

Effect of microbial load on therapeutically active constituent glycyrrhizin of *Glycyrrhiza glabra* L.

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Contamination of herbal drugs by microbes is a very common phenomenon as many reports are available, but what alteration occurs on secondary metabolites of plants due to these has very few records. In the present study, it has been established that the concentration of glycyrrhizin (active constituent) decreases by microbial contamination. The contamination of ten fungal species, viz. *Aspergillus niger* Tiegh., *A. flavus* Link, *A. fumigates* Fresenius, *A. sydowii* Thom & Church, *Penicillium chrysogenum* Thom, *P. purpurogenum* Stoll, *Alternaria alternata* (Fr.) Keissl., *Cladosporium sphaerospermum* Penz., *Absidia corymbifera* Sacc. & Trott., *Rhizopus oryzae* Went & Prins. Geerl. and three bacteria, viz. *Escherichia coli* (Migula) Castell. & Chalm., *Pseudomonas aeruginosa* (Schroeter) Migula and *Salmonella* Lignieres has been perceived in the samples of liquorice. Besides, a correlation between moisture, sugar, starch and microbial load has also been established. It was found that the sample having maximum moisture percentage had heavy microbial load. On the contrary, sugar and starch percentage were minimum in the same sample.

Keywords: Glycyrrhizin, Herbal drugs, Liquorice, Microbial contamination, HPTLC

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Despite the rapid growth of the herbal drug sector, there is a lack of sufficient awareness about the quality, safety and efficacy of the herbal drugs and their products. In general, medicinal plants may be associated with various kinds of microbial contaminants, in which bacterial and fungal infections are regarded as the two dominating groups, found either in or on the plant material¹⁻⁴. These microorganisms, including pathogens, limit the utilization of these plants^{2,5}. According to World Health Organization (WHO)⁶ and European Pharmacopoeia⁷, herbal drugs must meet the modern hygienic standards, which aim at low microbial load or the absence of pathogenic microorganisms. However, due to post harvest techniques and faulty storage conditions, the microbial load cannot be excluded and the herbal drugs may be infected by a number of saprophytic and pathogenic microorganisms. The presence of *Escherichia coli* (Migula) Castellani & Chalmers, *Salmonella* Lignieres spp. and moulds may indicate poor quality of production and harvesting practices⁶. Thus, the evaluation of microbial

contamination has increasingly become an integral part of quality control of herbal drugs.

The therapeutic efficacy of plants depends on their active constituents which are effective against the specific ailments. In the case of high microbial load in plants it may be possible that active constituents of the plants may deteriorate. The contamination of herbal drugs by microbes is a very common phenomenon as many reports are available on the occurrence of microbial contamination in herbal drugs⁸⁻¹⁰ but very little work has been carried out on the effect of microbial load on the active constituents of the medicinal plants. Efforts are being made by our group to develop TLC/HPTLC methods for qualitative and quantitative estimation of chemical constituents¹¹⁻¹⁴ in herbal drugs/formulations.

G. glabra L. (Fabaceae) is the principal source of commercial drug Liquorice. The drug consists of peeled or unpeeled roots and stolon, having faint odour and sweet taste almost free from bitterness. It contains triterpenoid saponin- Glycyrrhizin (2-9%)¹⁵ - a mixture of potassium and calcium salts of glycyrrhizic (glycyrrhizinic) acid¹⁶ and other minor constituents. The drug possesses potent demulcent,

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expectorant and anti-inflammatory properties and these are attributed to glycyrrhizin, which is 50 times sweeter than sucrose¹⁷. Besides, Glycyrrhizin is also credited with antihepatotoxic activity¹⁸. Recognition of deoxycorticosterone effects of liquorice extracts and glycyrrhetic acid has led to its use for the treatment of rheumatoid arthritis, Addison's disease¹⁹. The flavonoid component of root possess antimicrobial activity, beneficial in the treatment of peptic ulcer and also effective against gastric and duodenal ulcers¹⁹.

Keeping this in view, the present investigation has been undertaken with the aim to investigate microbial status of an important plant *Glycyrrhiza glabra* L., used in various preparations in Ayurveda and other indigenous systems of medicines and to observe the effect of microbes on its therapeutically important constituent- glycyrrhizin.

Methodology

Sample preparation

Samples of *Glycyrrhiza glabra* were procured from four different drug markets of the country, viz. Delhi (Sample I), Dehradun (Sample II), Kanpur (Sample III) and Lucknow (Sample IV) and their Botanical identity was confirmed by matching pharmacognostically with the genuine roots of *G. glabra*. Drug samples were initially washed thoroughly with fresh running water to remove dust and possible surface contaminants and then dried in an oven at 45°C. Moisture content was determined by placing samples in an oven at 110°C for four hours until weight was stabilized⁶. Sugar and starch were estimated by the method of Mont Gomery²⁰.

Fungal load

For fungal load, 100 replicates of each sample were sterilized by 2% sodium hypochlorite solution for 10 minutes and washed three times with sterilized distilled water. Thin sections of these samples (approximately 1 cm²) were placed on petri dishes containing (a) Three layered moist blotter paper, (b) 2% agar media without any nutrients, (c) Potato Dextrose Agar (PDA, HIMEDIA) containing 0.5 mg chloramphenicol/ml (HIMEDIA) for suppressing the bacterial growth. These plates were incubated at 25°C for seven days and samples were examined daily after fourth day for fungal growth. The fungi were isolated, purified and identified. The identity of fungal species was confirmed from Indian Agricultural Research Institute, New Delhi, India.

Bacterial load

WHO⁶ guidelines were used for the assessment of bacterial load and their identity was confirmed with different biochemical methods described in the aforesaid document.

Total aerobic count and coliform count were determined by using MPN (Most Probable Number) technique on soybean casein digest medium (HIMEDIA) and Enterobacteriaceae enrichment broth (HIMEDIA), respectively.

For *Escherichia coli*, homogenized pretreated plant material was transferred to Mac-Conkey broth, incubated at 43-45°C for 18-24 hrs. Broth was then transferred to Mac-Conkey agar (HIMEDIA), incubated at 43-45°C for 18-24 hrs. Presence of *E. coli* was confirmed by *Indole test* using Kovac's reagent⁶.

For *Salmonella*, homogenized pretreated plant material was transferred to tetrathionate bile brilliant green broth (HIMEDIA), incubated at 43-45°C for 18-24 hrs, broth was then transferred to plates of deoxycholate citrate agar (HIMEDIA), xylose, lysine deoxycholate agar (HIMEDIA) and brilliant green agar (HIMEDIA) incubated at 35-37°C for 24-48 hrs. The positive plates were sub cultured on triple sugar iron agar (HIMEDIA) slants using a deep inoculation technique⁶ for identification.

For *Pseudomonas aeruginosa* (Schroter) Migula, homogenized pretreated plant material was transferred to soybean-caesin digest medium (HIMEDIA) incubated at 35-37°C for 24-48 hrs, then placed on centrimide agar (HIMEDIA) and incubated at 35-37°C for 24-48 hrs. Presence of *P. aeruginosa* was confirmed by oxidase test⁶ using N, N, N/, N/-tetra methyl p-phenylenediamine dichloride (HIMEDIA).

Quantitative estimation of Glycyrrhizin

For quantitative estimation of Glycyrrhizin in *G. glabra*, sample was prepared by extracting 1 gm powdered drug by refluxing for 1 h in 10 ml methanol²¹, consecutively three times. The extraction was done in triplicate. The filtrate was taken as test solution. Two mg of glycyrrhizin (SIGMA), as reference, was dissolved in 1 ml methanol. Standard solutions of 10 - 50 µg/ml concentration were prepared by transferring aliquots of stock solution to 10 ml volumetric flask and adjusting the volume to 10 ml with methanol. Twenty µl of filtrates along with reference was applied on HPTLC precoated silica gel plates (E. Merck 60 F254) with the help of CAMAG Linomat IV applicator.

The plate was developed in the solvent system chloroform: acetic acid: methanol: water (6: 3.2: 1.2: 0.8) in previously saturated twin trough chamber (CAMAG). The developed plate was observed under UV 254 and under visible light after derivatization with anisaldehyde-sulphuric acid reagent (Fig. 1). The plate was scanned at the wavelength 600 nm using CAMAG TLC Scanner 3 with software CATS4. Photographs of TLC plate were taken by the DESAGA video documentation unit III.

Results and discussion

Herbal drugs normally carry a great number of bacteria and fungi which are directly dependent on several environmental factors, current practices of harvesting, handling, processing and storing. Quality of herbal drugs may be decreased by the microbial contamination. Therefore, WHO⁶ laid down the limits for microbial contamination depending on the use of plant material as a drug. The maximum limit for fungi is 10^5 while the maximum limit for *E. coli* is 10^2 to 10^4 per gram, for aerobic bacteria, it is 10^5 - 10^7 and it should be free from *Salmonella*. Therefore, assessment of microbial load on herbal drugs should be mandatory; particularly it is highly essential to observe the effect of microbial contamination on the active constituents of herbal drugs.

From the ongoing studies it was found that all the samples were found to be contaminated with different fungal species (Table 1). The fungal contamination belongs to group Deutromycotina dominating over other groups, i.e. Ascomycotina and Zygomycotina and these were identified as *Aspergillus niger* Tiegh., *A. flavus* Link, *A. fumigatus* Fresenius, *A. sydowii* Thom & Church, *Penicillium chrysogenum* Thom, *P. purpurogenum* Stoll, *Alternaria alternata* (Fr.) Keissl., *Cladosporium sphaerospermum* Penz., *Absidia corymbifera* Sacc. & Trott., *Rhizopus oryzae* Went & Prins. Geerl. Maximum percent incidence of fungi was observed on PDA, however, some fungi like *Aspergillus flavus*, *A. niger*, *Alternaria alternata*, *Rhizopus oryzae* were also grown on 2% agar, without any nutrients. Maximum fungal contamination was present in sample II and minimum in sample III. The fungal species found in the highly contaminated sample II were *Aspergillus flavus*, *A. niger*, *Penicillium chrysogenum* and *Alternaria alternata*. *Aspergillus flavus*, *A. candidus* Link, *A. niger*, *A. luchuensis* Inui, *A. ochraceus* Wilhelm, *A. nidulans* Winter, *Fusarium moniliforme* Sheld., *F. oxysporum* Snyder & Hansen, *Alternaria alternata*, *Curuvularia*

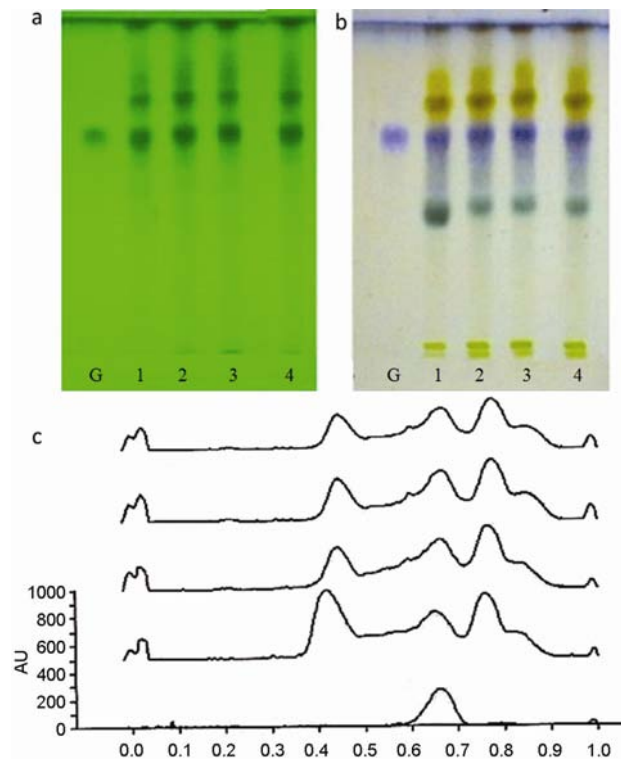


Fig.1- HPTLC finger print profiles of crude drug samples of *Glycyrrhiza glabra* L. (a) Under UV 254nm; (b) Under visible light after derivatization; (c) Densitometric scanning profile at 600 nm [G-reference (glycyrrhizin), 1-Delhi sample, 2- Dehradun sample, 3-Kanpur sample, 4- Lucknow sample].

Boedijn spp., *Chaetomium* Kuntz spp., *Penicillium citrinum* Thom and *Rhizopus stolonifer* Vuill. were the most common fungi isolated from different drug plants²²⁻²⁶.

It is quite evident from the present study that the magnitude of mycotoxin producing fungi *A. flavus* was very low and found only in sample II. This finding was also supported by Lutomski and Kedzia²⁷ that moulds of the genera *Aspergillus* and *Penicillium* comprised 50% of all moulds isolated from examined crude drugs and toxinogenic strains which produce mycotoxins occurred merely in 2% cases. The results provided by Hitokoto *et al.*²⁸ were also similar. Although the occurrence of mycotoxin producing fungi is very low, but one can not effectively say that these are safe and can be used without any doubt, unless it has been established that the microbial load does not alter the presence of secondary metabolites. Therefore, an attempt has been made to observe the effect of microbial load on active constituent of *G. glabra*- Glycyrrhizin and it was observed that the maximum Glycyrrhizin content was in sample III and minimum in sample II, i.e. 3.18 and 2.55 mg/gm

Table 1- Occurrence of various fungi in samples of *Glycyrrhiza glabra* L.

Media used	Sample I			Sample II			Sample III			Sample IV		
	2% agar	PDA	Moist blotter paper	2% agar	PDA	Moist blotter paper	2% agar	PDA	Moist blotter paper	2% agar	PDA	Moist blotter paper
DEUTROMYCOTINA												
<i>Aspergillus flavus</i> Link	-	+	-	-	+++	-	-	+	-	-	+	-
<i>A. fumigatus</i> Fresenius	-	-	-	-	-	-	-	+	-	-	-	-
<i>A. niger</i> Tiegh.	+	++	-	+	++	-	+	++	-	+	+	-
<i>A. sydowii</i> Thom & Church	-	-	-	-	-	-	-	+	-	-	+	-
<i>Penicillium chrysogenum</i> Thom	+	+	-	++	+++	++	-	+	-	+	++	-
<i>P. purpurogenum</i> Stoll	-	-	-	-	-	-	-	-	-	-	+	-
<i>Alternaria alternata</i> (Fr.) Keissl.	+	+	-	++	+++	-	-	+	-	+	+	-
<i>Cladosporium sphaerospermum</i> Penz.	-	-	-	-	-	-	-	-	-	++	+++	-
ZYGOMYCOTINA												
<i>Absidia corymbifera</i> Sacc. & Trott.	-	-	-	-	-	-	-	-	-	-	+	-
<i>Rhizopus oryzae</i> Went & Prins. Geerl.	+	+++	++	+	++	-	-	-	-	-	-	-

Explanation of symbols: - No contamination, +Low contamination, ++Medium contamination, +++High contamination

respectively (Table 2). This change in Glycyrrhizin content is directly proportional to the percent incidence of fungi (Table 2). Similarly, Dutta & Roy²⁹ also investigated that the microbial load deteriorated the percentage of strychnine and brucine in *Strychnos nux-vomica*. Therefore, it seems to be reasonable to continue such investigations, as if the number of *Aspergillus* and *Penicillium* strains exceed more than 10000 per gm of a crude drug, then it is probable that the moulds may be grown in the plant tissues²⁷. Such a situation may be responsible for degradation of the active constituents.

The total aerobic viable count of bacteria in herbal drugs is an important quality control parameter to assess the hygienic conditions. In this context, the frequency (colony forming unit/gram) and occurrence of different bacterial species were also detected in all the samples, and it was observed that highest total aerobic count, *E. coli* and *Salmonella* was present in sample II (Table 3). Baxter and Holzapfel⁸ also conducted a microbial investigation in selected spices and herbs and observed that the total number of colony forming units (CFU) varied from several hundred to several millions per gram, depending upon the source, the manufacturing process, age and type of condiments. It is interesting to note that the sample having maximum bacterial load has minimum Glycyrrhizin content. Hence, it can also be concluded

that the percentage of Glycyrrhizin may be affected by bacterial contamination.

An attempt has also been made for establishing correlation between moisture, sugar, starch and microbial load (Table 2). The maximum moisture was found in sample II (6.50%) and minimum in sample III (4.56%). Thus the higher percentage of moisture may be promoting the microbial growth as sample II has maximum microbial load too. Similarly, maximum sugar and starch percentage was in sample IV (5.85±0.28 and 27.77±1.50) and minimum in sample II (3.56±0.52 and 24.55±0.50, respectively). It seems that microbes also degrade sugars and starch components of plants. Similar results were also reported in *Terminalia bellerica* Roxb. and *T. chebula* Reiz. by three fungi, viz. *Aspergillus flavus*, *Curuvularia lunata* and *Fusarium moniliforme*^{30,31}. Hence, these changes in sugar and starch percentage may be due to the presence of microbial load.

Glycyrrhiza glabra is an age old plant used in traditional medicine across the world for its ethno-pharmacological values to cure varieties of ailments from simple cough to hepatitis more complex like Severe Acute Respiratory Syndrome (SARS) and cancer³². Microbial contamination in the crude drug may occur through handling by personnel who are infected with pathogenic bacteria during harvest/collection and post-harvest processing. The

Table 2- A comparative account of physico-chemical parameters, total percent contamination of fungi and glycyrrhizin content of *Glycyrrhiza glabra* L. samples

Sample	Moisture (%)	Sugar (%)	Starch (%)	Total percent contamination of fungi on			Glycyrrhizin (mg/gm)
				2% Agar	PDA	Filter paper	
I	5.58	4.60±0.66	25.60±1.20	56.00	72.00	30.00	2.84
II	6.50	3.56±0.52	24.55±0.50	56.00	84.00	33.00	2.55
III	4.56	5.50±0.27	26.85±1.22	19.00	50.00	-	3.18
IV	5.30	5.85±0.28	27.77±1.50	50.00	75.00	-	2.74

 Table 3- Bacterial count of samples of *Glycyrrhiza glabra* L. (CFU/gm)

Sample	Total aerobic count	Coliform count	<i>E. coli</i> (Migula) Castell. &	<i>P. aeruginosa</i> (Schroeter)	<i>Salmonella</i> Lignieres
			Chalm.	Migula	
I	9.1x10 ³	7.5x10 ⁷	1.8x10 ³	4.5x10 ³	<90
II	8.0x10 ⁷	2.5x10 ⁹	4.2x10 ³	2.7x10 ³	1.1x10 ³
III	6.9x10 ⁵	5.0x10 ⁸	1.0x10 ³	-	-
IV	5.2x10 ⁴	5.5x10 ⁷	3.0x10 ²	5.0x10 ²	8.0x10 ²

presence of *E. coli*, *Salmonella* spp. and moulds indicate poor quality. This risk can be reduced by ensuring that herbal medicines with harmful contaminants do not reach the public, by assessing the quality of the medicinal plants, herbal materials and finished herbal products before they reach the market. This should be controlled by implementing best practice guidelines. WHO has developed a series of technical guidelines and documents relating to the safety and quality assurance of medicinal plants and herbal drugs. These include, Guidelines on good agricultural and collection practices (GACP) for medicinal plants³³, Guidelines for assessing quality of herbal medicines with reference to contaminants and residues⁶, Quality control methods for medicinal plant³⁴ and Guidelines on good manufacturing practices (GMP) for herbal medicines³⁵.

Conclusion

From the ongoing study, it can be concluded that the concentration of glycyrrhizin in *G. glabra* decreases with the increased microbial load. The microbial findings from this study reiterate the need for constant quality assessment of commercial herbal medicines to ensure that they are suitable for human consumption and do not impose risk on human health. The crude drug samples in which microbial load is higher than WHO norms, either be less beneficial to the human health as the concentration of therapeutically active constituents reduces or harmful instead of curing the disease due to the presence of toxic substances.

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