Antibacterial effect of ethanol and aqueous root extracts from *Glycyrrhiza glabra* (Licorice) against *Streptococcus pyogenes* isolated from throat infection

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**Abstract**

*Glycyrrhiza glabra* commonly known as Licorice is a soothing, moist and sweet herb. It has been used as medicine since ancient times by physicians and herbalists in many parts of the world including Pakistan. The aim of present study was to evaluate the antibacterial effect of Licorice on *Streptococcus pyogenes* isolated from throat infection (tonsillitis) among patients of tonsillitis ranging 4 months to 64 years of age. Two extraction methods were used in two different solvents, ethanol and distilled water. The antibacterial effect of aqueous and ethanol extracts was investigated using agar well diffusion method. Diameters of zone of inhibition were measured in millimeters. It was observed that *S. pyogenes* tonsillitis was more prevalent in age group 15-40 years. Ethanol extraction method was found superior to aqueous extraction. The aqueous and ethanol extracts of *G. glabra* showed statistically significant antibacterial activities against *S. pyogenes* isolates in both extracts where ethanol extracts were found two-fold more effective as compared to aqueous extracts (*p* < 0.05). These results indicate that the *G. glabra* have more active compounds (phytochemicals) against *S. pyogenes* soluble in ethanol than water and that could be used for remedial purpose.

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belongs to class Equisetopsida, subclass Magnoliidae, order Fabales, suborder Rosanae, family Liguminosae and the genus Glycyrrhiza (Valli and Giardina, 2002). The genus includes species G. uralensis, G. inflate and G. glabra (Shirazi et al., 2007). G. glabra is native to Eurasia, western Asia and northern Africa (Fukai et al., 2003), where it grows up to 1,200 m above sea level. Though antibacterial effects of G. glabra are well documented (Dhanya and Sidhu 2011; Irani et al., 2010), a few (Zaidi et al., 2013; Tanaka et al., 2001) antibacterial studies against S. pyogenes have been carried out so far.

We report the antibacterial activity from G. glabra Licorice aqueous and ethanol extract against clinical isolates of S. pyogenes. To the best of our knowledge, this is the first report to provide evidence of antibacterial activity of G. glabra extracts against clinically isolated indigenous human strains of S. pyogenes from the study area.

Objective of Research

The objective of this research was to investigate the antibacterial effect of Licorice aqueous and ethanol root extracts against Streptococcus pyogenes (S. pyogenes) isolated from throat infection.

Justification of Research

Licorice has been used traditionally as treatment of sore throat and tonsillitis in Pakistan. S. Progenies is one of the etiological agents causing tonsillitis in human therefore we attempted to investigate the effect of Licorice against the clinical isolates of S. progenies. The results will be a valuable addition to the data on medicinal plants and open the avenues for research on pharmaceutics.

2. Materials and Methods

The study was undertaken to investigate the effect of aqueous and ethanol extracts from Licorice root on clinical isolates of S. pyogenes from different age groups. The samples were collected from City Branch of Civil Hospital Khairpur Sindh Pakistan. The extracts (aqueous and ethanol) were prepared using standard extraction methods. The inhibitory effect was tested by agar well diffusion method and zone diameter of inhibition of S. pyogenes growth was measured in mm. Penicillin was used as positive control where extraction solvent was used as negative control. The results were interpreted and statistical significance was calculated using SPSS (version 10).

2.1 Study settings and procedures

Ethical clearance was obtained from the District ethical review committee. A total of 100 throat specimens (N=100) were collected from February 2013- January 2014 from the suspected patients of tonsillitis aged 4 months to 64 years with fever, sore throat, headache and tonsillar hyperemia. An informed verbal consent was sought (in case of children under 18 years, from their care takers). Sampling site was the OPD (out patient department) City Branch of Civil Hospital Khairpur and all the patients were examined by a physician. The samples were collected with sterile cotton swabs (Transwabs) using tongue depressor; kept in icebox and brought to the Microbiology research laboratory within 1h of collection and processed.

2.2 Inoculation of specimen

The specimens were inoculated on blood agar (Oxoid Ltd, Hampshire, UK) containing 5% defibrinated sheep blood and incubated aerobically for 24 h at 37° C. On the next day the plates were observed for growth of hemolytic colonies typical of S. progenies.

2.3 Gram staining

Under sterile condition the smear of selected β hemolytic colonies were made on a clean oil free glass slide, Gram-stained and observed under oil immersion objective (x100)

2.4 Isolation of pure culture

Only those colonies which were identified as Gram positive Streptococci and β hemolytic on blood agar were streaked on nutrient agar (Oxoid Ltd, Hampshire, UK) plates and incubated aerobically for 24 h at 37° C.

2.5 Identification of bacterial cultures

The strains of S. pyogenes were identified according to standard microbiological and biochemical techniques (Bergey’s Manual 2012) using Gram Staining, Capsule Staining, Blood Hemolysis, Bacitracin Sensitivity, Penicillin Sensitivity, Catalase test, Glucose fermentation, Galactose fermentation, and Xylose fermentation.

2.6 Preparation of plant extracts

2.6.1 Plant material

The plant material was purchased from local market and identified by centre for biodiversity and conservation herbarium Shah Abdul Latif University Khairpur. The Licorice root was
washed with distilled water to remove physical impurities and dried in shade. After complete drying, the plant material was ground to powder in grinder (Anex, Germany) then stored in an air tight sterile jar.

2.6.2 Aqueous extraction
The aqueous extracts were obtained using percolation technique (Nair and Chandra, 2004, Nair et al., 2005). A 25 grams Licorice powder was boiled separately with 500ml of Distilled water for 6 h till the volume reached to 100ml. The extract then filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 minutes at room temperature. The supernatant was collected. Finally 25g was extracted in 100ml of distilled water giving a concentration of 25%. It was then autoclaved (121° C and 15 lbs pressure) and stored at 4° C.

2.6.3 Ethanol extraction
Ethanol extract was obtained using maceration technique (Ekpo and Etim, 2009). A 10g of licorice powder was measured and placed into sterile conical flasks, homogenized with 30ml of distilled water and 70ml of absolute ethanol was added (ethanol/water ratio = 70:30, v/v). The air tight flask was placed on shaking incubator at 360 rpm for 72h at 25° C. After 72h the contents were centrifuged at 5000rpm for 15 minutes at room temperature and supernatant was collected and stored at 4° C in airtight sterile bottle.

2.7 Preparation of Bacterial suspension of S. pyogenes strain
Pure culture of each bacterial strain was grown in nutrient broth at 37° C overnight. McFarland’s turbidity standard scale of 0.5 was prepared to give a turbid solution (NCCLS, 2011). Normal saline was used to make a turbid suspension of the S. pyogenes strains, diluted in sterile normal saline until the turbidity matched that of McFarland’s 0.5 scales by visual comparison; at that point suspension had a concentration of bacterial suspension approximately 1.5x 10⁸ CFU/mL.

2.8 Agar well diffusion method (Ahmed and Baig 2001)
The susceptibility of the test bacteria to plant extracts was determined using agar well diffusion method on Mueller-Hinton agar plates, following the method described in NCCLS manual (NCCLS, 2003). Diluted bacterial cultures were adjusted to a 0.5 McFarland turbidity standard (1.5 x 108 CFU/mL) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 min under laminar air flow system at room temperature before wells were cut into the agar using sterile cork-borer (8mm). A100µl, 50µl and 30µl of the extract were introduced into each labeled respective wells. The plates were incubated aerobically at 37° C for 24h. Anti microbial effect was determined by measuring the diameter of the zones of inhibition of microbial growth in mm. For each bacterial strain, the control was maintained where pure solvent was used instead of extract as negative control and Penicillin (10 units) as positive control.

2.9 Statistical analysis
Experiments were carried out in triplicates. Results were expressed as mean ± standard errors of mean and examined for significance using unpaired two- tailed Hest (SPSS version 10) for differences of means of zone of inhibitions against aqueous and ethanol extracts.

3. Results
3.1 Isolation of S. pyogenes from throat specimens
On blood agar, three types of colonies were observed 1) non hemolytic, 2) γ hemolytic 3) β hemolytic. Only β hemolytic colonies were selected for Gram staining because the organism of our interest was β hemolytic S. pyogenes. A total of 08 confirmed strains of S. pyogenes were isolated from 100 throat specimens of tonsillitis. They were designated as Sp 9, 24, 25, 28, 39, 45, 59 and 66. All strains except 24 and 25 grown well when sub-cultured on nutrient agar where the strain 24 and 25 were exceptionally slow growing therefore these strains were abandoned. The results of Gram staining and hemolysis are shown in Figure 1 and 2.

Age-wise distribution of tonsillitis
The prevalence of streptococcal tonsillitis was determined in the population (N=100). The test population was divided in three groups and positive cases of Streptococcal tonsillitis were evaluated on age wise bases. It was observed that the tonsillitis was more prevalent in adults (as compared to children and elderly). The data is depicted in Figure 3.

3.2 Agar well diffusion assay
All the strains showed sensitivity against Licorice extracts in dose-dependent manner. The strains however differed in their degree of inhibition and a varying degree of sensitivity was noticed among the strains of S. pyogenes.
Highest sensitivity against aqueous Licorice extract was showed by strain 66 (16.6 mm zone size). The dose dependent sensitivity of all the strains was found approximately twofold in Licorice ethanol extract (Figure 4). Highest sensitivity was again showed by strain 66 (30 mm zone size). The negative controls (distilled water and ethanol) did not exhibit any antimicrobial activity whereas zone of inhibition against Penicillin (positive control) was observed in all the strains tested. The P-value from two-tailed t was <0.05 and difference was statistically significant. The results are summarized in Table 1 and 2.

4. Discussion

As shown in the results, the extracts of Glycyrrhiza glabra exhibited a strong activity against clinical isolates of S. pyogenes from throat specimens. Several reports are available on the antibacterial activity of Glycyrrhiza against Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, E. coli (Nitalikar et al., 2010), while aqueous extracts of Glycyrrhiza were applied on B. subtilis, S. aureus, P. aeruginosa and E. coli and significant antibacterial potential was found in each case (Patil et al., 2009). Highest activity against S. pyogenes exhibited by licoricidin has been reported (Tanaka et al., 2001).

Figure 1: Isolation and identification of β hemolytic S. pyogenes on blood agar and Gram stain reaction

Figure 2: β hemolytic S. pyogenes on blood agar

This figure shows β hemolysis of blood agar by the S. pyogenes isolated in present study.
Figure 3: Age wise distribution of Streptococcal Tonsillitis in the study population. The samples were collected and processed as described in material and methods.

This figure shows age wise distribution of tonsillitis in the study population. The samples were collected and processed as described in material and methods.

Figure 4: Dose-dependent sensitivity of Licorice ethanol extract against S. pyogenes strain 66

The figure shows the dose dependent antibacterial effect of Licorice ethanol extract. The inhibitory effect (wells in orange color) was observed and zone diameter measured using well diffusion method as described in material and methods.

In present study, significantly higher antibacterial activity was observed in ethanol extracts, whereas activity observed in case of aqueous extract was also prominent. Ates et al. (2003) have reported a range of antibacterial activities (7-11 mm/20 µL inhibition zone) of the alcohol, acetone, and chloroform extracts of Glycyrrhiza glabra roots against the microorganisms tested; the alcohol extracts inhibited B. cereus, K. pneumoniae, S. aureus; the acetone extracts inhibited B. cereus, B. subtilis, K. pneumoniae, S. aureus; the chloroform extracts showed inhibition effect against B. cereus, B. subtilis, E. faecalis, K. pneumoniae, S. aureus. In a related investigation, Zaidi et al., (2013) reported that aqueous extract of Licorice roots inhibited the growth of S. pyogenes. In the present study, we report that extraction with ethanol appeared to be more effective as compared to water ($P< 0.05$). In a study on extraction solvents for extracting phytochemicals Glycyrrhizic acid (GA) and glabridin from Licorice, GA showed the highest extracted amount by water while glabridin was easily extracted by ethanol and it showed higher extracted amount by ethanol (Tian et al., 2008). This indicates that glabridin extracted by ethanol in our study appeared to have showed enhanced anti-S. pyogenes activity where GA extracted by water also possessed...
anti-S. pyogenes action. These extracts shall provide an effective and safe alternative to multidrug resistant S. pyogenes throat infections and could be used in development of oral mouth washes and gargle preparations.

**Conclusion**

To sum up the whole work it was concluded that herbs have substantial antimicrobial compounds that could be used for remedial purpose, as supportive therapy in MDR tonsillitis infection.

Throat infection was more prevalent in the age group of 15-40 years.

The Licorice root showed significant in vitro anti-S. pyogenes effect by agar well diffusion method.

Ethanol extraction method was found superior to aqueous extraction that indicate the G. glabra have more active compounds (phytochemicals) against S. pyogenes soluble in ethanol than water.

To the best of our knowledge, this is the first report of antibacterial effect of ethanol root extract from G. glabra against human strains of S. pyogenes.

Our study substantiates previous findings regarding antibacterial effects of Licorice against diverse species of microorganisms including Streptococci. Our findings further extend the data and report that the ethanol extracts appeared to be more effective than the aqueous extracts of Licorice against S. pyogenes. Further studies aimed at the identification of substances producing the antibacterial effect are planned.

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**Table: 1 Effect of Licorice root aqueous extract by well diffusion method**

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Key: A = Aqueous (D/water= negative control), Penicillin (Positive control)
L.A = Licorice Root Aqueous Extract, D/w= distilled water (negative control)
Mean = Arithmetic mean, SEM = Standard error of the mean

**Table: 2 Effect of Licorice root ethanol extract by well diffusion method**

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Research Highlights

In this study area, tonsillitis is more prevalent in age group 15-40 years than children and elderly.

The Licorice root has significant antibacterial effect against clinical isolates of *S. pyogenes*

Ethanol extract have two fold higher antibacterial effects than aqueous extract against the clinical isolates of *S. pyogenes*

Limitations

Our study has some limitations. The test population has been randomly selected from the local hospital where the data for antibiotic treatment, previous to the sampling was unavailable. The less number of *S. pyogenes* isolated in the present study could have been due to the reason of antibiotic treatment.

Recommendations

The findings of our research provide the evidence for use of medicinal plants as alternative medicine. It is recommended that the health policy makers should take interest in facilitating the research and development in phytomedicine. Drug regulatory authorities should strengthen and encourage evidence-based research on traditional medicines through clinical trials on selected medicinal plants

Funding and Policy Aspects

The *Unani* Tib (herbal medicines) system has been accepted and integrated into the national health system. Pakistan is the only country in the eastern Mediterranean region where formal *Unani* teaching institutions are registered. There has been momentous progress at the policy level in terms of regulation. The government of Pakistan has in place a number of organizations such as W.H.O aimed at strengthening and coordinating various aspects of the medicinal plants. However, stronger organization at the national level under a strategic plan is essential. There is a definite need to design training and capacity building programs; implement a national policy on traditional medicines and to raise awareness on the implication of development of necessary legislation in response to it. We are planning to seek funds to identify phytochemicals from Licorice and perform molecular level study to find target genes of *S. pyogenes* responding to these phytochemicals.

Authors' Contribution and Competing Interests

Kazi Yasmeen Faiz conceived the research idea supervised the entire research and prepared this manuscript.

Khatoon Safia performed the experiments

Kumar Pardeep performed the statistics of the experiments

Qazi Nasreen interpreted the results and helped in preparation of manuscript.

The authors declare that they have no competing interests.

Acknowledgments

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Conflict of interest

The authors have no conflict of interests

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