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Antioxidant, antimicrobial and cytoprotective action of ethanolic extract of *Glycyrrhiza glabra* root against ccl4 induced damage on *Saccharomyces cerevisiae*

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Abstract

Ethanolic extract of *Glycyrrhiza glabra* root was investigated with antioxidant, antimicrobial and Cytoprotection assays in different *in vitro* models. Diphenyl picrylhydrazyl (DPPH) radical, inhibition of Reactive oxygen species (ROS), Lipid peroxidation (LPO), Reducing power, total phenolic content were some of the antioxidant assays performed. From the results obtained it was observed that the DPPH radical activity and ROS showed IC_{50} of 55 and 20µg respectively and LPO with IC_{50} of 6 µg. The extract also showed good reducing activity, which is an index of hydrogen donor. Total phenolic content was recorded 100mg/g of the extract. Further the extract showed cytoprotective effect on xenobiotic induced (CCl_4) oxidative stress in *Saccharomyces cerevisiae* (yeast cells). Cell viability, ROS, TBARs, LDH leakage in yeast cells were studied. From the obtained results it shows that the ethanolic extract of *Glycyrrhiza glabra* is having potent radical scavenging molecules which minimized the damage and protected cell, which was evident with decrease in the level of ROS, LPO and LDH leakage. The extract also showed antimicrobial activity with inhibition in growth of different Microorganism, *B. cereus, S. aureus*, *and E. Aerogens and E. coli*. The broad range of activity of the extract suggests that *Glycyrrhiza glabra* is a source of natural antioxidant, antimicrobial and cytoprotective, which could be considered as nutraceutical with health promoting properties in the prevention, and amelioration of degenerative diseases.

Keywords: Glycyrrhiza *glabra*, CCl_4, antioxidant, antimicrobial, Cytoprotection

Introduction

Oxidative stress induced free radicals, harm cells causing degenerative diseases, viz., atherosclerosis, diabetes, malignant growth, and aging. (Halliwell & Gutteridge, 1999; Yu, 1994) [1, 2]. Nutraceuticals present in fruits and vegetables are good scavengers of free radicals, considered as a good nourishment for health benefits (Klein *et al*., 2000) [3]. Phytochemicals are involved in reducing the free radical induced degenerative ailments (Chen & Ho 1995) [4]. There is tremendous scope for the plant inferred biomolecules viz., phenolics, flavanodis, lignin, saponins glycosides in neutralizing the radicals and protecting the cells (Kinsella *et al.,* 1993) [5]. Products containing phytochemicals are known to ameliorate oxidative stress and to counteract degenerative illnesses. It is reported that higher intake of foods rich in antioxidants is related with diminished level of degenerative ailments (Ames *et al.,* 1993: Joseph *et al*., 1999) [6-7].

In vitro cell culture is valuable for studying cytotoxicity induced by free radicals and to test the cytoprotective activity of antioxidants. Cell damage mediated by lethal chemicals is protected by antioxidants have been reported (Karnick 1993) [8]. Cytotoxic injury is accepted to be integral to toxicological appearance and cell pathobiology (Anup srivastava *et al*., 2007) [9]. Therefore compounds that ameliorate cytotoxic injury are likely to exhibit health-promoting potential.

Over the past two decades plants have been considered as a significant source of nutraceuticals towards humans, for treating different diseases (Artizzu *et al.,* 1995) [10]. Plant derived molecules has been utilized broadly in pharmaceuticals. According to the World Health Organization, numerous medicinal plants are source to obtain drugs and cure several diseases. In developed countries most of the individuals are using medicines in the form of herbs as they are rich in phenolic, essential oils, tannins (Jansen, *et al.,* 1986, Scalbert, 1991) [11-12]. The utilization of plant derived or photochemical has antimicrobial properties. There are a several reports on plants for their antimicrobial activity (Saxena *et al*., 1994) [13].

Glycyrrhiza glabra is one of the most extensively used medicinal herbs from the ancient medical history of ayurveda. *Glycyrrhiza glabra* Linn, commonly known as 'Liquorice or 'sweet wood' belongs to leguminosae family. It contains important phytoconstituent such as *Glycyrrhizin* which is a saponin which is sixty times sweeter than sugar. This plant species is to have many biological activities such as anti-inflammatory, hepatoprotection and hormonal imbalance effects (Adianti *et al.*, 2014) [14]. Medicinally, it is used for asthma, peptic ulcer, arthritis, allergic complaints (Zeng *et al.*, 1998) [15]. It is consumed as an energy tonic as it is having antiviral, antimalarial activities (Matsumoto *et al.*, 2014) [16]. Its root was also demonstrated to have antidepressant, memory strengthening activity (Lakshmi & Geetha 2011) [17]. Liquorice extract can be efficiently used to formulate cosmetic products for the protection of skin and hair against oxidative damage. Since this plant is reported with several biological activities in literature, study was conducted to evaluate its free radical scavenging potential, antimicrobial action and cytoprotection effect of *Glycyrrhiza glabra* root extract on yeast cells by creating oxidative stress using CCl_4.

Materials and Methods

Extraction

Glycyrrhiza glabra root powder was procured from local ayurveda store. Powdered material was extracted with ethanol using Sox let apparatus. The extractant was subjected to flash evaporator to dry the solvent and the extract obtained was stored at 4^0C till use.

Antioxidant assays

DPPH radical scavenging assay

The DPPH assay was carried out as described by (Guohua *et al* 1997) [18] with some modifications. Different concentrations of ethanolic extracts were mixed with of 1 ml DPPH solution (0.1 mmol/l, in 95% ethanol (v/v)), and the reaction mixture incubated for 30 min at room temperature. The optical density was measured spectrophotometric ally at 517 nm against a blank. BHA was used as a positive control. Decrease in the absorbance of DPPH indicates a higher radical scavenging activity.

Superoxide radical scavenging assay

Superoxide anion was generated by the reaction of NADH and phenazine methosulphate (PMS) Coupled with a reduction of Nitro Blue Tetrazolium chloride (NBT) (Nishikimi *et al.,* 1972) [19]. The reaction mixture contained NBT (100 lM), NADH (300 lM) with or without extract in a total volume of 1 ml Tris buffer (0.02 M, pH 8.3). The reaction was measured spectrophotometric ally at 560 nm every 30 sec for 1 min by adding PMS to the mixture

Reducing power

The reducing power of the extracts was measured according to the method described by (Oyaizu 1986) [20]. 1 ml of reaction mixture containing extracts in phosphate buffer 0.2 mol/l, pH 6.6 was incubated with 3 ml of 1% potassium ferricyanide at 50 °C for 20 min. After incubation, the reaction was stopped by adding 1 ml of 10% TCA solution and the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was mixed with distilled water (2.5 ml) and ferric chloride solution (0.1 g/ 100 ml), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance indicated increased reducing power.

Antimicrobial activity

Antimicrobial activity was measured using paper disc method. The paper disc for antimicrobial test was prepared by taking aliquots of extracts with different concentrations in separate appendorf tubes. The sterilized paper disc prepared from the Whatman paper was dipped in the extracts with different concentrations for 1 hr. After 1 hr the paper discs were incubated in the oven at 45^oc overnight to evaporate the solvent from the paper disc. Autoclaved nutrient agar plates was inoculated with 0.1 ml of the inoculums (*E.coli, Klebsiella, B. cereus, S. aureus*) by spread plate method using a spreader. To the inoculated plates, paper discs dipped with extract was placed in the plate and incubated at 37^oC for 12-24 hrs.

Cytoprotection

Cell viability

CCl_4 was used as toxicant, to check the Cytoprotection of the extract. The concentration of CCl_4 was used at 50 percent lethality (LC_{50}). Cytoprotection investigations were performed by incubating 1.0 ml of yeast cells (10 X 10^6) suspended in YEPD with CCl_4, (dissolved in DMSO) at LC50 concentration 1mM with/without the extract for 1 hr in a shaking water shower at 37^0C. After the incubation period, an aliquot of cells was taken for viability test by trypan blue exclusion method (Anup *et al.,* 2007) [9].

Lactate dehydrogenase leakage

The supernatant obtained after incubation followed by centrifugation from the reaction mixture of yeast cells in the presence of xenobiotics with/without extract was assayed for LDH with sodium lactate as the substrate (Bergmeyer 1974) [21].

Lipid peroxidation of yeast cell

The cells were centrifuged after incubation, with CCl_4 along with the extract and the cell pellet was washed in saline and the pellet was boiled in TCA (5.5%) and TBA (0.34%) for 15 min, cooled and centrifuged. The supernatant was measured in a spectrophotometer at wavelength of 535 nm (Cereser *et al.,* 2001) [22].

Reactive oxygen species (superoxide anion)

The cells (10 X 10^6) suspended in 1.0 ml YEPD were incubated with NBT (0.2 mM) with or without xenobiotics (in DMSO) and extracts in a shaking water bath at 37^0C. The generation of ROS by cells (respiratory burst) was measured by the formation of coloured formazan due to reduction of NBT (Pompeia *et al.,* 2003) [23].

Isolation of genomic DNA from yeast cells & Induction of DNA damage by CCl_4 and its protection by the extract

Yeast cells (10 X 10^6) was treated with 0.3ml of 4% SDS and keep in boiling water bath for 15 minutes and centrifuged at 10000 rpm for 5 minutes. Supernatant collected was treated with 15µl of 3M sodium acetate and 0.5ml of ethanol and kept in freezer for 15minute and centrifuge at 12000rpm for 10 min. Pellet obtained was dissolved in 0.1M TE buffer (Adianti *et al.,* 2014) [14]. The isolated DNA was induced damage with CCl_4 (1mM) co treated with different concentration of the *Glycyrrhiza glabra* root extract (20-140µg), incubated at 37^0c for 1 hr respectively. After incubation the DNA sample was loaded to 1% agarose gel and subjected to electrophoresis at 50-100v.

Statistical analysis
Data are expressed as mean ± S.E. of three separate experiments

Results and Discussion
DPPH Radical Scavenging Activity
DPPH is a stable free radical which has the tendency to accept electrons to become stable molecules. Basically, it is used to screen the antioxidant activity of various samples. DPPH radical can be measured at absorbance maxima at 517 nm. Decrease in the absorbance indicates the acceptance of electrons which is induced by the antioxidants. The results are shown in Table 1. This represents ethanolic extracts of *Glycyrrhiza glabra* extract is potent in scavenging the free DDPH radical with an IC_{50} of 55μg/mL Scavenging effect was concentration dependent of the extract. The antioxidant activity of the extracts is credited to their hydrogen donating ability (Yamaguchi *et al.,* 1998) [24]. Results suggest that extracts have strong potential in scavenging the free radical, which could be attributable to its hydrogen donating ability.

Table 1: Antioxidant activity of ethanolic extract of *Glycyrrhiza glabra* root

Ethanolic extract	Free radical Scavenging activity IC_{50} μg/ml			Phenol mg/g
	DPPH	ROS	LPO	100 ±1.4
	55	20	06	

Superoxide radical scavenging
The inhibition of reactive oxygen species (ROS) by ethanolic extract of *Glycyrrhiza glabra* with an IC_{50} of 20 μg/ml is represented in Table 1. Extract showed the highest activity compared to standard antioxidant molecule, BHA, which was not able to prevent the inhibition of ROS. The ROS which is produced under electron transport system of normal physiological process is harmful to the living system, though it is a minute oxidant but it leads to oxidation chain reaction producing more threatening free radicals such as hydroxyl radical and singlet oxygen which are unsafe, promotes to oxidative damage (Dahl and Richardson 1978) [25]. Since extract is showing good inhibition of ROS activity, consumption of green vegetable and fruits in daily diet will definitely influence in scavenging the ROS that are generated also protects body from the endogenous radical sources

Lipid Peroxidation
Inhibition of lipid peroxidation was observed in ethanolic extracts of *Glycyrrhiza glabra* root with an IC_{50} of 6 μg/mL (Table 1). Oxidation of polyunsaturated fatty acids in the cell membrane produces Malondialdehyde (MDA), which is the index of lipid peroxidation and marker of cell injury. Cell damage can occur in any internal organ by free radicals, leading to various disorders, viz., atherosclerosis, hepato and nephro damage (Janero, 1990; Rice-Evans and Burdon 1993) [26-27]. Our results showed inhibition of lipid peroxidation with increase in concentration of the extracts, indicating ethanolic extracts have certain antioxidant molecules which are able to repair the damage caused by the free radicals. The mechanism in inhibiting the lipid peroxidation by the extracts could be by preventing the chain initiation of polyunsaturated fatty acid chain by donating the hydrogen atom to the damaged lipid bilayer.

Reducing Power
The reducing power of the extracts is shown in Fig.1. The extracts showed increasing activity with increase in the concentration of extracts. Reducing capacity of the extracts could be considered an indicator towards its potential antioxidant properties. Total potential of the antioxidant activity has been attributed to various mechanisms viz., prevention of chain initiation, binding of transition metal ion, inhibition of hydrogen abstraction, radical scavenging and preventing lipid bilayer damage (Diplock, 1997; Duh 1998) [28, 29] has shown that reducing properties are associated with the presence of reductones. Reductones play a major role in exerting antioxidant action by donating hydrogen atom and preventing the free radical chain damage. It also reacts with precursors of peroxideand prevents the peroxide formation. Results suggest that both the extracts of have potential to free radical damage by donating hydrogen atom thereby preventing oxidative stress.

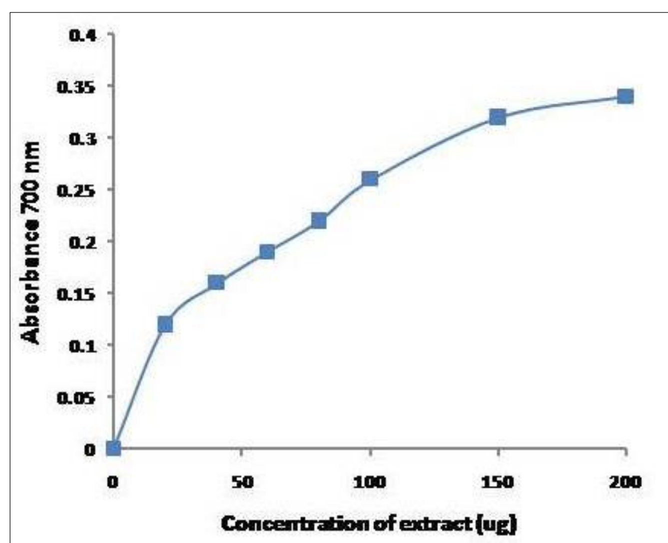

Fig 1: Reducing power of ethanolic extract of *Glycyrrhiza glabra* root

Phenolic Content
Phenolic content in the ethanolic extract of Glycyrrhiza glabra was 100±3.7 mg guaicol equivalent per gram (Table 1). Phenols play a major role in radical scavenging because of their hydroxyl groups. Total phenolic content present in the extract is directly related to antioxidant activity (Duh *et al*., 1999) [29]. It is reported that polyphenolic compounds protect humans from mutagenesis and carcinogenesis (Diplock, 1997) [28]. In this study, there is a correlation between antioxidant activity and phenol content.

Cytoprotection of *Glycyrrhiza glabra* root on yeast cells against CCl_4, induced damage
In vitro cell culture play a model role in evaluating the phytochemical in ameliorating the level of oxidative stress induced by xenobiotic in cells, which is measured by cell viability. Earlier studies on photochemical have shown Cytoprotection in both *in vitro* and *in vivo* models (Anup *et al*., 2007) [9]. In this test we have demonstrated the *Glycyrrhiza glabra* root extract in protecting xenobiotic induced cell death in yeast cells. CCl_4, inducer of oxidative stress in cells is utilized as toxicant. Outcomes of result indicated depletion of xenobiotic induced lipid peroxidation, inhibition of ROS by preventing cell death and reduction in level of LDL leakage with increase in concentration of extract. Cytoprotection observed as cell viability, was observed for cells co treatment with 50–200μg/ml of extract

and 1mM CCl_4, Fig.2. LDH leakage in the cells was altogether decreased when cells co treated with increasing concentration of extract, compared with CCl_4, treated Fig.3. Lipid peroxidation was depleted in the cells treated with high convergence of extracts, in which the development of Malondialdehyde was measured as marker record of lipid bilayer damage Fig 4. Reactive oxygen species (ROS), level increases when cells exposed to stress condition. The level of ROS was reduced when cells co treated with the extracts Fig 5. Further the DNA damage by CCl_4 was reversed with concentration dependent of root extract, which indicates that the extract has potential molecules which activate topoisomerase and Polymerases family which repair the DNA Fig.6. These outcomes demonstrate that the unrefined extract might contain cocktail of phytochemicals, which improve the level of oxidative stress instigated by the CCl_4, by protecting the cell from undergoing death. Further the photochemical responsible from protecting cell death, work under progress.

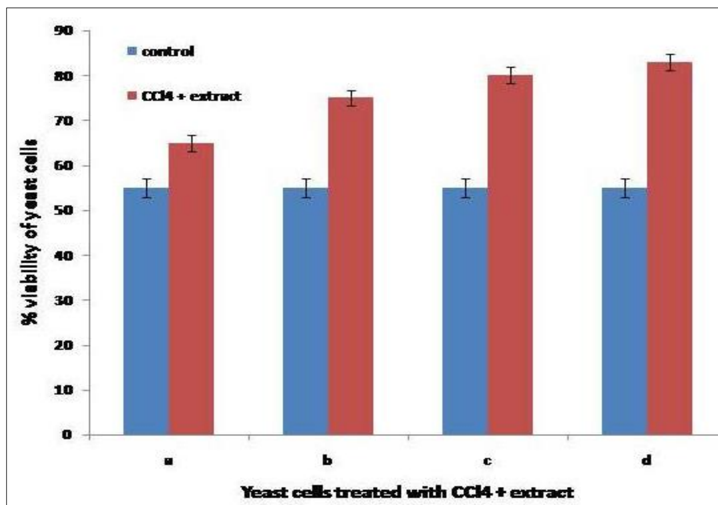

Fig 2: Cell viability of yeast cells co treated with different concentration of extracts (a- 50µg, b-100 µg, c- 150 µg, d- 200 µg) and CCl4 (1mM).

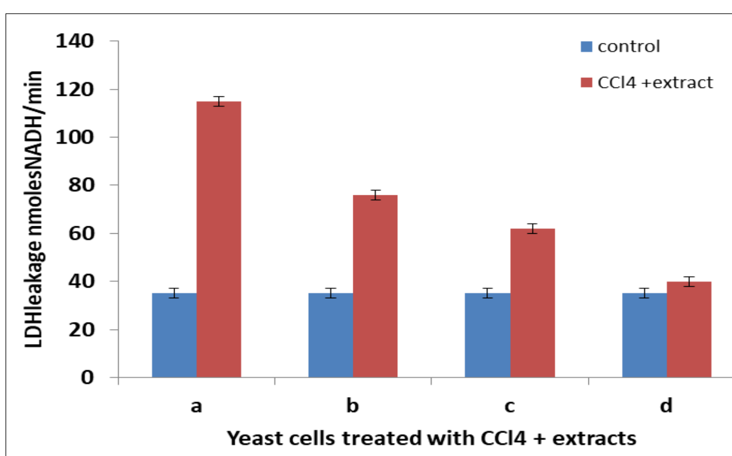

Fig 3: LDH leakage of yeast cells co treated with different concentration of extracts (a- 50µg, b-100 µg, c- 150 µg, d- 200 µg) and CCl4 (1mM).

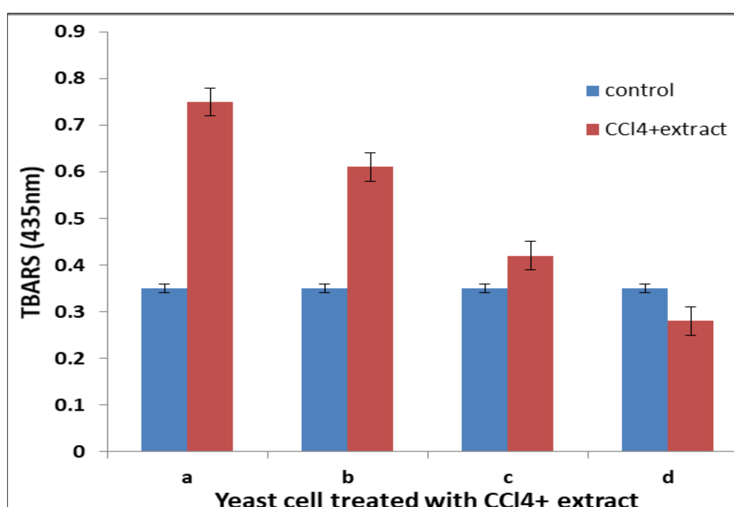

Fig 4: Lipid peroxidation of yeast cells co treated with different concentration of extracts (a- 50µg, b-100 µg, c- 150 µg, d- 200 µg) and CCl4 (1mM).

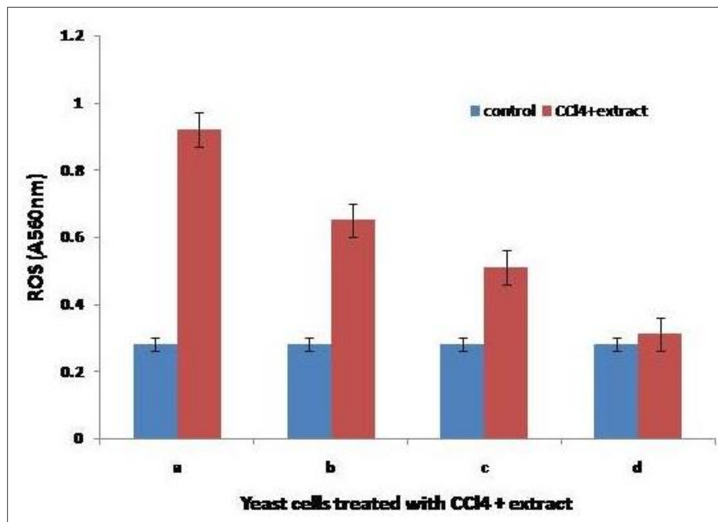

Fig 5: ROS of yeast cells co treated with different concentration of extracts (a- 50µg, b-100 µg, c- 150 µg, d- 200 µg) and CCl4 (1mM).

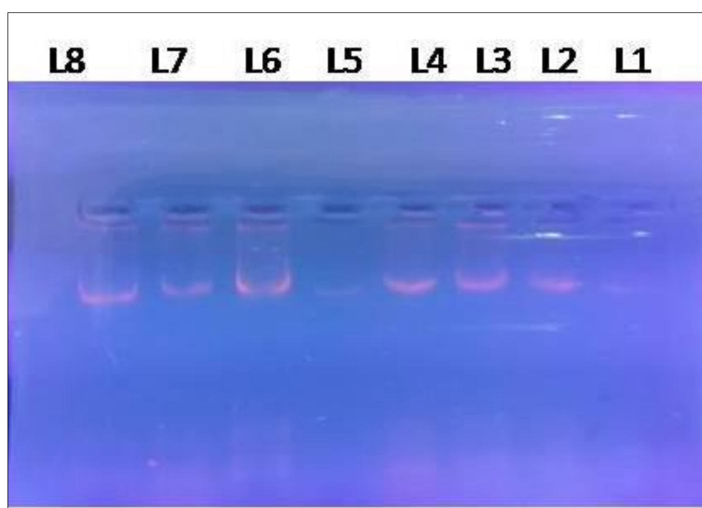

Fig 6: DNA of yeast cells (L1- DNA+CCl_4 (1mM), L2- L8 (DNA + CCl4 + extract with different concentration (20-140µg)

Antimicrobial assay

The antibacterial assay of *Glycyrrhiza glabra* root extracts against different bacteria were assessed by the presence or absence of inhibition zones and recording the zone of inhibition diameter (mm) (Matsumoto *et al*., 2014) [16]. The zone of inhibition recorded is shown in Table 2, Fig.7 the zone of inhibition was concentration dependent 0.3-0.8mm with volume of extract 10-20µl.

Table 2: Antimicrobial activity of ethanolic extract of *Glycyrrhiza glabra* root

Organisms	Vol of extract 10 µl	Vol of extract 20 µl
E. coli	0.3mm	0.5mm
E. aerogen	0.5mm	0.7mm
S. aureus	0.6mm	0.8mm
B. cereus	0.3mm	0.5mm

Fig 7: Antimicrobial activity of *Glycyrrhiza glabra* root extract

With increase in antibiotic resistance has resulted in an urgent need for alternative therapies to treat diseases the best strategy is to use Phyto formulation. In recent years, many studies have shown that *Glycyrrhiza glabra* aqueous extract, ethanol extract and supercritical fluid extract have potent effects in inhibiting the activities of gram positive and negative bacteria, such as *S. aureus, E. coli, P. aeruginosa* and *B. subtilis* which is correlating with the results that we have obtained (Awandkar *et al*., 2012; Irani *et al*., 2010) [30-31]. These extracts are also being considered as potential alternatives to synthetic fungicides or as lead compounds for new classes of synthetic fungicides (Messier & Grenier 2011; Treutwein *et al*., 2010) [32-33]. Based on the above inhibitory activities against bacteria licorice may serve as an alternative therapy for treating several diseases viz., dental diseases, digestive anabrosis and tuberculosis etc.

Conclusion

The results obtained in the present study clearly suggest that the ethanolic extract of *Glycyrrhiza glabra* root may contain numerous antioxidant compounds which can effectively scavenge various ROS/free radicals under *in vitro* conditions. Further the extracts have shown the cytoprotective action on xenobiotic induced toxicity in yeast cells, caused by the toxicant and preventing the cell death. Extract also showed antimicrobial activity. The broad range of activity of the extract suggests that *Glycyrrhiza glabra* root is a source of

natural antioxidants which could be considered as nutraceutical with health promoting properties in the prevention and amelioration of degenerative diseases. Although we have not isolated and characterized the antioxidant molecules responsible for the antioxidant activity and cytoprotection, we speculate that it could be related to the phenolic and nonphenolic compounds present in the extract. Therefore, further work will be carried out to isolate and identify the effective cytoprotective molecules.

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