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Affectivity of *Zataria multiflora* Boiss Alcoholic Extracts Against Bacteria

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Abstract: The alcoholic extracts of *Zataria multiflora* Boiss were evaluated for prospective antibacterial activity against gram negative and gram positive bacteria. Cefoperazone is used as antibacterial reference drug. The antibacterial activity of ethanol and methanol extracts varied from organism to organism. The inhibitory effect of both the extracts and their 50/50 combination was more pronounced against gram positive bacteria. The MIC and MBC values were in the range of 1.718-6.25 and 2.832-6.25 mg/ml, respectively. Significant (p=0.05) synergistic effect of combination of EtOH and MeOH extracts was recorded against *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923 while additive effect against rest of the bacterial strains. The present studies provide evidences for presence of antibacterial elements in alcoholic extracts and recommends for more exploration for it use against bacterial diseases.

Key words: Extracts % *Zataria multiflora* Boiss % Antibacterial % MIC % MBC % Synergistic.

INTRODUCTION

Zataria multiflora locally known as "SAATAR" belongs to a family Labiatae possessing fragrant odor like lemon and thyme. The plant consists of small ovate or nearly round dotted, leathery leaves mixed with numerous minute flowers [1]. It is extensively used in folk medicines of Pakistan. The most effective compounds of *Zataria multiflora* Boiss are thymol and carvacrol. Its infusion is valued as an aromatic stimulant, cure for stomach ache and gastrointestinal infections [2-4].

The medicinal plants are being used for treatment of infections is an age-old practice especially in developing countries. Plants generally act to stimulate and supplement the healing forces and are the natural food for human beings [5,6]. The medicines of plant origin are used for a variety of diseases [7,8]. The interest in use of plants and their antimicrobial activity has revived due to the problems associated with the current use of antibiotics [9,10]. In recent years, human pathogen had developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. The undesirable side effect of certain antibiotics and the emergence of previously uncommon infections, led the scientists to look for new antimicrobial substance from various sources especially medicinal plants [11,12]. The screenings of plant extracts and products presents potential source of new antimicrobial agents [13,14]. Keeping in view of the above concerns, the present study was conducted to evaluate the potential of alcoholic extracts of *Zataria multiflora* Boiss for inhibition and elimination of gram negative and positive bacteria.

MATERIALS AND METHODS

Plant Material: The aerial part of *Zataria multiflora* was used in this study. The plant material was collected from Hazargangi area of Quetta, Balochistan, Pakistan and the taxonomic identity was confirmed from Department of Botany, University of Balochistan, Quetta, Pakistan. The plant material was air dried by protecting it from direct exposure of sunlight and homogenized to fine powder. The prepared plant material was stored in air tight glass bottles.

Preparation of Extract: 100 gm of powdered plant material was soaked in 1000 ml each of ethanol (EtOH) 80%, methanol (MeOH) 80%, kept on rotatory shaker for 24 hours. Thereafter, it was filtered through Whatman No. 2 filter paper under suction. The filtered extracts were concentrated in vacuum using rotatory evaporator, weighed and saved in screw capped tubes.

Microbial Strains: Five strains of gram negative bacteria i.e., *Salmonella typhimurium* ATCC-14028, *Escherichia coli* ATCC-8739, *Escherichia coli* ATCC-25922, *Pseudomonas aeruginosa* ATCC-27853, *Pseudomonas*

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aeruginosa ATCC-9027 and three gram positive bacteria i.e., *Staphylococcus aureus* ATCC-25923, *Staphylococcus aureus* ATCC-29213, *Bacillus subtilis* ATCC-6633, were used in the present studies. These strains were procured from OXID in the form of cultiloops, revived and maintained on nutrient agar.

Inoculum Preparation: All the bacteria used in the present studies were grown to exponential phase in nutrient broth at 37ºC for 18 hrs and adjusted to final density 1 to $2X10^8$ cfu/ml by diluting fresh cultures and comparing with McFarland density.

Antibacterial Assay: The antibacterial activity of individual extracts (Ethanol, methanol extracts) and their 50/50 combination was measured by using modified agar well diffusion method according to NCCLS [15]. Nutrient agar was inoculated with the inoculums (200 Fl/ 20ml medium) of given microorganism and poured in to sterile Petri plates. After allowing the medium to solidify at room temperature, wells of 6 mm diameter were bored in agar and filled with 50 Fl of 200 mg/ml of each solvent extract and combination of extracts. Control wells received 50 Fl neat solvent (negative control) and 50 Fl standard antibiotic solution (positive control) viz., Cefoperazone (1.5 mg/ml) were also run parallel in the same plate. The plates were allowed to stand at room temperature for 1 hour for extract to diffuse into the agar and then they were incubated at 37ºC for 18 hours. Subsequently the plates were examined for growth inhibition by measuring the inhibition zone formed around the well in millimeter. Three independent experiments represented by 5 replicates for each extract and combinations of extract were carried out.

Determination of Minimum Inhibition Concentration (Mic) and Minimum Bactericidal Concentration (Mbc): The minimal inhtionibitory concentration (MIC) of extracts were determined based on modified microdilution method in 96 multi-well microtiter plates [16]. The extracts were diluted to highest concentration to be tested (25 mg/ml). 50Fl of nutrient broth was distributed from the 2nd to the 12th well, a volume of 100 Fl from each of alcoholic extract initially prepared was pipette into the 1st well of each microtiter line and then 50 Fl of scalar dilution was transferred from 2nd to 12th well. 10 Fl of reazurin indicator (6.75 mg/ml) was added to each well. Finally 10 Fl of bacterial suspension was added to each well. The final concentration of alcoholic extracts adopted for antibacterial activity was from 50 to 0.024 mg/ml. Three columns in each plate were used for control; 1 column for standard antibiotic (50 to 0.024 mg/ml) as positive control and 2 columns containing solvents methnol and ethanol as negative control. The microtiter plates were covered with microtiter plate cover to avoid dehydration of bacteria and 10 replicates were prepared. The plates were incubated at 37ºC for 18-24 hrs. The change in color from purple to pink or colorless was visually assed and recorded as positive. The highest dilution (least concentration) showing change in color was recorded as the MIC value. The average and standard deviation of 10 values was calculated.

MBC was determined by sub culturing the test dilution on to fresh drug-free solid medium and incubating further for 18-24 hours. The highest dilution that yielded no single bacterial colony on a solid medium was taken as MBC.

The effect of combination of extracts was expressed after following calculation:

Calculated zone size/MIC/MBC= Sum of Zone size/MIC/MBC of both extract ÷ 2

If

1. Observed zone size/MIC/MBC = Calculated zone size/MIC/MBC than the effect will be additive
2. Observed zone size/MIC/MBC > Calculated zone size/MIC/MBC than the effect will be Synergistic
3. Observed zone size/MIC/MBC < Calculated zone size/MIC/MBC than the effect will be antagonistic

Statistical Analysis: Statistical analysis of the data was carried out by using computer programme SAS statistical software package [17,18].

RESULTS AND DISCUSSION

Yield of Extraction: The extractive yield of EtOH and MeOH form *Zataria multiflora* Boiss was 15.74 and 13.52 %, respectively.

Antibacterial Activity: The inhibitory effects of alcoholic extracts of *Zataria multiflora* Boiss against gram negative and positive bacteria are presented in Table 1. The activity of both the extracts was weaker against gram negative (inhibition zone 11.8 to 15.2 mm) than gram positive bacteria (inhibition zone 24.0 to 30.0 mm). Among gram negative bacteria *Pseudomonas aeruginosa* ATCC 27853 was found to be most sensitive. The zone sizes recorded for *Pseudomonas aeruginosa* ATCC 27853 were 15.2, 15.0 mm for ethanol (EtOH) and methanol (MeOH) extracts, respectively.

Table 1: Inhibitory effect (inhibition zone diameter in mm) of *Zataria multiflora* extracts against gram negative and positive bacteria

Mean of 15 values ± Standard deviation

S.N	Test Organism	Cefoperazone	*Zataria multiflora* Extracts		L.Sd at p=0.05
			Ethanol Extract	Methanol extract	
1	*Salmonella typhimurium* ATCC-14028	33.2±0.61	14.0±0.37	14.2±0.54	1.505
2	*Escherichia coli* ATCC-8739	33.2±0.40	12.4±0.49	11.8±0.40	1.285
3	*Escherichia coli* ATCC-25922	30.2±0.65	11.8±0.40	12.2±0.40	1.488
4	*Pseudomonas aeruginosa* ATCC-27853	25.0±0.79	15.2±0.40	15.0±0.63	1.814
5	*Pseudomonas aeruginosa* ATCC-9027	24.0±0.37	13.8±0.65	11.8±0.75	1.867
6	*Staphylococcus aureus* ATCC-25923	27.0±0.82	26.6±0.61	29.6±0.71	2.069
7	*Staphylococcus aureus* ATCC-29213	24.6±0.71	24.8±0.40	26.0±0.73	1.876
8	*Bacillus subtilis* ATCC-6633	22.4±0.49	23.0±0.52	30.0±0.37	1.376

Table 2: Inhibitory effect (inhibition zone diameter in mm) of combination of *Zataria multiflora* extracts against gram negative and positive bacteria

Mean of 15 values ± Standard deviation

S.No.	Test Organism	Expected calculated Zone size	Observed Zone size	L.Sd at p=0.05	Remarks
Gram negative bacteria					
1	*Salmonella typhimurium* ATCC-14028	14.10±0.45	14.40±0.80	Not significant	Additive effect
2	*Escherichia coli* ATCC-8739	12.10±0.44	12.00±0.37	Not significant	Additive effect
3	*Escherichia coli* ATCC-25922	12.00±0.40	12.20±0.65	Not significant	Additive effect
4	*Pseudomonas aeruginosa* ATCC-27853	15.10±0.50	15.00±0.52	Not significant	Additive effect
5	*Pseudomonas aeruginosa* ATCC-9027	12.80±0.70	14.20±0.40	Not significant	Additive effect
Gram positive bacteria					
1	*Staphylococcus*	*28.10±0.66*	*31.40±0.89*	*2.367*	*Synergistic*
2	*Staphylococcus*	*25.40±0.56*	*28.60±0.49*	*1.969*	*Synergistic*
3	*Bacillus subtilis ATCC-6633*	*26.50±0.44*	*28.40±0.81*	*Not significant*	*Additive effect*

Table 3: Antibacterial activity (MIC & MBC, mg/ml) of different solvent extracts of Zataria multiflora against gram negative and positive bacteria

S.No.	Test Organism	Zataria multiflora Extracts: Ethanol Extract MIC	Ethanol Extract MBC	Methanol Extract MIC	Methanol Extract MBC	Cefoperazone MIC	Cefoperazone MBC	L.Sd. atP=0.05 MIC	L.Sd. atP=0.05 MBC
Gram negative bacteria									
1	*Salmonella typhimurium ATCC-14028*	2.812±0.625	5.938±0.938	2.969±0.469	5.938±0.938	0.0185±0.029	0.0185±0.029	1.472	2.714
2	*Escherichia coli ATCC-8739*	3.438±0.938	5.938±0.938	3.125±0.781	5.938±0.938	0.045±0.007	0.045±0.007	1.372	2.737
3	*Escherichia coli ATCC-25922*	2.969±0.489	6.250±0.540	2.969±0.469	6.250±0.540	0.092±0.01	0.092±0.01	1.376	0.025
4	*Pseudomonas aeruginosa ATCC-27853*	2.832±0.625	5.938±0.938	3.125±0.469	6.250±0.469	0.185±0.092	0.185±0.092	0.944	1.392
5	*Pseudomonas aeruginosa ATCC-9027*	2.969±0.469	6.250±0.244	3.428±0.938	6.250±0.244	0.195±0.01	0.195±0.01	2.474	0.024
Gram positive bacteria									
6	*Staphylococcus aureus ATCC-25923*	2.812±0.625	2.969±0.469	2.344±0.781	6.250±0.540	0.087±0.019	0.087±0.019	2.015	0.687
7	*Staphylococcus aureus ATCC-29213*	3.438±0.938	3.438±0.938	2.969±0.469	6.250±0.244	0.090±0.014	0.090±0.014	1.804	1.373
8	*Bacillus subtilis ATCC-6633*	1.718±0.469	2.969±0.469	1.718±0.469	2.832±0.625	0.370±0.058	0.370±0.058	1.315	1.663

Significantly (p=0.05 level) larger zone sizes of 50:50 EtOH and MeOH extract combination were observed than calculated zone sizes in case of both the strains of *Staphylococcus aureus* ATCC-29213 and ATCC-25923 thus showing synergistic effect. Whereas, non significant (p=0.05 level) difference between calculated and observed zone sizes of 50:50 EtOH and MeOH extract combination were recorded for all the gram negative bacteria under

Table 4: Effect of combination of extracts of *Zataria multiflora* extracts on antibacterial Activity (MIC & MBC mg/ml).

Mean of 10 values ± Standard deviation

S.No.	Test Organism	Expected calculated MIC /MBC		Observed MIC/ MBC		L.Sd atP=0.05		Remarks	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram negative bacteria									
1	*Salmonella typhimurium* ATCC-14028	2.890±0.547	5.938±0.938	5.938±0.938	6.250±0.469	2.371	Not significant	Antagonistic effect	Additive effect
2	*Escherichia coli* ATCC-8739	3.281±0.469	5.938±0.938	3.125±0.244	3.438±0.938	Not significant	Not significant	Additive effect	Additive effect
3	*Escherichia coli* ATCC-25922	2.969±0.244	6.250±0.547	3.438±0.938	6.25±0.547	Not significant	Not significant	Additive effect	Additive effect
4	*Pseudomonas aeruginosa* ATCC-27853	2.978±0.312	6.094±0.469	6.250±0.244	12.50±0.346	2.260	2.364	Antagonistic effect	Antagonistic effect
5	*Pseudomonas aeruginosa* ATCC-9027	3.198±0.703	6.250±0.547	3.125±0.450	6.250±0.547	Not significant	Not significant	Additive effect	Additive effect
Gram positive bacteria									
6	*Staphylococcus aureus* ATCC-25923	2.890±0.547	4.609±0.234	0.865±0.253	1.718±0.469	1.987	2.469	Synergistic effect	Synergistic effect
7	*Staphylococcus aureus* ATCC-29213	3.203±0.703	4.844±0.469	2.832±0.450	3.125±0.452	1.203	1.519	Synergistic effect	Synergistic effect
8	*Bacillus subtilis* ATCC-6633	1.718±0.469	2.900±0.547	1.718±0.469	2.832±0.346	Not significant	Not significant	Additive effect	Additive effect

investigation and gram positive bacteria *Bacillus subtilis* ATCC-6633 to register its additive effect against the aforesaid strains (Table 2). The inhibitory effect of EtOH extract (200 mg/ml) against gram positive bacteria was more or less the same as compared with Cefoperazone (1.5mg/ml). Whereas, MeOH extract and combination of extracts (200 mg/ml) showed stronger inhibition than that of Cefoperzone (1.5 mg/ml). *Zataria multiflora* is commonly used in folk medicine as stimulant, curing agent for stomach ache, tooth ache, healing of wounds and other purposes [19-22]. Jaferi [23] has also reported the effectivity of *Zatari multiflora* against recurrent aphthous stomatitis. The antibacterial activity and antifungal effects of *Zataria multiflora* have also been reported [24, 25]. Its most effective compounds are carvacrol and thymol [26, 27] whose concentrations are 57.40 and 15.50 %, repectively. In the year 2004 Ramezani and his colleagues [28] confirmed that the said compounds have spasmodic and antibacterial effects. Khalili and Vahidi [25] have reported activity of *Zataria mtiultiflora* against S.entertis, S. dysenteriae and E.coli.

The results of MIC and MBC of EtOH and MeOH extracts of *Zataria multiflora* Boiss are summarized in table 3. The strongest activity of *Zataria multiflora* Boiss extracts was found against *Bacillus subtilis* ATCC 6633 (MIC 1.718 mg/ml) among all the bacterial strains under investigation. The range of MIC value for gram negative bacteria was 2.812-3.438 mg/ml whereas, for gram positive bacteria was 1.718-3.438 mg/ml.

The observed MIC and MBC values of combination of extracts (Table 4) confirmed the results of inhibitory effect presented in Table 2 by showing significantly (p=0.05 level) low values (synergistic effect) for *Staphylococcus aureus* ATCC-25923 & ATCC-29213. The MIC and MBC results of combination of extract also show significant (p=0.05) antagonistic effects against *Salmonella typhimurium* ATCC-14028 and *Pseudomonas aeruginosa* ATCC-27853 (Table 4).

One of the important finding of the study was that MIC were lesser than MBC values of extracts as well as their combination against some of the bacterial strains under investigation. These findings imply that although these extracts inhibit bacteria at lower concentration but higher concentrations are required for their elimination (Table 3&4). Abu-Shanab et al. [29] have also reported lower MIC than MBC values for ethanolic extracts of *Althaea officinalis*, *Mentha longifolia*, *Melissa officinalis* and *Rosa damascene* against *Staphylococcus aureus*.

The standard drug Cefoperazone was active against all the bacteria under investigation. The inhibition zones were in the range of 24.0-33.2 mm for gram negative bacteria and 22.4-27.0 mm. Cefoperazone demonstrated strongest activity against *E.coli* ATCC 8739 with MIC value 0.045 mg/ml. The MBC values were same as that of MIC values for all the bacteria.

CONCLUSION

The current studies provides the evidences for the presence of active and effective constituents in the alcoholic extracts of *Zataria multiflora* Boiss, those can inhibit as well as eliminate gram positive and negative bacteria.

The results of present study recommends that *Zataria multiflora* Boiss should be explored for its potential use in treatments of bacterial infectious diseases.

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