Evaluation of Root and Leaf Extracts of Glycrriza Glabra for Antimicrobial Activity

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***Abstract*—*Glycyrrhiza glabra* is a traditional herb which grows in various parts of the world. It is a very sweet, moist, soothing herb that detoxifies and protects the liver. It also has a powerful anti-inflammatory action and also finds applications in arthritis and mouth ulcers. In the present study, antibacterial activity of *Glycyrrhiza glabra* root and leaf extracts were tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Entereobacter cloacae* and *Klebsiella* sp. using agar well/disc diffusion assay. The extracts obtained by cold maceration method and Soxhlet method could not inhibit the test bacteria. Both the leaf and root extracts prepared using reflux extraction method exhibited strong antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*. However these were found to be ineffective against *Enterobacter cloacae* and *Klebsiella* sp. The MIC of *Glycyrrhiza glabra* roots were 3µg/ml and 1µg/ml for *E. coli* and *P. aeruginosa*, respectively. The phytochemical analysis revealed the presence of tannins in leaf and root extracts and the concentration was found to be 3.161 and 2.455mg/ml, respectively at 530nm. This could be the possible explanation for the inhibitory effect of the extracts against the test organisms.**

***Index Terms*—anticmicrobial activity, glycyrrhiza glabra, MIC, extract, zone**

I. Introduction

Nature has been a source of a variety of medicinal remedies since human existence on earth and an equally impressive number of modern drugs have been isolated from natural sources, many based on their uses in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world. In fact, plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Higher plants, as a source of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times [1]. The World Health Organization (2003) has estimated that 80% of the populations of developing countries being unable to afford pharmaceutical drugs and thus, rely on traditional medicines. Use of herbal medicines in Asia represents a long history of human interactions with the environment. In recent years, apart from increasing infections, antibiotic resistance has becomes a chief therapeutic crisis world-wide. This has led to an increased thrust towards identification of novel antimicrobial agents from the natural sources. Besides, globally there is a patient - driven drift towards "natural remedies". This is due to increased awareness of the limited ability of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from the plant kingdom. This burgeoning worldwide interest in medicinal plants reflects recognition of the validity of many traditional claims regarding the value of natural products in health care [2].

India has a rich flora that is widely distributed throughout the country. Herbal medicines have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [3]. Several plant species are used for the treatment of various ailments ranging from minor infections to dysentery, skin diseases, asthma, malaria and a horde of other infections [4].

Glycyrrhiza glabra is a traditional herb which grows in various parts of the world. The term Glycyrrhiza has been derived from ancient Greek word glykos, meaning sweet and rhiza, meaning root [5]. *Glycyrrhiza glabra*, also known as liquorice and sweet wood, is native to the Mediterranean and Middle East. It was one of the most widely known medicines in ancient history, and records of its use include Assyrian tablets of around 2000 BC [6]. *Glycyrrhiza* is part of both western and eastern herbal traditions. In the traditional system of medicine, the roots and rhizomes of *Glycyrrhiza glabra* (family: Leguminosae) have been in clinical use. *Glycyrrhiza glabra* consists of polysaccharides, flavonoids, triterpene, saponins, pectins, simple sugars, mineral salts, amino acids, and various other substances [7]. Glycyrrhizin, a triterpenoid compound, accounts for the sweet taste of licorice root. Traditional uses of *Glycyrrhiza glabra* include the treatment of asthma, pharyngitis, malaria, arthritis, abdominal pain, and infections [8]. *Glycyrrhiza glabra* roots have anti-ulcer, anti-inflammatory [9], antioxidant [10], expectorant, diuretic, laxative, and sedative [11] properties. They also possess antipyretic, antiherpes, antiviral, antimicrobial and anxiolytic activities. [7], [12], [13].*Glycyrrhiza* glabra has also been

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shown to have a direct hepatoprotective and free-radical quenching effect [14]. *Glycyrrhiza* exerts antiviral activity *in vitro* toward a number of viruses, including hepatitis A [15], varicellazoster [16], HIV [17], herpes simplex type 1, Newcastle disease, and vesicular stomatitis viruses [13]. It has been suggested that *Glycyrrhiza* should not be used in patients with a history of hypertension, renal failure, or current use of cardiac glycosides [18].

The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection fighting strategies [19], [20]. Thus new prototype antimicrobial agents are needed to address this situation. In the present investigation, the leaf and root extracts of *Glycyrrhiza glabra* prepared by various extraction processes using different solvents was screened for potential antibacterial activity against both gram positive and gram negative strains of bacteria.

II. Material and Methods

A. Plant Material

Leaves and roots of *Glycyrrhiza glabra* were obtained from Medicinal Plant Nursery, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Plant leaves and roots were washed thoroughly under running tap water followed by a wash with sterile water, dried in oven, powdered and used for extraction. The solvents used for the extraction were water and methanol.

B. Bacterial Strains

The test organisms used in this study were *Escherichia coli*, *Pseudomonas aeruginosa*, *Entereobacter cloacae* and *Klebsiella* sp. *Escherichia coli* and *Pseudomonas aeruginosa* (MTCC 1688) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, *Enterobacter cloacae* and *Klebsiella* sp. were obtained from Genei, Bangalore and Mangalam Laboratory, Hisar, respectively.

C. Preparation of Extracts (Aqueous/Organic Solvent)

Three different methods employed for extraction were cold maceration method, soxhlet extraction and reflux extraction. In routine the plant samples (leaf and root) were dried in oven for 15 days at 45 ℃ and powdered using pestle and mortar. For cold maceration method, 45g of the powder was placed in 450ml of 80% ethanol (Qualigens Chemical, India)/water in a conical flask, plugged with cotton and then kept on a rotary shaker at 180-200rpm for 72h. After 72h, the extract was filtered. The final dried extract (600mg) was dissolved in 500 µl of Dimethylsulfoxide (DMSO, S. D. Fine Chemicals Ltd., India). For soxhlet extraction, 200g of the sample powder was extracted using solvent (750ml methanol/water) at 65 ℃ for 16h. The extracted sample was dried using distillation or in water bath at 80 ℃. The final dried extract (2.5g) was dissolved in 1ml DMSO. During reflux extraction, 60g of the powder was refluxed in 300ml methanol/water at 65 ℃ for 8h. After 8h, the extract was filtered and the residue was refluxed again. The procedure was repeated 3 times. All the filtrates were collected and the solvent was evaporated in water bath at 80 ℃. The final dried extract (7670mg) was dissolved in 9ml DMSO. All the extracts finally dissolved in DMSO, were stored at 4 ℃ and subjected to antibacterial activity assay.

D. Bacterial Cultures

Nutrient broth (Hi Media) was used for liquid culture and nutrient agar (Hi Media) for solid. 13.5g of nutrient broth was dissolved in 1000ml distilled water (pH 7.4 ±0.2). All solutions were sterilised in an autoclave for 15min at 121 ℃. A loopful of cultures were streaked on nutrient agar plates and incubated at 37 ℃ for 24h for appearance of discrete colonies for diffusion assays whereas for growth curve studies in liquid cultures single colonies were inoculated in 5ml nutrient broth and allowed to grow for 18h (overnight) at 37 ℃ in a rotary shaker at 180rpm.

E. Assays to Determine Antimicrobial Activity and Minimum Inhibitory Concentration (MIC)

The screening for antimicrobial activity of *Glycyrrhiza glabra* extract (GGE) was performed by three methods, agar disc diffusion method, agar well diffusion method as well as by studying effect of extracts on the growth of the bacterial strains in liquid cultures. In routine a suspension of (100 µl) bacterial culture grown in nutrient broth for 18h was used for seeding the nutrient agar to form a lawn. For agar disc diffusion method, the discs (5mm) were saturated with 50 µl of the GGE (test plant root/leaf extract) then allowed to dry and were finally placed on the upper layer of the seeded agar plate. For agar well diffusion method, a well was cut in to the plates with the help of a sterile 200 µl pipette tip. 50 µl of the GGE was introduced into the well. The plates were incubated overnight at 37 ℃. Antibacterial effect of the extract on microbial growth was determined by measuring the diameter of clear zone around the well/disc (zone of inhibition). For each bacterial strain, negative and positive controls were maintained where 12.5 µl of 200 µg/µl ampicillin (as positive control) and pure solvent (DMSO) or empty well (as negative control) were used instead of the GGE in the well/disc on same seeded plate. To study the effect of GGE on growth of bacterial strains in liquid cultures, the growth curves (OD 600nm was measured at an interval of 1-2 h after the addition of the test and control samples in the log phase bacterial cultures) for each individual cultures along with all control cultures were monitored. The MIC value of GGE against *E. coli* and *P. aeruginosa* MTCC1688 was measured using decreasing concentrations of GGE in the range of 2.50mg/ml to 0.125mg/ml with the help of agar disc diffusion assay. All the values reported in this paper are the average of a minimum of three sets of experiments performed independently.

III. Results and Discussion

The antibacterial activity of *Glycyrrhiza glabra* root and leaf extracts was initially evaluated by the agar well diffusion assay. Among the three different extraction methods namely, cold maceration method, soxhlet extraction and reflux extraction that were employed for the extraction of bioactive components from the leaves and roots of the *Glycyrrhiza glabra*, the reflux extraction was found the best. Both the leaf and root extracts prepared using reflux extraction method exhibited strong antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*. However these were found to be ineffective against *Enterobacter cloacae* and *Klebsiella* sp. Taking into account that the two strains growth of which were unaffected upon treatment with *Glycyrrhiza glabra* leaf and root extracts were clinical isolates, it is possible that these strains could be the resistant/multi drug resistant ones. The results of the well diffusion assay are shown in Table I. The susceptible bacteria were again tested by disc diffusion assay and the results are shown in Table II.

TABLE I. ANTIBACTERIAL ACTIVITY OF *GLYCYRRHIZA GLABRA* LEAVES AND ROOTS EXTRACTS (WELL DIFFUSION ASSAY)

Bacteria	**Zone of inhibition (mm)**			
	Leaves	**Roots**	**Ampicillin**	**Negative control**
Escherichia coli	23	22	13	0
Pseudomonas aeruginosa	22	20	13	0
Enterobacter cloacae	0	0	12	0
***Klebsiella* sp.**	0	0	11	0

TABLE II. ANTIBACTERIAL ACTIVITY OF *GLYCYRRHIZA GLABRA* ROOTS AND LEAVES (DISC DIFFUSION ASSAY)

Bacteria	**Zone of inhibition (mm)**			
	Leaves	**Roots**	**Ampicillin**	**Negative control**
Escherichia coli	13	14	11	0
Pseudomonas aeruginosa	11	10	12	0

The extracts were diluted and minimal inhibitory concentration was determined. The MIC of *Glycyrrhiza glabra* leaf extract was found to be 1 µg/ml for both *E. coli* and *P. aeruginosa*. The MIC of *Glycyrrhiza glabra* root extract was 3 µg/ml and 1 µg/ml for *E. coli* and *P. aeruginosa*, respectively. The inhibition zones at different concentrations are shown in Table III. The size of the inhibition zone for the same bacterium may be influenced by multiple factors like, diffusion capacity of bioactive components (present in the extracts) in the agar medium, antimicrobial activity of diffused substances, pH of substrates in plates, growth and metabolic activity of microorganisms in the medium etc. Therefore the inhibitory activity might not necessarily be proportional to the inhibition zone diameter, especially when comparing different extracts but it still can safely be considered as one of the best and efficient method of identifying bioactive components from crude plant extracts [21].

TABLE III. ESTIMATION OF MINIMAL INHIBITORY CONCENTRATION (MIC) OF *GLYCYRRHIZA GLABRA* ROOT AND LEAF EXTRACTS

Concentration (µg/ml)	**Zone of inhibition (mm)**			
	Root Extract		**Leaf Extract**	
	E. coli	***P. aeruginosa***	***E. coli***	***P. aeruginosa***
2000	22	20	23	22
1500	21	19	22.5	21
1000	20	18	22	20.5
500	17.5	17.5	20.5	20
400	17	17	20	19.5
200	16.5	16.5	19.5	18.5
100	16	16	19	18
50	15	15.5	18	17
25	13	15	16	16
20	12.5	14	13.5	14.5
5	12	13.5	13	14
3	12	11.5	12.5	12
1	0	9	12	11
0.2	0	0	0	0

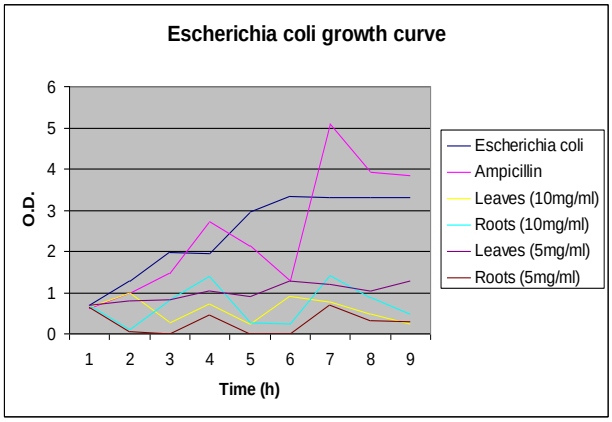

Figure 1. *E. coli* growth curve

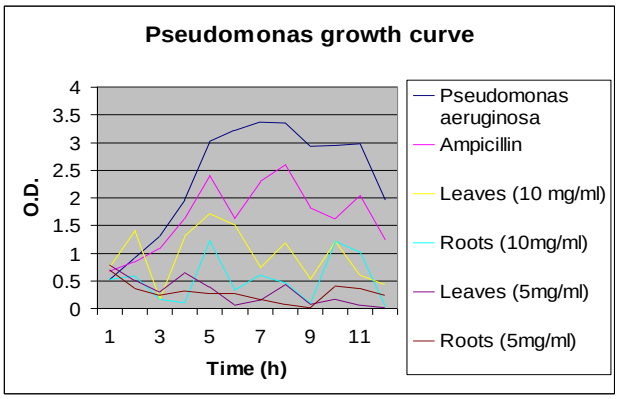

Figure 2. *P. aeruginosa* growth curve

Finally, the results obtained from agar disc/well diffusion assay were corroborated by the inhibitory effects of *Glycyrrhiza glabra* extracts (at concentration 10mg/ml and 5mg/ml) as observed on the growth curves of the liquid cultures of susceptible bacterial strains *E. coli* and *P. aeruginosa*, respectively (Fig. 1 & Fig. 2). The growth inhibition patterns observed for bacteria in liquid culture also suggests that *Glycyrrhiza glabra* extracts at a concentration of 5-10mg/ml is primarily bacteriostatic in nature and not bacteriocidal in which

case the OD 600 should have becomes static and never rise again. The reproducibility of the results in liquid culture proves the efficacy of the extracts. In case of *P. aeruginosa*, the 5mg/ml concentration of GGE was found more inhibitory to the growth of bacteria as compared to 10mg/ml concentration. This could be due to the fact that the extracts used were not pure and crude preparations were tested. So some compounds working antagonistically to the biologically active compounds might also be present. The reduction in concentration of such compounds may lead to better results at lower concentration.

The extracts were also screened for the presence of tannins using the method described by Haggerman and Butler [22]. Both the extracts showed positive results for tannins. The concentration of tannin was found to be 3.161 and 2.455mg/ml for leaf and root extracts respectively. Tannins have been found to form irreversible complexes with proline rich proteins. This could be the possible explanation for the inhibitory effect of the extracts against the test organisms.

The microorganisms selected for this study are important entero-pathogenic bacteria. All the test bacteria selected were gram negative. Considering the fact that the most of the plant antibiotic substances appear to be more inhibitory to gram positive organisms than to the gram negative type, it may also be taken into the account that penicillin and some of the other prominent antibiotic agents of fungal origin are also rather selective in their inhibitory action, most of them being inhibitory to gram positive organisms. *P. aeruginosa* is an important pathogen with high resistance to different compounds and also several antimicrobials. Infections caused by P. *aeruginosa* are among the most difficult to treat with conventional antibiotics [23]. The growth of *P. aeruginosa* was completely inhibited by both the test extracts. These extracts may thus be a source which could yield drugs that could improve the treatment of infections caused by this organism.

The well diffusion assay shows that the leaf extracts (2mg/ml) were more active against both *E. coli* and *P. aeruginosa* showing 23mm and 22mm inhibition zones, respectively. On the other hand, disc diffusion assay data indicates that roots were more effective against *E. coli* and leaves against *P. aeruginosa*. The minimal inhibitory concentration (MIC) of the extracts was found to be very low. The MIC of 1 µg/ml proves that the extracts were effective even at a concentration as low as 1 µg/ml. Such a low MIC value is an indicator of the high antimicrobial activity of the extracts.

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