pathogenic bacteria (disk agar diffusion method).

extract	Microorganism	The concentration of *Dorema aucheri* extracts (mg/ml)			
		10	20	30	40
Aqueous	*P. aeruginosa*	7.00±0.58[a]	10.10±0.54[b]	13.60 ±.50[c]	15.90±0.28[d]
Aqueous	*P. vulgaris*	8.60±0.58[a]	10.50±0.54[b]	13.90 ±.54[c]	16.20±0.28[d]
Aqueous	*E. coli*	8.10 ±0.58[a]	10.10 ±0.54[b]	13.10± 0.28[c]	16.50± 0.50[d]
Aqueous	*B. cereus*	9.80 ±0.58[a]	12.60 ±0.58[b]	14.90± 0.28[c]	17.90± 0.50[d]
Aqueous	*B. subtilis*	11.00 ±0.54[a]	13.00 ±0.54[b]	16.00± 0.28[c]	18.00± 0.54[d]
Aqueous	*S.aureus*	11.40 ±0.54[a]	14.10 ±0.58[b]	16.00± 0.28[c]	18.90± 0.54[d]
Aqueous	*S. pyogenes*	13.00 ±0.54[a]	15.00 ±0.58[b]	17.00± 0.28[c]	19.00± 0.54[d]
Ethanolic	*P. aeruginosa*	8.00±0.58[a]	11.20±0.57[b]	15.10 ±.55[c]	18.90±0.28[d]
Ethanolic	*P. vulgaris*	9.50±0.58[a]	12.10±0.54[b]	15.90 ±.55[c]	19.30±0.28[d]
Ethanolic	*E. coli*	9.10 ±0.54[a]	11.80 ±0.58[b]	15.20± 0.50[c]	17.80± 0.58[d]
Ethanolic	*B. cereus*	11.30 ±0.58[a]	13.90 ±0.50[b]	16.50± 0.28[c]	19.90± 0.57[d]
Ethanolic	*B. subtilis*	12.20 ±0.58[a]	15.80 ±0.50[b]	18.90± 0.28[c]	21.00± 0.58[d]
Ethanolic	*S.aureus*	14.00 ±0.58[a]	16.90 ±0.50[b]	19.80± 0.28[c]	23.00± 0.58[d]
Ethanolic	*S. pyogenes*	14.30 ±0.58[a]	17.40 ±0.50[b]	21.20± 0.28[c]	24.00± 0.58[d]

[a]Values are means ± standard deviations, n=3.

Table 3. Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of aqueous and ethanolic extract of *Dorema aucheri* on some pathogenic bacteria.

Extract	Bacteria species	MIC (mg/ml)	MLC (mg/ml)	Negative	Positive
Aqueous	*P. aeruginosa*	64	256	-	+
Aqueous	*P. vulgaris*	16	64	-	+
Aqueous	*E. coli*	32	128	-	+
Aqueous	*B. cereus*	8	32	-	+
Aqueous	*B. subtilis*	8	32	-	+
Aqueous	*S.aureus*	8	16	-	+
Aqueous	*S. pyogenes*	4	8	-	+
Ethanolic	*P. aeruginosa*	32	128	-	+
Ethanolic	*P. vulgaris*	8	32	-	+
Ethanolic	*E. coli*	16	64	-	+
Ethanolic	*B. cereus*	4	16	-	+
Ethanolic	*B. subtilis*	4	16	-	+
Ethanolic	*S.aureus*	4	8	-	+
Ethanolic	*S. pyogenes*	2	4	-	+

+: Grow –: Not grow, N=3.

The results the antimicrobial effects of ethanolic and aqueous *Dorema aucheri* extracts, by "the agar diffusion method" were presented in Tables 2. The zone of inhibition for the ethanolic extract varied from 8 mm for Pseudomonas aeruginosa to 24 mm for Streptococcus pyogenes and from 7 mm for Pseudomonas aeruginosa to 19 mm for Streptococcus pyogenes in the

aqueous extract. The ethanolic and aqueous extracts inhibited the growth of all the test organisms. The results the MIC and MLC of ethanolic and aqueous *Dorema aucheri* extracts were presented in Tables 3. The MIC of ethanolic extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 32, 8, 16, 4, 4, 4 and 2 mg/ml, respectively. But MIC of the aqueous extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 64, 16, 32, 8, 8, 8 and 4 mg/ml, respectively. The MLC of ethanolic extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 128, 32, 64, 16, 16, 8 and 4 mg/ml, respectively. But MLC of aqueous extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 256, 64, 128, 32, 32, 16 and 8 mg/ml, respectively.

DISCUSSION

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity. Iran is a varietal emporium of medicinal plants and is one of the richest countries in the world in regard to genetic resources of medicinal plants. It exhibits a wide range in topography and climate, which has a bearing on its vegetation and floristic composition [17]. Therefore, in this study investigation the antimicrobial effect of medicinal plant *Dorema aucheri* on Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes in vitro.

Results obtained in the present study relieved that the tested two extracts possess potential antibacterial activity against Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes (Table 1 and 2). The zone of inhibition for the ethanolic extract varied from 8 mm for Pseudomonas aeruginosa to 24 mm for Streptococcus pyogenes and from 7 mm for Pseudomonas aeruginosa to 19 mm for Streptococcus pyogenes in the aqueous extract. The ethanolic and aqueous extracts inhibited the growth of all the test organisms. Successive isolation of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure [18]. The traditional healers use primarily water as the solvent but we found in this study the plant extracts by ethanol provided more consistent antimicrobial activity compared to those extracted by water. This might have resulted from the lack of solubility of the active constituents in aqueous solutions while ethanol extract showed some degree of antibacterial activity. Further trials using solvents of various polarities will explore the effects of solvent composition on extract efficacy.

The presence of some of the phytochemical components like saponins, tannins and phenolic compounds have been attributed to the antibacterial activity of the crude drugs observed. The presences of these bioactive components in the crude drugs have been linked to their activeties against disease causing microorganisms and also offering the plants them-selves protection against infection by pathogenic microorganisms [19].

The results showed 2 mg/ml concentration of ethanolic extract (extract on medium surface method), were quite effective on reduce of growth Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes and were had prevent growth over the medium, However, 2 mg/ ml concentration ethanolic extracts, have no significant antibacterial effect on Pseudomonas aeruginosa and it is not able to prevent the growth of bacteria on culture. The aqueous extract, only had antimicrobial effect in 2 mg/ml concentration on growth of, Proteus vulgaris, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes Table (1). On the basis of the above results, it showed that ethanol extract of *Dorema aucheri* exhibited a greater inhibition compared with aqueous extract. Alizadeh Behbahani et al. (2015) reported that most of the antimicrobial active compounds were soluble in polar solvent such as ethanol instead of water [20]. This result is comparable to the study by Alizadeh Behbahani et al. (2015) using ethanolic extract of *Myrtu s communis* that

showed effective antibacterial activity on *S. epidermidis*, *E. faecalis*, *E. coli* and *S. flexneri* [21].

Tabatabaei Yazdi et al. (2015) the antimicrobial effect of ethanol and aqueous extracts of *Mespilus germanica* against some pathogenic bacterial strains (*Streptococcus pyogene*, *Listeria innocua*, *Enterobacter aerogenes* and *Klebsiella pneumoniae*) were determined using agar well diffusion and paper disk methods. The results showed that ethanol was the best extractive solvent for antimicrobial properties of *Mespilus germanica* followed in order aqueous [22]. This was also reported by Doughari et al. (2008), the antibacterial screening of the water and ethanolic extracts of the various plant materials were carried out against pathogenic bacteria including Pseudomonas aeruginosa, Klebsiealla pneumonia, Escherichia coli, Staphylococcus aureus and Shigella dysenteriae. Ethanolic extracts were more potent than aqueous extracts and activity were concentration dependent. The Gram positive bacteria were more sensitive to the ethanolic extracts of both plants [23].

Among the selected bacteria studied, *Dorema aucheri* samples inhibited the growth of Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes better than Gram-negative against Pseudomonas aeruginosa, Proteus vulgaris and Escherichia coli. Previous studies also reported that Gram-negative bacteria were less susceptible to lower minimal inhibitory concentrations (MIC) than Gram-positive strains [16]. The MIC of ethanolic extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 32, 8, 16, 4, 4, 4 and 2 mg/ml, respectively. But MIC of the aqueous extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 64, 16, 32, 8, 8, 8 and 4 mg/ml, respectively. The MLC of ethanolic extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 128, 32, 64, 16, 16, 8 and 4 mg/ml, respectively. But MLC of aqueous extract of *Dorema aucheri* for Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and Streptococcus pyogenes were 256, 64, 128, 32, 32, 16 and 8 mg/ml, respectively.

By referring to Tables 1 and 2, the extracts were found to be more effective on Gram positive than Gram negative bacteria, which is in conformity with a number of earlier studies where compounds derived from plants often show considerable activity against Gram positive bacteria but not against Gram negative species [24]. Gram negative bacteria have effective permeability barrier, comprised of the outer membrane, which restricts the penetration of amphiphatic compounds and multidrug resistance pumps that extrude toxins across this barrier. It is possible that the apparent ineffectiveness of the plant antimicrobial activity is largely due to this permeability barrier [24].

CONCLUSION

In this study, aqueous and ethanolic extracts of *Dorema aucheri* were assessed. The results seem to justify their continued use in the treatment of microbial infections. The present study demonstrated that the ethanol leaf extract of *Dorema aucheri* hold an excellent potential as an antibacterial agent.

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