ORIGINAL ARTICLE

In Vitro Assessment of Antimicrobial Potential of Ethanolic and Aqueous Extract of *Phlomis Umbrosa* Against Some Highly Resistant Pathogens

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ABSTRACT

Objective: To find out the antibacterial potential of ethanolic and aqueous roots extract of *Phlomis umbrosa L.* against both Gram positive and Gram negative isolates

Methodology: Disk diffusion method according to Clinical Laboratory and Standards Institute (CLSI) standard was used to examine the in vitro antibacterial activity of *P. umbrosa* extract while minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using broth dilution technique. Miles and Misra technique was also utilized to count the number of colonies CFU/mL of bacteria at different concentrations of extract.

Results: All the studied strains showed a diverse range of vulnerability against both ethanolic and aqueous plant extract. Among all tested isolates, ethanolic extract of *P. umbrosa* showed highly significant activity against Gram positive isolates i.e. *S. aureus* (20.1 mm) and *B. subtilis* (22.9 mm) with least MIC (12.5 mg/mL) and MBC (12.5 mg/mL) as compared to Gram negative isolates. A progressive decline in bacterial colonies (CFU/mL) was observed in Miles and Misra technique. One way ANOVA followed by postHoc Tukey test showed the significant differences in antimicrobial activities of plant extract with two tested antibiotics i.e. Amoxicillin and Erythromycin (10 µg/disc) as positive control at p-value of 0.05. The antimicrobial activity of this plant exhibit may be due to the presence of such chemical constituents namely monoterpenoids and sesquiterpenoids compounds.

Conclusion: It is concluded that roots ethanolic extract of *P. umbrosa* has a promising antibacterial potential so it can also be used as an alternative medicine to treat different infections for reducing bacterial resistance and side effects associated with antibiotics.

Key words: *Phlomis umbrosa;* amoxicillin; erythromycin; antimicrobial activity; Miles and Misra method

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INTRODUCTION

For centuries, the use of medicinal plants as an essential source of medicinal agents for maintaining good health is well known and a number of novel compounds have been isolated from such plants[1]. Around 80% of population relies on traditional medicines primarily from herbal origins. Secondary metabolites as tannins, flavonoids, terpenoids, and alkaloids extracted from plants with high structure diversity possess strong antimicrobial significance[2].

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Moreover, rising credence is being given to the extraction and augmentation of distinct medicines from plants in modern culture[3]. Studies show that large populations heavily rely on medicinal plants to fulfill their primary health care needs[4].

Antimicrobial resistance (AMR) against pathogenic bacteria or multidrug resistant bacteria (MDR or superbugs), is a serious global threat for humans, animals, environmental health, and one of the major causes for endangering the worth of antibiotics. Reduction in financial inducement, inadequacy of newer drugs, over the counter availability, poor hygiene and sanitation, and misuse of antibiotics are attributable to the crisis of antimicrobial resistance. Comprehensive efforts are needed to minimize the pace of resistance by studying emergent microorganisms, resistance mechanisms, and antimicrobial agents[5].

This dilemma features the immediate demand for advance strategies and latest classes of antibiotics. In developing countries, plants are widely used as a source of medicine where traditional medicine plays a major role in primary healthcare. About 80% of individuals from these countries still use plants as remedies for many diseases, using their own personal recipes which have been passed down through generations. Clinical plant utilization provides a steady inspiration of bioactive antimicrobial agents with less toxicity, broad microbial coverage and good pharmacokinetics without chemical modification[6,7].

The genus *Phlomis* belonging to family *Lamiaceae* is a perennial herb. More than 100 species are reported from this genus widely distributed throughout Asia, Europe and Africa. Number of species are well known for their aromatic and medicinal function. Many species of this genus have usage for medicinal and aromatic purposes. Folk uses of different species of this plant include as stimulant, carminative, tonic, appetizer, antidiuretic, and in herbal tea. A number of biological activities are reported such as antimicrobial, anti-ulcerogenic, immunosuppressive and free radical scavenging, and anti-inflammatory[8,9].

Previously, several studies reported on screening of antimicrobial activities of several medicinal and traditional plants[10-12]. The antimicrobial activity of essential oil of flowers extract of *Phlomis umbrosa* was also evaluated[13]. However, according to the literature survey, no studies have been conducted on screening of antimicrobial potential of root extract of *P. umbrosa.* Therefore the aim of this study was to evaluate the antimicrobial activity of aqueous and ethanolic roots extract of *P. umbrosa* in comparison with standard marketed antibiotics.

METHODOLOGY

Collection of antimicrobial agents and chemicals
Dried powder roots extract of *Phlomis umbrosa* were recieved from a manufacturer and supplier M/S. HUNAN NUTRAMAX INC. (Changsha, China) with a batch number of PUE-160419 and was identified by a pharmacognosist and meritorious professor of University of Karachi, Pakistan. The standard antibiotics disc such as erythromycin and amoxicillin were purchased from distributor Musaji Adam and Sons (Karachi, Pakistan). Methanol was purchased from Sigma-Aldrich (St. Louis, USA). DMSO was procured from Merck (Darmstadt, Germany), nutrient agar and Mueller Hinton broth (MHB) were collected from Oxoid LTD (Hemisphere, England). Both antibiotics discs were of 10 µg.

Collection of different clinical and standard ATCC strains
Gram positive and Gram negative highly resistant clinical strains including *Bacillus subtilis* (MT 0250), *Staphylococcus aureus* (MT 0484), *Streptococcus pyogenes* (MT 0258), *Salmonella enterica* (MT 0691) were obtained from pathological laboratories of Dr. Essa's Laboratory and Kutiyana Memon Hospital in Karachi, Pakistan. ATCC standard cultures strains used in this study were *Bacillus subtilis* (ATCC 04262), *Staphylococcus aureus* (ATCC 08854), *Streptococcus pyogenes* (ATCC 10258), *and Salmonella enterica* (ATCC 10691). The received bacterial strains from different laboratories were identified by pathologists based on their cultural, morphological, and biochemical reactions.

Antibacterial activity
Inoculation of bacterial strain: Antibacterial activity against collected isolates was performed using well reputed disc diffusion method which was first reported in 1940. The Clinical Laboratory and Standards Institute (CLSI) have approved standards for testing of different clinical and highly resistant pathological strains[14]. Two different antibiotics (Amoxicillin 10 µg and Erythromycin 10 µg/disc) as positive control and *Phlomis umbrosa* ethanolic and aqueous extract disc (6 mm in diameter) were used to evaluate their antibacterial activity. The discs of plant extract were prepared using concentrations of (20 mg/mL, 30 mg/mL, 40 mg/mL and 50 mg/mL) dried powder of *P. umbrosa* obtained by both aqueous and alcoholic solvents. Whereas, Dimethyl sulfoxide (DMSO) was used as negative control in whole study because, it is considered as a non-toxic solvent[15]. These prepared discs were placed on the pre-inoculated Mueller Hinton Agar (MHA) plates with different collected bacterial cultures and were placed in incubator for 24 h at 37^{O}C.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The concentration of extract at which the growth of clinical isolates was inhibited is refered to as minimum inhibitory concentration (MIC) while the concentration used for complete killing of clinical isolate is known as minimum bactericidal concentration (MBC). The MIC and MBC of ethanolic and aqueous root extract of *P. umbrosa* were determined using broth dilution method[10]. As much as 100 µL crude root extract of plant was taken to prepare 50 mg/mL concentration, then further dilution was made using serial dilution method up to 0.5 mg/mL. Tween 20 was used to solubilize the different concentrations of plant extract in nutrient broth. Each bacterial strain was adjusted to the concentration of 1×10^8cfu/mL in

respective media. The plates of different bacterial strains were incubated at 37°C for 24 h. The growth of bacterial strains was examined by the turbidity found in their respective test tubes. The prepared broth culture was incubated at 37°C for 24 h in Tyramide Signal Amplification (TSA) system.

Miles and Misra method: The technique of Miles and Misra was used to examine the antibacterial potential of ethanolic and aqueous root extract of *P. umbrosa* at different concentrations by counting the number of colonies forming units (CFU) of bacteria after exposure to extract[16]. The test isolates suspension was prepared in pH 7 PBS buffer and the inoculums of tested organisms defined at 1.5×10^8 cells/ml using the 0.5 McFarland index.

Statistical analysis

All the above defined experiments were performed in triplicates. The obtained results of all experiments were presented as mean ± standard deviation (SD). Statistical software (SPSS version 23) was used to analyze the obtained data by applying one way ANOVA with Tukey's post Hoc Test at minimal level of significance of $p < 0.05$. Furthermore, Pearson correlation was also applied to evaluate the relationship between the concentrations of plant extract with their antimicrobial effects.

RESULTS

The extract of *Phlomis umbrosa* roots with ethanol and also in water was light brown in colour and exhibited great antibacterial activity against some highly resistant clinical pathogens including *B. subtilis, S. aureus, S. pyogenes* and *S. enterica*. The obtained antibacterial activities of ethanolic and aqueous extract of plant are dose dependent and found within ranges of (5.9 ± 1.074 mm to 22.6 ± 1.195 mm) in ethanolic and (4.0 ± 0.821 mm to 17.9 ± 0.995 mm) in aqueous extract at different concentration as presented in Table 1 and 2 respectively. Among all the tested clinical isolates, Gram positive isolates i.e. *S. aureus* and *B. subtilis* were highly susceptible to the ethanolic plant extract at 50 mg/mL i.e. 25.6 ± 2.009 and 24.8 ± 1.371 respectively. However, the obtained zones of inhibitions from plant extract, primarily ethanolic extract, were much greater and significant than two tested antibiotics. The susceptibility of ethanolic and aqueous extract of plant also tested antibiotics against some ATCC cultures presented in Table 3 and 4. The obtained values of ZIs were significantly different at different concentrations of ethanolic plant extract against clinical isolates i.e. *B. subtilis (P = 0.016); S. aureus (P = 0.021); S. pyogenes (P = 0.027)* and *S. enterica (P = 0.015)*. Moreover, highly significant differences (*P>0.005*) were found in ZIs of amoxicillin against *B. subtilis* and *S. enterica* while significant differences (*P>0.05*) were found in antibiotics against tested isolates compared to extract at 30 mg/mL concentration. The values of MIC and MBC of ethanolic root extract of *P. umbrosa* indicated the positive results in screening test of different clinical highly resistant microbes (Table 5). The lowest and same MIC and MBC values were observed against *B. subtilis* i.e. 12.5 mg/mL while the highest were against *S. enterica* as 20.0 mg/mL. The Miles and Misra test showed a gradual decrease in log of CFU/mL of bacteria with increasing concentration of both aqueous and ethanolic root extract of *P. umbrosa* as indicated in Figure 1. Among all tested organisms, log of CFU/mL of *S. aureus* and *B. subtilis* were decrease up to 3.7 and 3.9 respectively in ethanolic extract.

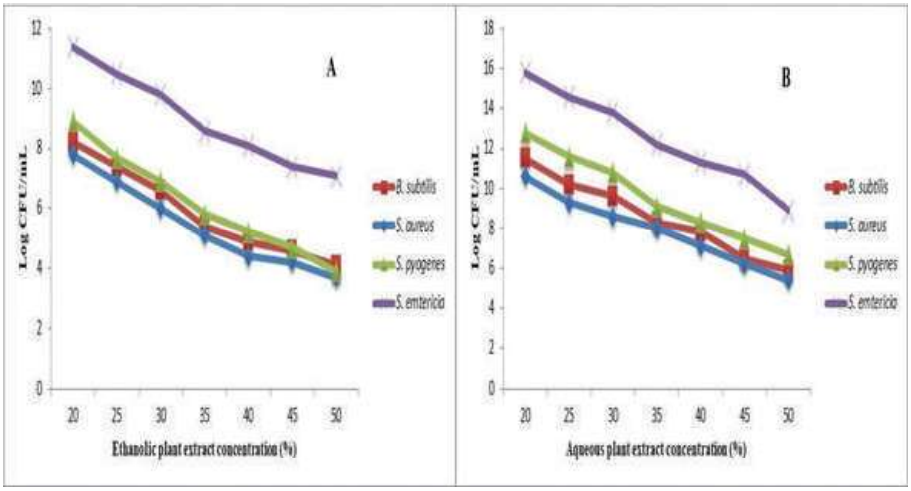

Figure 1: (A) Log of CFU/ml at different concentrations of ethanolic extract (B) Log of CFU/ml at different concentrations of aqueous extract

Table 1: Antibacterial Activity of Ethanolic Plant Extract and Different Antibiotics Against Clinical Isolates

Clinical isolates	Zone of inhibitions in mm							
	Plant extract (Ethanolic)						Standard drugs	
							Amoxicillin	Erythromycin
	20 mg/mL	30 mg/mL	40 mg/mL	50 mg/mL	Pearson correlation	P-value	10 µg/discs	10 µg/discs
B. subtilis	13.8 ± 1.326	15.5 ± 1.461	18.1 ± 0.513	20.1 ± 1.254	0.998	0.016	9.1 ± 1.224**	12.1 ± 1.724*
S. aureus	14.5 ± 0.862	17.0 ± 0.472	21.7 ± 1.316	22.9 ± 1.614	0.985	0.021	12.6 ± 1.375*	11.5 ± 1.215*
S. pyogenes	10.8 ± 1.246	14.2 ± 1.725	17.6 ± 1.258	20.7 ± 1.290	0.994	0.027	11.6 ± 1.224*	12.0 ± 1.552*
S. enterica	5.9 ± 1.514	8.5 ± 0.914	12.6 ± 1.413	15.7 ± 1.309	0.985	0.015	7.9 ± 1.256**	11.6 ± 0.646*

n=10, Average values ± SD
*p = 0.05 significant as compared to control
**p = 0.005 highly significant as compared to plant extract at concentrations of 50 mg/mL

Table 2: Antibacterial Activity of Aqueous Plant Extract and Different Antibiotics Against Clinical Isolates

Clinical isolates	Zone of inhibitions in mm							
	Plant extract (Aqueous)						Amoxicillin	Erythromycin
	20 mg/mL	30 mg/mL	40 mg/mL	50 mg/mL	Pearson correlation	P-value	10 µg/discs	10 µg/discs
B. subtilis	10.0 ± 1.248	12.2 ± 1.215	14.2 ± 1.214	15.6 ± 0.825	0.992	0.024	9.1 ± 0.813*	12.1 ± 1.226
S. aureus	9.4 ± 0.925	12.6 ± 1.425	15.0 ± 1.242	17.9 ± 0.635	0.993	0.014	12.6 ± 1.074	11.5 ± 0.673*
S. pyogenes	7.2 ± 0.725	8.7 ± 1.346	11.6 ± 1.220	13.0 ± 0.971	0.987	0.021	11.6 ± 1.577	12.0 ± 0.804
S. enterica	4.0 ± 0.901	5.9 ± 1.250	7.3 ± 1.457	9.1 ± 0.729	0.994	0.047	7.9 ± 0.703	11.6 ± 0.750*

n=10, Average values ± S.D
*p = 0.05 significant as compared to control
**p = 0.005 highly significant as compared to plant extract at concentrations of 50 mg/mL

Table 3: Antibacterial Activity of Ethanolic Plant Extract and Different Antibiotics Against Standard ATCC Strains

Clinical isolates	Zone of inhibitions in mm							
	Plant extract (Ethanolic)						Amoxicillin	Erythromycin
	20 mg/mL	30 mg/mL	40 mg/mL	50 mg/mL	Pearson correlation	P-value	10 µg/discs	10 µg/discs
B. subtilis (ATCC 04262)	16.3 ± 1.451	19.3 ± 1.126	21.6 ± 1.341	24.8 ± 1.157	0.996	0.014	16.8 ± 0.742*	20.4 ± 1.265
S. aureus (ATCC 08854)	18.2 ± 1.625	20.2 ± 1.134	22.4 ± 1.327	25.6 ± 1.709	0.994	0.021	19.4 ± 0.912*	22.3 ± 1.524
S. pyogenes (ATCC 10258)	13.2 ± 0.936	16.3 ± 1.615	19.7 ± 1.184	23.4 ± 1.525	0.991	0.018	17.3 ± 0.742*	22.0 ± 0.814
S. enterica (ATCC 10691)	8.4 ± 1.604	11.5 ± 1.260	14.0 ± 1.610	18.2 ± 0.723	0.988	0.009	14.7 ± 1.459	15.3 ± 1.230

n=10, Average values ± S.D
*p = 0.05 significant as compared to control
**p = 0.005 highly significant as compared to plant extract at concentrations of 50 mg/mL

Table 4: Antibacterial Activity of Aqueous Plant Extract and Different Antibiotics Against Standard ATCC Strains

Clinical isolates	Zone of inhibitions in mm							
	Plant extract(Aqueous)						Amoxicillin	Erythromycin
	20 mg/mL	30 mg/mL	40 mg/mL	50 mg/mL	Pearson correlation	P-value	10 µg/discs	10 µg/discs
B. subtilis (ATCC 04262)	12.3 ± 0.743	14.1 ± 1.424	16.8 ± 1.472	18.7 ± 1.273	0.995	0.012	16.8 ± 0.736	20.4 ± 1.363*
S. aureus (ATCC 08854)	10.5 ± 0.642	13.8 ± 0.757	16.8 ± 1.635	18.0 ± 1.356	0.988	0.026	19.4 ± 1.324	22.3 ± 1.642*
S. pyogenes (ATCC 10258)	8.6 ± 0.853	10.9 ± 0.681	13.0 ± 1.635	16.7 ± 1.635	0.986	0.024	17.3 ± 1.206	22.0 ± 0.752*
S. enterica (ATCC 10691)	6.2 ± 0.692	8.4 ± 1.624	9.9 ± 1.344	12.3 ± 0.843	0.992	0.031	14.7 ± 1.526	15.3 ± 1.532

n=10, Average values ± S.D
*p = 0.05 significant as compared to control
**p = 0.005 highly significant as compared to plant extract at concentrations of 50 mg/mL

Table 5: MIC and MBC of Ethanolic and Aqueous Extract Against Clinical Isolates

Clinical Isolates	Ethanolic Extract		Aqueous Extract	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
B. subtilis	12.5 ± 1.381	12.5 ± 1.513	15.0 ± 1.424	15.0 ± 1.753
S. aureus	12.5 ± 1.514	15.0 ± 1.361	17.5 ± 1.467	17.5 ± 1.646
S. pyogenes	15.0 ± 0.724	15.0 ± 1.525	20.0 ± 1.624	20.0 ± 1.632
S. enterica	20.0 ± 0.510	20.0 ± 1.424	25.0 ± 0.585	30.0 ± 0.745

n=10, Average values ± S.D

DISCUSSION

One of the most pressing issues around the world is anti-microbial resistance. In order to overcome this problem, there is an immediate need of new antimicrobial agents with novel systems of activity. Scientific strategies are being adopted based on continuous planning and processing by researchers to find out new antimicrobial agents with minimum side effects and maximum efficacy. In developing countries, traditional medicines are gaining importance persistently. A number of plant and herb species are available in the market with strong antimicrobial potential[17].

In the present study, the antibacterial potential of *Phlomis umbrosa* was determined using ethanolic and aqueous crude root extract at different concentrations (Table 1 and 2). It was observed that with the increase in concentration, sensitivity of organism towards sample also increased. Pearsons correlations was used to show dose dependent effect of plant extract on their antibacterial efficacy. The correlation values of Pearson correlations analysis also indicated the dose dependent antibacterial activities of plant extract in both ethanolic and aqueous medium.

However, ethanolic extract was found to be more effective compared to aqueous extract against Gram positive isolates than Gram negative isolates. Miscibility of ethanol with water is well known because of its low polarity compared to water and active microbial compounds present in plants are mostly saturated and non-polar. Lipophilic compounds are difficult to extract in water, while ethanol and methanol are good choices of solvents in such cases[18,19]. Earlier, Guang-hui et al studied the phytochemical components accounted for 91.53% of the all peak area in *P. umbrosa* flowers extract. They reported the antimicrobial activity of this plant occuring due to the presence of such chemical constituents as monoterpenoids and sesquiterpenoids compounds, the major components were toluene, phthalic acid, diisobutyl ester, á-linalool, diphenylamine, and 1-octen-3-ol[13].

A number of antibiotics reported resistance against these collected pathogenic strains. Literature illustrates that Penicillin G, Gentamycin, lincosamide and tetracycline show resistance pattern against these organisms. In case of both aqueous and ethanolic extracts, maximum activity was found against *Staphylococcus aureus* followed by *Bacillus subtilis* with zone of inhibition (22.9 ± 1.195) and (20.1 ± 0.755) at concentration of 50 mg/mL respectively. These findings are in line with the study performed by Hui et al., in 2008 on antimicrobial activity[20]. Moreover, Morteza-Semnani et al., in 2006, reported the antibacterial activity of *Phlomis* species against some highly resistant pathogen including *E. coli, K. pneumonia, S. aureus, S. sanguis*, and *P. aeruginosa*[21]. However, limited data is available on antimicrobial studies of this plant in Pakistan, but according to our study, this plant would be a promising future candidate against microbes.

S. enterica was least sensitive isolate against test sample except at 50 mg/mL with zone of inhibition (15.7 ± 1.419). Erythromycin (10 µg/discs) was more effective compared to amoxicillin (10 µg/discs) used as positive control. Results were also noticed against ATCC cultures and it was observed that standard cultures were more sensitive towards plant extract in comparison to clinical isolates. Mounting graph of resistance among clinical isolates may be contributed to by a number of reasons playing their role in lack of antimicrobial potential. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also determined (Table 5). Least value of MIC was analyzed against *S. aureus* (12.5 ± 1.011 mg/ml) with MBC value of 15.0 ± 1.254 mg/mL. ANOVA followed by post Tukey showed significant differences in antimicrobial activities of plant extract with two tested antibiotics.

The results of Miles and Misra technique also showed the inhibitory effect of ethanolic as well as aqueous extract of *P. umbrosa* against both tested clinical and standard ATCC cultures by lowering down the log of CFU/ml at increasing concentrations of plant extract. Inhibitory effect of this plant even at low doses also supports its use in folk medicine for not only the treatment of infectious diseases but also for other ailments. Therefore, it appears to be promising for treatment of bacterial infections. Activity directed bioassay along with extensive activity of bioactive compounds is required for further investigation of compounds actually responsible for antimicrobial potential.

CONCLUSION

Study showed that crude root extract of *P. umbrosa* have potent antimicrobial potential and produce both bacteriostatic and bactericidal effects. The low values of MIC and MBC reflected the antibacterial potency of this studied plant. It is recommended that systematic and focused researches are required to get new chemical, biological and pharmacological findings.

Authors' contributions: Dr Yousra Shafiq designed the initial study, searched related literature, collected data and conducted the study. Dr. Muhammad Arif Asghar designed the initial draft of manuscript, reviewed and made corrections. Dr Huma Ali worked on literature search, review and finalized results and discussion. Dr Saima Abedien reviewed the literature, and contributed to the discussion. Dr Ahad Abdul Rehman reviewed the study outcomes and conclusion. All authors contributed to the final manuscript.

Conflict of interest: No conflict of interest associated with this work

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