

ESTIMATION OF TOTAL FLAVONOID, PHENOLIC CONTENT, AND FREE RADICAL SCAVENGING POTENTIAL OF *GLYCYRRHIZA GLABRA* ROOT EXTRACT

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ABSTRACT

Objective: The present study attempts to estimate the total flavonoid and phenolic content and to evaluate the free radical scavenging activity of the roots of *Glycyrrhiza glabra* Leguminosae family.

Methods: The various extracts, namely, petroleum ether, chloroform, ethyl acetate, aqueous, and methanol of *G. glabra* root were evaluated by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), hydroxyl radicals, superoxide, 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS), and non-radicals such as hydrogen peroxide (H₂O₂) and nitric oxide (NO) and assays. Total flavonoid and phenol contents were estimated as per the standard procedure.

Results: The effect of the methanolic extract of *G. glabra* root was found to be more in scavenging the free radicals such as DPPH, hydroxyl, H₂O₂, superoxide, NO, and ABTS when compared to other extracts. The presence of flavonoids and phenol in the plant is responsible for the free radical scavenging effects.

Conclusion: This *in vitro* assessment of the antioxidant properties of the various extracts of *G. glabra* roots validates and confirms its free-radical scavenging activity. Thus, the present data suggest that the *G. glabra* root extract can be used as a good source of natural antioxidants for health benefits.

Keywords: *Glycyrrhiza glabra*, 2, 2-diphenyl-1-picryl-hydrazyl, 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulfonic acid, Superoxide, hydroxyl, Hydrogen peroxide, Nitric oxide, Flavonoids, Phenol.

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INTRODUCTION

Glycyrrhiza glabra Linn. is one of the most extensively used medicinal herbs from the ancient medical history of Ayurveda. It is also used as a flavoring herb. The word *Glycyrrhiza* is derived from the Greek term glykos (meaning sweet) and rhiza (meaning root) [1]. This plant is a tall perennial herb, up to 2 m high found cultivated in Europe, Persia, Afghanistan, and too little extent in some parts of India [2].

Glycyrrhiza plays important parts in Hindu medicine and is one of the principle drugs of the "susruta." In ancient Egypt, Greece, and Rome, *Glycyrrhiza* was also frequently used. Liquorice is referred by Theophrastus. It is interesting to find that even to this day liquorice is maintaining its place in medicine and pharmacy [3].

It is popularly used worldwide in food, confectionery, and pharmaceutical products, such as cough syrups, herbal supplements, chewing gums, drinks, and candies. Liquorice roots are extensively used in herbal medicines for their emollient, anti-inflammatory, antiviral, anti-allergic, gastroprotective, and anticancerous properties attributed mainly to the presence of wide range of flavonoids. Various antioxidant properties of *G. glabra* roots have also been reported which are attributed to the phenolic compounds such as glabridin, licoisoflavone B, licochalcone, and liquiritigenin [4].

Reactive oxygen species (ROS) are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. ROS includes free radicals such as O₂⁻, hydroxyl radicals (OH·), and non-free-radical species such as hydrogen peroxide (H₂O₂) and singlet oxygen (¹O₂) [5].

Antioxidants can protect the human body from free radicals and ROS effects. They retard the progress of many chronic diseases as well as lipid peroxidation. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties [6]. Hence, a need for identifying alternative natural and safe sources of food antioxidants has been created, and the search for natural antioxidants, especially of plant origin, has notably increased in recent years. The objective of this study was to estimate the total phenol and flavonoid content and to investigate the free radical scavenging activity of the different extract of *G. glabra* root sample.

METHODS

Collection and extraction of plant materials

G. glabra plants were collected from Tami Nadu Agricultural University and authenticated by Taxonomist from Botanical Survey of India. The plant roots were shade-dried at room temperature and then ground into powder. The sample was extracted into solvents of increasing polarity using a Soxhlet apparatus. About 20 g of each powdered sample was extracted with 250 mL of respective solvents such as petroleum ether (PE), chloroform (CH), ethyl acetate (EA), and methanol (ME). The extracts were filtered and concentrated in rotary evaporator to obtain crude extracts. They were stored at -20°C until use. Apart from the solvent extracts, a fresh aqueous extract (AQ) was also prepared. The AQ extract was prepared in distilled water and centrifuged at 2000 rpm for 5 min, and the supernatant was used for the further assay. In the present study, 10-100 µg/ml of the PE, CH, EA, ME, and AQ extracts roots of *G. glabra* were used for the *in vitro* free radical scavenging activity. Ascorbic acid was used as a standard.

Estimation of total phenol and flavonoid content

Estimation of total phenol

Phenolic compounds are a class of antioxidant agents which act as free radical terminators. The amount of total phenol present in the roots of *G. glabra* was estimated by the method described by Baba and Malik, 2015 [7].

The Folin-Ciocalteu reagent was used for determining the TPC. Using 100, 200, 300, 400, and 500 µg/ml concentrations of gallic acid, a standard calibration plot was generated at 760 nm. The concentrations of phenol in the test samples were calculated from the calibration plot. Total phenolic content was expressed as mg gallic acid equivalents (GAE). All determinations were performed in triplicates, and the results were expressed as mg GAE/g sample extract.

Estimation of flavonoids

Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants. Flavonoids are known to be antimutagenic, anticarcinogenic, anti-inflammatory, and anti-allergic with antioxidant properties. A standard calibration plot was generated at 415 nm using different and known concentrations of quercetin.

The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent/g of sample. The flavonoid content in different parts of the plant was assessed by the method of Chang *et al.* [8].

Determination of the free radical scavenging activities

The *in vitro* free radical scavenging activities of different extracts of *G. glabra* were evaluated by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), hydroxyl, H₂O₂, superoxide, nitric oxide (NO), and 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) assays.

DPPH radical scavenging assay

DPPH free radical method is an antioxidant assay based on electron transfer that produces a violet solution in ethanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. DPPH radical scavenging activity was measured according to the method of Mensor *et al.* [9].

Hydroxyl radical scavenging assay

Hydroxyl radical is one of the potent ROS in biological system. It reacts with polyunsaturated fatty acid and causes damage to the cell. The hydroxyl radical scavenging activity was measured in terms of the amount of thiobarbituric acid reactive substances (TBARS) formed on reaction of deoxyribose with H₂O₂. The amount of TBARS formed is indirectly proportional to the hydroxyl radical scavenging. The scavenging ability of hydroxyl radicals is measured by the method of Elizabeth and Rao [10].

H₂O₂ scavenging assay

H₂O₂ itself is not only very reactive but also could be toxic to cells. The H₂O₂ scavenging activity was determined according to the method described by Ruch *et al.* [11].

Superoxide radical scavenging assay

Superoxide anion radical is generated by four-electron reduction of molecular oxygen into water. This radical is also formed in aerobic cells due to electron leakage from electron transport chain. In living organisms, superoxide is removed by superoxide dismutase. Superoxide radical scavenging activity of various extracts of *G. glabra* was determined by the method of Misra and Fridovich [12].

NO scavenging assay

NO produced at the cellular level from L-arginine catalyzed by NO synthase is a very important signaling molecule. NO scavenging activity

of various extracts of *G. glabra* roots was determined by the method described by Green *et al.* [13].

ABTS radical scavenging assay

The ABTS is the simple, fast, reliable, inexpensive method to assess the total antioxidant capacity of the medicinal herb extracts on a large scale, which is also very adaptable to both hydrophilic and lipophilic antioxidant systems. This effective and efficient method could be used for systematic screening of medicinal herbs and edible plants for their relative antioxidant content. The ABTS radical scavenging activity was determined by the method described by Shirwaiker *et al.* [14].

RESULTS

The total flavonoid content (TFC) and TPC were calculated from the absorbance calibration curve generated with different concentrations of quercetin and gallic acid (standards), respectively, which is shown in Fig. 1.

DPPH radical scavenging assay

DPPH scavenging activity is one of the best methods to screen the free radical scavenging activity of medicinal plants and its compounds. DPPH is scavenged by antioxidants by donating hydrogen ion and forming reduced DPPH. As a result of the formation of reduced DPPH, the color changes from purple to yellow and the intensity of the color increases with increasing percentage of the radical inhibition [15].

DPPH is a free radical that keeps its stability in AQ or methanolic solutions. It accepts an electron or hydrogen ion to become a stable diamagnetic molecule. The percent DPPH scavenging ability by the *G. glabra* root extract was carried out spectrophotometrically, and the results are presented in Fig. 2.

DPPH radical scavenging activity was found to be increased with increasing concentration of the root extract. At a concentration of 64 µg/ml, the methanolic extract of *G. glabra* recorded 50% of

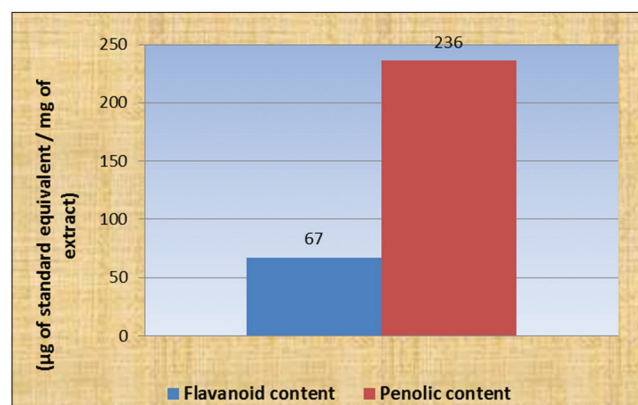


Fig. 1: Estimation of total flavonoid and total phenol content

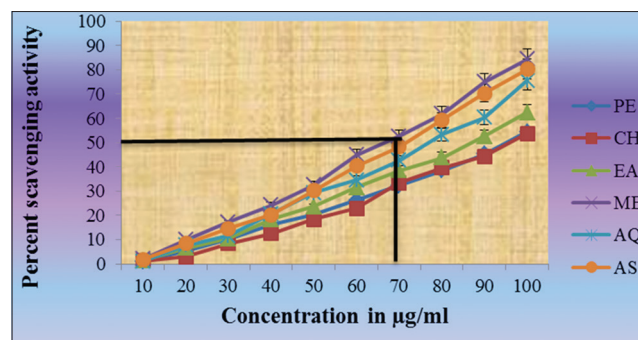


Fig. 2: 2, 2-diphenyl-1-picryl-hydrazyl radical scavenging activity of the *Glycyrrhiza glabra* root extract of various solvents

scavenging activity and it is increased to 80% at a concentration of 100 $\mu\text{g/ml}$. The least scavenging activity was found in CH extract of roots of the plant assessed.

Hydroxyl radical scavenging assay

Hydroxyl radicals are the most reactive free radical formed during the metabolism among the ROS which could be formed by superoxide and H_2O_2 radicals in the presence of metal ions such as copper or iron [16]. Hydroxyl radical is capable of damaging almost every molecule found in living cells. This radical has the capacity to induce carcinogenesis and rapidly initiate lipid peroxidation [17]. The percent inhibition of hydroxyl radical by various extracts was concentration dependent, and the methanolic extract of *G. glabra* proved to be more effective than the standard ascorbic acid in scavenging hydroxyl radical which is shown in the Fig. 3.

H_2O_2 radical scavenging assay

H_2O_2 exposure significantly increased the TBARS formation, which was effectively counteracted by the presence of the *G. glabra* root extracts. The IC_{50} value of methanolic extract was 68 $\mu\text{g/ml}$. All the other solvent extracts also showed good H_2O_2 scavenging activity. H_2O_2 radical scavenging activity of various solvent extracts was shown in Fig. 4.

Inhibition of superoxide radical generation

Superoxide is a highly toxic species and can be generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme which catalyzes the breakdown of superoxide radical [18]. It was found that the methanolic extracts of *G. glabra* showed the highest inhibitory activity when compared with other extracts at 100 $\mu\text{g/ml}$ concentration. The superoxide radical scavenging activity of all the extracts was found to be concentration dependent, i.e. as the concentration of the extracts increased the radical scavenging activity was also found to be increased. Superoxide generation inhibitory potentials of *G. glabra* were shown in Fig. 5.

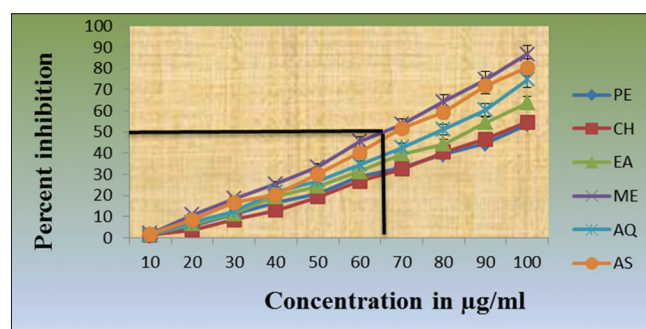


Fig. 3: Hydroxyl radical scavenging effect of the *Glycyrrhiza glabra* root extract of various solvents

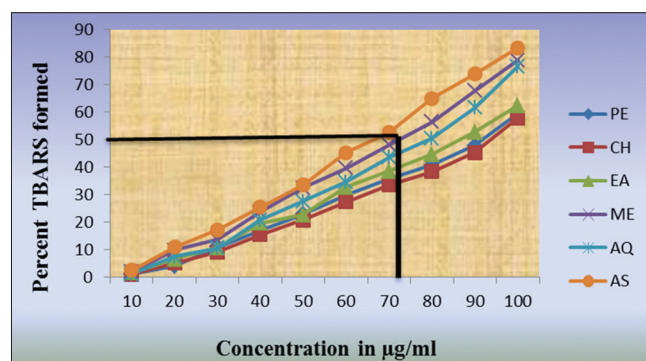


Fig. 4: Hydrogen peroxide radical scavenging effect of *Glycyrrhiza glabra* root extract of various solvent

Inhibition of NO radical generation

NO is a potent pleiotropic mediator of physiological process such as smooth muscle relaxation, neuronal signaling inhibition of platelet aggregation, and regulation of cell-mediated toxicity [19]. An excess amount of NO may lead to tissue damage and cause various degenerative diseases, namely, cancer, diabetes mellitus, multiple sclerosis, arthritis, and ulcerative colitis. It is also a potent inhibitor of physiological processes such as smooth muscle relaxation, neural signaling, inhibition of platelet aggregation, and regulation of cell-mediated toxicity [20]. All the extracts of *G. glabra* root extract effectively reduced the generation of NO from sodium nitroprusside. Standard ascorbic acid and methanolic extract showed highest NO radical inhibition compared to other solvent extracts. The IC_{50} value of methanolic extract was 58 $\mu\text{g/ml}$. The effect of the inhibition of the generation of the NO radical potentials of the *G. glabra* root extracts of various solvents was depicted in Fig. 6.

ABTS radical scavenging assay

The maximum extent of ABTS scavenging ability was elicited by the methanolic extract (IC_{50} value was found to be 44 $\mu\text{g/ml}$) of *G. glabra* roots followed by standard ascorbic acid. The least scavenging activity was found in CH extract as shown in Fig. 7.

DISCUSSION

The total phenolic content of the methanolic extract was 236 GAE/mg. Similarly, the TFC was assessed as 67 quercetin equivalents/mg. Flavonoids, including flavones, flavanols, and condensed tannins, are plant secondary metabolites, the antioxidant activity of which depends on the presence of free hydroxyl groups. Plant flavonoids have antioxidant activity *in vitro* and also act as antioxidants *in vivo*. Plants rich in secondary metabolites, including phenolic, flavonoids, and carotenoids, have antioxidant activity due to their redox properties and chemical structures [21]. An earlier study reported that phenolic compounds are a major source of natural antioxidants in foods of plant origin which in turn exhibit a wide spectrum of biochemical

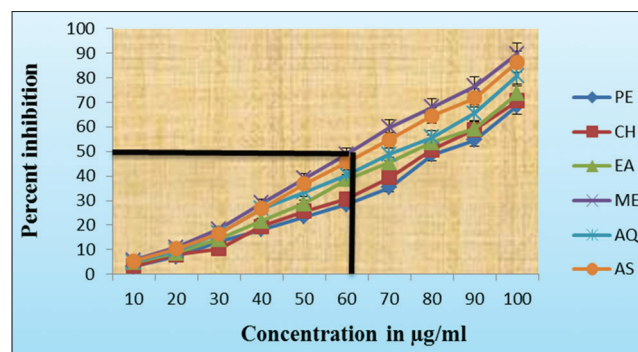


Fig. 5: Inhibition of the generation of the superoxide radical potentials of the *Glycyrrhiza glabra* root extracts of various solvents

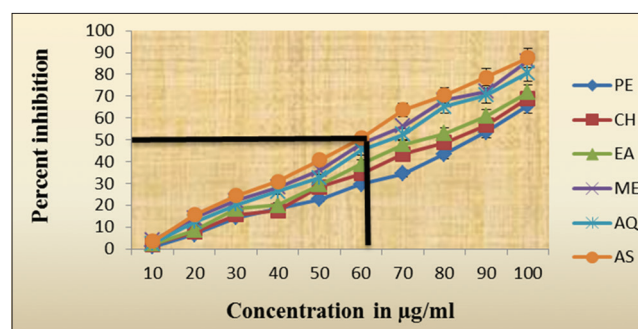


Fig. 6: Effect of the Inhibition of the generation of the nitric oxide radical *Glycyrrhiza glabra* root extracts of various solvents

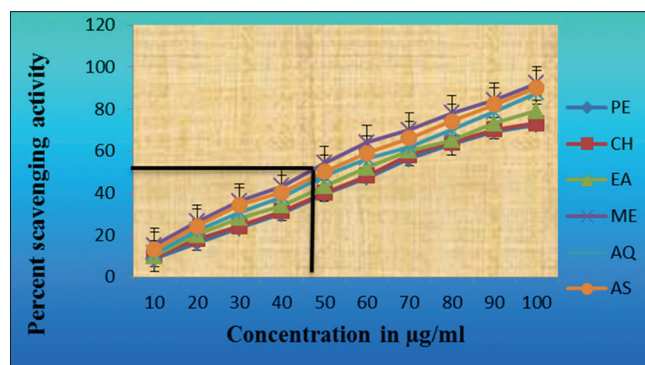


Fig. 7: 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid radical scavenging effect of *Glycyrrhiza glabra* root extract of various solvent extracts

activities such as antimicrobial, antimutagenic, anticarcinogenic as well as the ability to modify the gene expression [22].

Phenolic compounds were produced by plant as a defense mechanism against different stress conditions. Phenolic compounds included flavonoid compounds have various effects such as antioxidant activity, antibacterial activity, antidiabetic, and hepatoprotective [23].

The high phenolic and flavonoid content is responsible for the bioactivity of these crude extracts. Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several diseases.

Free radicals are responsible for causing a wide number of health hazards such as cancer, aging, heart diseases, and gastric problems. Oxygen free radicals disintegrate DNA and destroy cell membranes by enzymatic metabolic processes [24].

DPPH is a stable nitrogen-centered free radical commonly used for testing radical scavenging activity of the compound or plant extracts. When the stable DPPH radical accepts an electron from the antioxidant compound, the violet color of the DPPH radical was reduced to yellow-colored diphenyl picrylhydrazine radical which was measured colorimetrically. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [25].

Gulcin [5] reported that DPPH radical scavenging activity of *G. glabra* AQ and ethanol root extracts was 52.2% and 54.4%, respectively, at 30 µg/ml concentration. These results support the data reported by Chopra *et al.* [1] who observed that methanolic extracts of liquorice roots displayed good antioxidant activity (IC_{50} 359.45 mg/mL). Al-Bachir and Al-Adawi [26] also demonstrated that liquorice roots powder showed antioxidant activity with an IC_{50} value of 87.152 mg/mL versus 22.78 mg/mL for ascorbic acid. The reduction in the DPPH molecule can be correlated with the available number of hydroxyl groups present in the extracts, and it might be due to their hydrogen-donating ability. The high content of phenolic component in methanolic extract of liquorice (*G. glabra* L.) is responsible for its powerful antioxidant activity by means of significant free radical scavenging, hydrogen-donating, metal ion chelating, and anti-lipid peroxidative and reducing abilities [27].

Glycyrrhizin present in the roots of *G. glabra* is considered as quenching agent of free radicals and also as blocking agent of lipid peroxidation chain reactions. Glycyrrhizin showed chemopreventive, antioxidant, and anti-proliferative activity when tested on animal model [28].

Liquorice flavonoids have exceptionally strong antioxidant activity. Antioxidant activity of liquorice flavonoids was found to be over 100 times stronger than that of antioxidant activity of Vitamin E. A dose of 2.58 mg/ml liquorice flavonoids can scavenge more free radicals (20.6% scavenging) than 258 mg/ml of Vitamin E (11.2%

scavenging). The flavonoids from liquorice are currently the strongest natural antioxidants known [29]. The isoflavones, glabridin and hispaglabridins A and B, have considerable antioxidant activity. Both glabridin and glabrene have estrogen-like activity [30]. A previous study reported by Franceschelli [31] evidenced that the constituents of *G. glabra* were effective in preventing microsomal lipid peroxidation and licochalcone B and D exhibited potent antioxidative and superoxide scavenging activities. Barik *et al.* [19] demonstrated that the NO scavenging activity of AQ and ethanolic extract of *G. glabra* was found to be in the range of 20.63% which is similar to the findings of the present study. Kang *et al.* [32] demonstrated that glabridin a polyphenolic compound present in *G. glabra* inhibits NO production. There was a concentration-dependent inhibition against hydroxyl radical exhibited by *G. glabra* ethanolic extract and was in the range of 35.1% [33]. The percent scavenging of ABTS and H_2O_2 radicals was also dose dependent indicating that the root sample exhibited maximum radical scavenging activity which was supported by Mradu Gupta *et al.* [34]. The percentage inhibition as well as the low IC_{50} values of DPPH, hydroxyl, superoxide, H_2O_2 , NO, and ABTS assay of standard ascorbic acid and methanolic extract indicates that its very high antioxidant capacity in comparison with other solvent extract.

CONCLUSION

The evaluation of the free radical scavenging activity of various extracts of *G. glabra* showed substantial and significant free radical scavenging activity during the study which is comparable to that of the standard drug. The methanolic extract exhibits a higher level of activity than the other extracts in all the antioxidant assessment. This significant pronounced antioxidant activity of *G. glabra* roots may be due to the presence of higher concentrations of flavonoids and phenols in the plant sample. This *in vitro* assessment of the antioxidant properties of the methanolic extracts of *G. glabra* validates and confirms its free-radical scavenging activity. Thus, the present data suggest that the *G. glabra* root extract can be used as a good source of natural antioxidants for health benefits.

AUTHORS CONTRIBUTIONS

Annapurani S designed the experimental study and Velvizhi S carried out analysis and coordinated in preparing the manuscript and revision. Both the authors have read and approved the final manuscript.

CONFLICTS OF INTERESTS

All authors have none to declare.

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