

Original Article

ANTIOXIDANT ACTIVITY AND ANTIPROLIFERATIVE ACTION OF METHANOLIC EXTRACT OF LIQUORICE (*GLYCYRRHIZA GLABRA*) IN HEPG2 CELL LINE

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ABSTRACT

Objective: To evaluate the *in vitro* antioxidant activity of liquorice (*Glycyrrhiza glabra*) against H₂O₂ induced oxidative stress in HepG2 cell line.

Methods: Antioxidant activity of methanolic extracts of *Glycyrrhiza glabra* was investigated by measuring total phenolic content using folin-ciocalteu reagent (FCR), free radical scavenging activity by DPPH and ferric reducing antioxidant power (FRAP). The presence of phenolic compounds and flavonoids in the extract was confirmed by Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. Furthermore, the protective effect of methanolic extract of *Glycyrrhiza glabra* against oxidative stress induced by H₂O₂ in HepG2 cells was investigated by MTT assay. HepG2 cells were exposed with five different treatments viz. liquorice, H₂O₂, ascorbic acid, H₂O₂+liquorice and H₂O₂+ascorbic acid, to explore the effect of the extract on malondialdehyde (MDA) production, catalase activity, and glutathione reductase levels.

Results: The total phenolic content estimated in *Glycyrrhiza glabra* extract was found to be 241.47 µg per 1000 µg/ml of methanolic extract. It was found that as the concentration of the extract was increased both the free radical scavenging activity and ferric ion reducing power was also found to increase. LC-MS analysis confirmed the presence of eight different phenolic compounds in the methanolic extract which are possibly contributing to the antioxidant activity exhibited by the extract. It was also observed that liquorice treated HepG2 cells showed lower MDA and higher glutathione and catalase levels as compared to only H₂O₂ treated HepG2 cells where increased MDA production, decreased glutathione reductase and catalase production was observed.

Conclusion: Our results thus conclude that, the methanolic extract of *Glycyrrhiza glabra* can be used as natural supplements in various disease conditions where oxidative stress has been reported.

Keywords: Liquorice, Oxidative stress, H₂O₂, HepG2 cells, Antioxidant activity, Free radicals, MDA, SOD, Catalase

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INTRODUCTION

Liquorice is a general herb of the Asian region and is commonly used in the diet. Roots of liquorice have been found to be useful due to its pharmacological properties such as anti-inflammatory, anticarcinogenic, antiallergic, antimutagenic, antimicrobial, antiulcer activity along with cardioprotective, hepatoprotective and immune modulatory effects [1, 2]. Many studies have shown that the consumption of fruits and vegetables is associated with a reduced risk of developing chronic diseases, such as coronary heart diseases, cancer, diabetes and Alzheimer's disease. The protective effect of these foods is attributed to the presence of phytochemical compounds such as carotenoids, tocopherols, and polyphenols [3]. The yellow color of liquorice is due to high flavonoid content of the plant, which includes liquiritin, isoliquiritin (a chalcone) and other compounds responsible for its antioxidant activity. The isoflavones glabridin and hispaglabridins A and B have been reported to exhibit significant antioxidant activity [1]. Increase in oxidative stress as observed in lifestyle diseases and increasing use of allopathic medicines and chemical compounds there is an immediate need of identification of alternatives which can alleviate the altered health conditions [4].

Phytochemicals especially polyphenols constitute a major group of compounds that act as active antioxidants. They are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because of their stable radical intermediate activity [5]. The reported phytochemical studies showed that liquorice roots contain a saponin such as glycyrrhizin which is 60 times sweeter than cane sugar [6].

Many factors are responsible for inducing oxidative stress and enhancing production of free radicals such as radiation or exposure to heavy metals and xenobiotics. Reported literature also indicate that H₂O₂ induced oxidative stress leads to lipid peroxidation and causes cell damage because of its half-life is longer than that of other reactive

oxygen species (ROS) and it can easily transformed into a hydroxyl radical, which is one of the most destructive free radical [7, 29].

ROS production is a well-established physiological process which is controlled by the intrinsic system of antioxidants. Particularly liver is susceptible to toxic and oxidative insults because the portal vein brings blood to this organ after intestinal absorption. In a concentrated form absorbed drugs and xenobiotics can cause free radicals and ROS production which mediate damage that may result in inflammatory and fibrotic processes [8, 9].

The present study reports for the first time the investigation of the antioxidant properties of liquorice in an *in vitro* model viz. HepG2 cells.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals required in the study were purchased from Sigma: (DPPH), HiMedia: (Potassium ferricyanide, TBA, NADH, Ellmans reagent, Glutathione, MEM, TPVG, Antibiotics: Penicillin and Streptomycin solution, PBS, TBA), MERCK: (DMSO, Potassium chloride), Fisher Scientific: (Methanol, Ascorbic acid), SRL: (TCA, Ferric chloride, Sodium chloride Meta-phosphoric acid, Phenazine methosulphate, Tetrasodium pyrophosphate).

Preparation of plant extract

Liquorice roots were procured from the Department of Agricultural Botany, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India. Roots were washed with water and dried in the shade. The dried plant material was then blended into powder using an electric blender, 250 gm of dry powder was mixed with 500 ml of methanol, and the mixture was filtered through Whatman filter paper No.1. The filtrate was collected in a new sterile flask, and the extracts were concentrated using a rotary evaporator and was stored at refrigerator for further experiments.

Determination of total phenolic content

Total phenolic constituents of plant extracts was performed as described earlier [5, 10] which involves folin-ciocalteu reagent and ascorbic acid as standard. About 1.0 ml of plant extract (5 µg/ml to 1000 µg/ml) was taken in a test tube. Then 5 ml of folin-ciocalteu (diluted 10 fold) reagent solution and 4 ml of sodium carbonate solution (7.5%) was added into the test tube. The test tube was incubated for 30 min at 20°C to complete the reaction. Absorbance was measured at 765 nm using spectrophotometer against blank. The total content of phenolic compounds in methanol plant extracts was calculated as ascorbic acid equivalents.

Free radical scavenging activity by DPPH

Free radical scavenging activity of the extract was evaluated *in vitro* by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described by Hatano T. *et al.*, 1989 and Neeti S. *et al.*, 2014 with some modifications [11, 12]. DPPH solution was prepared by dissolving 24 mg DPPH in 100 ml methanol and stored at -20 °C till further use. 150 µl of this DPPH solution was mixed with 50 µl of the methanolic extract at various concentrations 25 µg/ml to 1000 µg/ml. The reaction mixture was mixed well and incubated in the dark for 30 min. The absorbance was measured at 517 nm using an ELISA plate reader. Blank was prepared without adding methanolic extract. The scavenging activity was calculated using the following formula based on the percentage of DPPH radical scavenged.

$$\text{Percent of inhibition} = \frac{\text{Blank} - \text{Sample}}{\text{Blank}} \times 100$$

Ferric reducing antioxidant power (FRAP)

Antioxidant activity of the extract was measured as described by Asan M. and Karakoca, 2013 with few modifications [13]. Briefly, 40 µl of methanolic plant extracts at various concentrations (1-10 mg/ml) was mixed with 100 µl of 0.2 M phosphate buffer (pH 6.6) and 100 µl of potassium ferricyanide (1%). After incubation at 50 °C for 20 min, 100 µl of 10% trichloroacetic acid (TCA) were added to the mixture followed by centrifugation at 10,000 rpm for 10 min to stop the reaction. 100 µl of the collected upper layer of the mixture was mixed with 100 µl of distilled water, and 20 µl of ferric chloride (0.1%) and the absorbance of the resulting solution was read at 700 nm against a blank. Ascorbic acid was used as positive control.

Liquid chromatography-mass spectrometry (LC-MS)

Identification of major phenolic compounds in the extracts was carried out using LC-MS method as described by Ibrahim K. *et al.*, (2010) with few modifications [14]. Briefly 1 mg root extract was suspended in methanol and dissolved thoroughly and centrifuged at 10,000 rpm for 10 min. 20 µl of the supernatant was suspended in 980 µl of MS grade methanol and used to perform LC-MS analysis. All the solvents used for LC-MS analysis were of MS grade. The LC-MS analysis was performed using thermo qExactive orbitrap mass spectrometry equipped with acela 1250 UHPLC systems. Separation of compounds was achieved using a reverse phase Thermo C-18 column (200 mm x 2.1 µm x 1.9 µm) and mobile phases with a gradient of water and acetonitrile. Data acquisition of the sample was done in a positive mode of ionization to obtain the chromatogram and the spectra. Using the obtained spectra, the identification of the compounds was performed.

Maintenance of cell line

The human hepatoma cell line HepG2 was procured from National Center for Cell Science (NCCS), Pune. Cells passaging was done every third day after cells were at least 80% confluent as per the method described by Jiying J. *et al.*, 2014 [15]. Cells were incubated in a humidified atmosphere and 5% CO₂ in a 37 °C incubator with minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml of penicillin.

Determination of cytotoxicity of plant extracts by MTT assay

MTT assay was carried out as per the method described by Jiying J. *et al.*, 2014 [15]. 2×10⁵ HepG2 cells per well were cultured in 96 well

microtiter plates in a final volume of 100 µl culture medium MEM (containing 10% FBS) per well. After incubation for 24 h at 37 °C and 5% CO₂, when a confluent monolayer was formed, the medium was removed, washed once with plane medium (MEM without serum) and 100 µl different concentrations of methanolic extract of liquorice (10-100 µg) were added to each well and the plate was again incubated for 24 h. 20 µl of MTT in phosphate buffer saline (PBS) solution (1 mg/ml) was added to each well and the plate was further incubated for 4 h in the dark after incubation MTT was removed and 100 µl of dimethyl sulphoxide (DMSO) was added into each well to solubilize the formazan crystals. The absorbance was measured at 490 nm using an ELISA plate reader. Cell viability against H₂O₂ and ascorbic acid was determined using the same protocol. Further, H₂O₂ treated cells were treated with different concentration of the liquorice and effect of methanolic extract of liquorice was then evaluated. The percent growth inhibition was calculated using the following formula.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of experimental group}}{\text{Absorbance of Blank}} \times 100$$

Determination of MDA

Cell lysates of treated and control groups were prepared in 1X PBS. MDA levels were estimated by the double heating method of Draper and Hadley as followed by Kerman M. and Nilgun S. 2012 [16]. 2.5 ml of 100 g/l trichloroacetic acid (TCA) solution was added to 0.5 ml cell lysate in each centrifuge tube and the tubes were placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 g for 10 min and 2 ml of the supernatant was added to 1 ml of 6.7 g/l thiobarbituric acid (TBA) solution in a test tube and the tube was placed in a boiling water bath for 15 min. The solution was then cooled in tap water, and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA-TBA complex (absorbance coefficient = Absorbance/1.56 × 10⁵/cm/M) and was expressed as nM/g units wet tissue.

Glutathione reductase assay

Glutathione reductase activity was determined by a colorimetric assay described by Akerboom TP and Sies H 1981 [17]. In short 2.5 ml of metaphosphoric acid (1.67 g glacial metaphosphoric acid; 0.2 g of EDTA, and 30 g of NaCl in 100 ml of deionized water) was mixed with 1.5 ml of distilled water and 0.5 ml of sample mixed well and 1 ml of mixture was taken in new tube and mixed with 4 ml of Na₂HPO₄. Then 0.5 ml of dithio-bisnitro benzoic acid (DTNB) was added and the reaction mixture was mixed properly. Blank was prepared without addition of cell lysate and absorbance was recorded at 412 nm.

Catalase activity

Catalase activity was determined in freshly sonicated extracts of cells treated with H₂O₂, liquorice and ascorbic acid as described by Jingxiang B *et al.*, 1999 [18]. Decomposition of hydrogen peroxide by catalase was followed by ultraviolet spectroscopy at 230 nm, at different time intervals from 30 to 180 s.

Statistical analysis

All experiments were carried out in at least 3 replicates. One-way ANOVA was applied to determine the significance of results between different treatments. Statistical analysis was carried out using Graph Pad Prism 5 for Windows version 5.03. p<0.05 was considered to be statistically significant.

RESULTS

Liquorice extract was evaluated for its antioxidant activity and cytotoxicity in HepG2 cell line by various *in vitro* assays. The total phenolic content of the methanolic extract of liquorice was measured using the folin-ciocalteu method, and it was found that 241.47 µg of total phenolic compounds was present in 1000 µg/ml of methanolic extract of liquorice.

The free radical scavenging activity of liquorice was assessed by DPPH method and compared with standard antioxidant ascorbic acid. It was observed that the free radical scavenging activity of liquorice

increased as its concentration was increased with respect to ascorbic acid which was used as a standard fig. 1(A). Ferric ion reducing power

of liquorice extract was also found to increase in a similar way as the reducing power of ascorbic acid increased fig. 1(B).

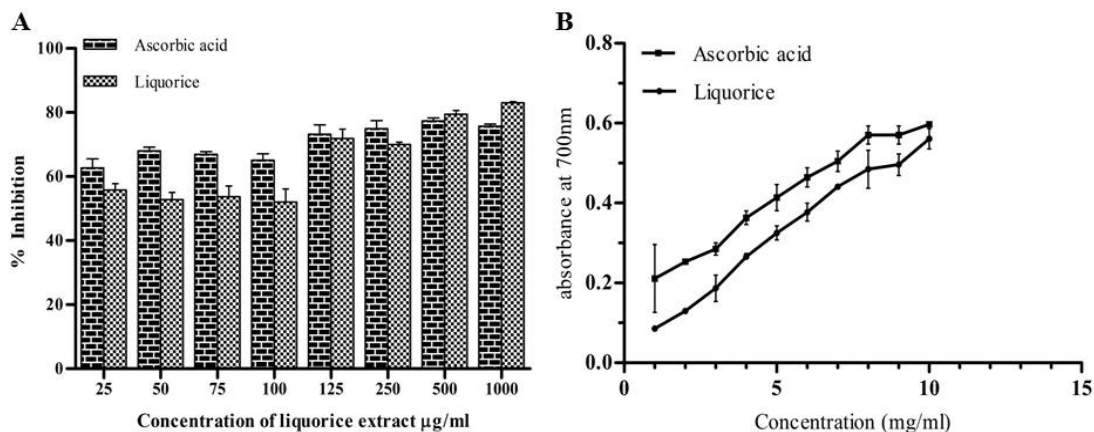


Fig. 1: A. Free radical scavenging activity of liquorice as compared with standard ascorbic acid measured by DPPH assay; B. Ferric reducing the antioxidant power of liquorice measured and compared with standard ascorbic acid. All the values are expressed as mean±SEM, n=3

Table 1: List of eight different phenolic group compounds identified in the methanolic extract of liquorice by LC-MS analysis (Abbreviations: m/z = mass/charge ratio, Sr. No = serial number, gm/mol = grams per mole)

| Sr. No. | Phenolic compounds | Molecular formula | Molecular mass gm/mol | Retention time (RT) min | m/z ratio | References |
|---------|--------------------|---|-----------------------|-------------------------|-----------|------------|
| 1 | Liquiritin | C ₂₁ H ₂₂ O ₉ | 418.39 | 10.77 | 419.30 | 24 |
| 2 | Isoliquiritin | C ₂₁ H ₂₂ O ₉ | 418.39 | 10.77 | 419.30 | 24 |
| 3 | Liquiritigenin | C ₁₅ H ₁₂ O ₄ | 256.25 | 11.56 | 257.08 | 24 |
| 4 | Formonetin | C ₁₅ H ₁₂ O ₄ | 268.26 | 11.81 | 269.08 | 4, 24 |
| 5 | Hispaglabridin-B | C ₂₅ H ₂₆ O ₄ | 390.41 | 13.09 | 391.19 | 4, 24 |
| 6 | Glabridin | C ₂₀ H ₂₀ O ₄ | 324.37 | 15.92 | 325.14 | 24, 25 |
| 7 | Glabrol | C ₂₅ H ₂₈ O ₄ | 329.49 | 16.60 | 393.20 | 4, 24 |
| 8 | Glycyrrhizin | C ₄₂ H ₆₂ O ₁₆ | 822.94 | 16.28 | 823.41 | 4, 24 |

Methanolic extract of liquorice was analyzed using LCMS which confirmed the presence of eight different phenolic compounds (fig. 2) (table. 1) which are known for their antioxidant activity.

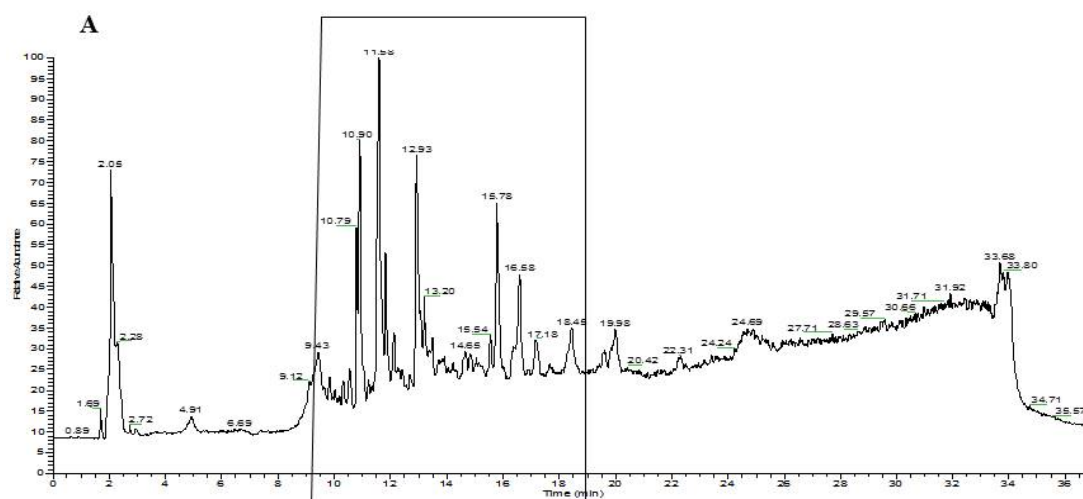


Fig. 2A: LC-MS chromatograms representing different phenolic compounds presents in the methanolic extract of liquorice

H₂O₂ is already known for its oxidant activity, and it tends to induce oxidative stress in the cells. To determine whether liquorice inhibits the H₂O₂-induced cell death in HepG2 cells MTT assay was performed by treating the cells with different concentration of H₂O₂

(50 to 100 µM) for 2 h. The viability of HepG2 was found to decrease by about 50% at the concentration of 30 µM H₂O₂ fig. 3(A). Therefore we exposed HepG2 cells to a concentration of 30 µM H₂O₂ for 2 h to induce oxidative stress for further experiments.

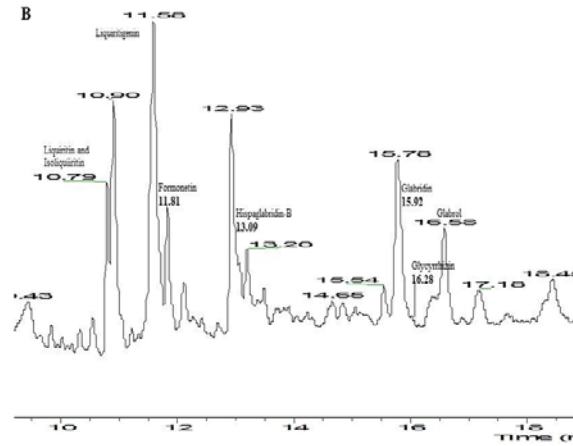


Fig. 2B: Enlarge form of square section of fig 2A representing eight different phenolic compounds presents in the methanolic extract of liquorice

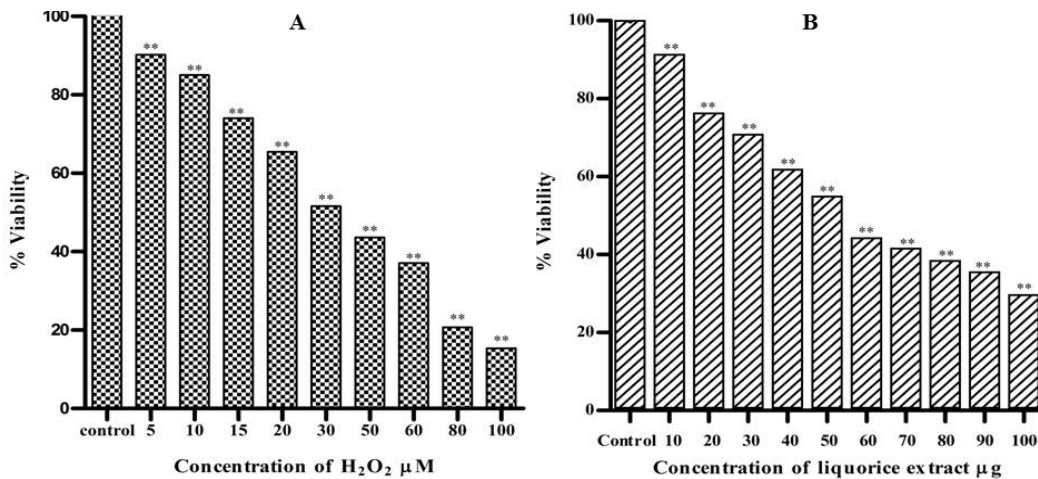


Fig. 3: A. MTT assay of HepG2 cells treated with different concentrations of H₂O₂ (5, 10, 15, 20, 30, 50, 60, 80 and 100 µM); B. HepG2 cells treated with different concentrations of liquorice (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg). All the values are expressed as mean±SEM, n=3. (**p<0.01)

To evaluate the most effective concentration of liquorice, HepG2 cells were treated with different concentration of liquorice (10-100 µg), and found cell viability decreased by about 50% [15] at the concentration of 60 µg fig. 3(B) which was used for further experiments.

The viability of HepG2 cells in the presence of liquorice or in response to H₂O₂ was studied and was compared with standard ascorbic acid. The cell viability of liquorice treated group was found to be almost similar to the standard ascorbic acid. Similar protective effect of liquorice was also found to increase significantly in the presence of H₂O₂ (fig. 4).

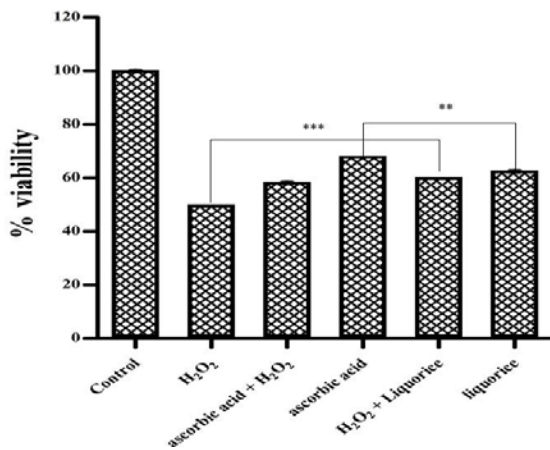


Fig. 4: MTT assay cells treated with treated with 30 µM H₂O₂, 60 µg of liquorice and 60 µg of ascorbic acid. Comparative effect of H₂O₂ with H₂O₂+liquorice and liquorice with ascorbic acid as a standard was determined in HepG2 cells. All the values are expressed as mean±SEM, n=3, (**p<0.01, ***p<0.001)

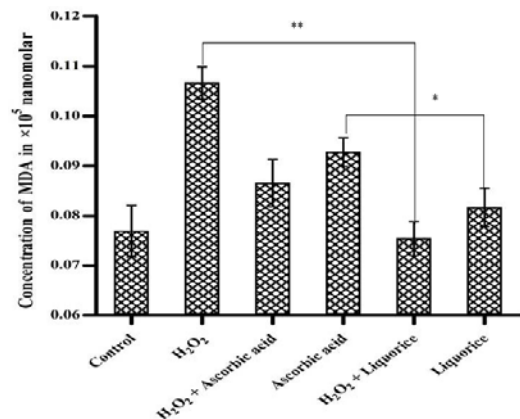


Fig. 5: MDA assay: effect of liquorice extract on MDA production in HepG2 cells treated with H₂O₂ as compared with standard ascorbic acid. All the values are expressed as mean±SEM, n=3. (*p<0.05, **p<0.01)

Malondialdehyde (MDA) is an organic compound which is the end product of the lipid peroxidation of polyunsaturated fatty acids known to induce oxidative stress [19, 20]. The level of lipid peroxidation in liquorice treated cells was evaluated and it was found that the level of lipid peroxidation declined significantly in liquorice treated cells as compared to H_2O_2 treated cells (fig. 5).

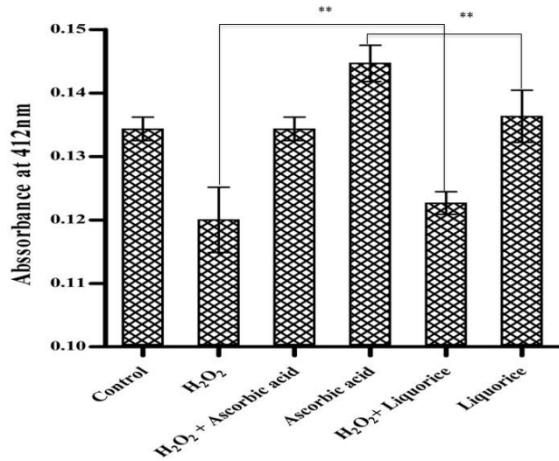


Fig. 6: Glutathione reductase activity of liquorice extract in HepG2 cells treated with H_2O_2 as compared with standard ascorbic acid. All the values are expressed as mean \pm SEM, n=3, (p<0.01)**

Glutathione reductase is an antioxidant enzyme which is responsible for the detoxification process reduces oxidative stress and maintains the reducing environment of the cell [21, 22]. Hence the levels of enzyme glutathione reductase were evaluated in the presence of liquorice and it was found that glutathione reductase levels increased in liquorice treated cells as compared to H_2O_2 treated cells (fig. 6).

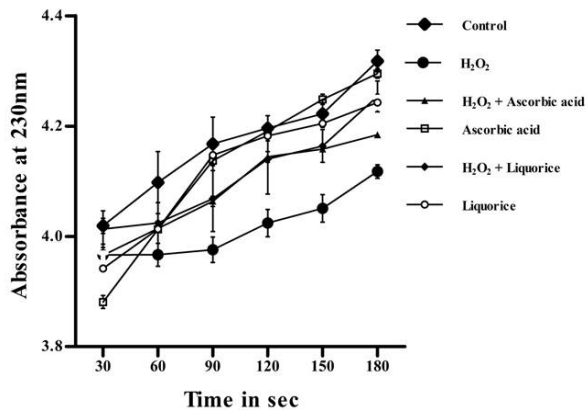


Fig. 7: Catalase activity of liquorice extract in HepG2 cells treated with H_2O_2 as compared with standard ascorbic acid. All the values are expressed as mean \pm SEM, n=3

Catalase is an antioxidant enzyme. The level of catalase also determined in the presence of H_2O_2 and it was found that there was a reduction in catalase production in H_2O_2 treated cells. However, it significantly increased in liquorice treated cells (fig. 7).

DISCUSSION

Many studies have constantly shown that the consumption of fruits and vegetables is associated with a reduced risk of developing chronic diseases, such as coronary heart diseases, cancer, diabetes

and Alzheimer's disease [3, 28]. The protective effect of these foods is attributed to the presence of phytochemical compounds such as carotenoids, tocopherols and polyphenols provided with antioxidant properties. Gupta VK. 2006 [22] reported that the plant extract is an ayurvedic source, and it does not have a side effect on humans.

It is a known fact that the root of *Glycyrrhiza glabra* is one of the richest sources of biological active compounds such as phenolic and flavonoid compounds which are act as primary antioxidants or free radical scavengers [2, 23]. Several investigations [10, 13] have revealed the presence of a variety of bioreactive polyphenols in liquorice extract which was confirmed by our results of total phenol content assay, DPPH assay, reducing power assay and LC-MS. In the present study, the antioxidant activity of methanolic extract of *Glycyrrhiza glabra* was evaluated by various biochemical and *in vitro* assays. The total phenolic content of liquorice methanolic extract was estimated by the folin-ciocalteu method and was found to be 241.47 μ g. The presence of phenolic compounds such as flavonoids, alkaloids, saponins, tannins, phenols indicates that liquorice plant exhibits good antioxidant activity [11, 12]. Determination of free radical scavenging activity in the study was done by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method. The different concentrations of liquorice were compared with standard ascorbic acid and it was found that the free radical scavenging activity of liquorice was higher than the ascorbic acid at higher concentration of liquorice such as 500 and 1000 mg/ml, and on decreasing the concentration the free radical scavenging activity was found to be less as compared to ascorbic acid which are similar to results as reported earlier by Gaitry C. et al., 2013 [2].

The ferric reducing power antioxidant assay (FRAP) assay was also carried out to measure, the reduction of Fe^{3+} to Fe^{2+} by donating an electron. It was observed that increase in the concentration of liquorice increased the reducing power as compared with ascorbic acid. To further confirm the presence of polyphenolic compound LC-MS was carried out, and the presence of eight different known polyphenolic compounds (Liquiritin, Isoliquiritin, Liquiritigenin, Formononetin, Glabridin, Glabrol, Glycyrrhizin, Hispaglabridin-B) was confirmed by LC-MS. Similar findings have been reported earlier by Mohammad AF. 2012 [24]. The isoflavones, glabridin and hispaglabridin A and B which were confirmed by LC-MS analysis have significant antioxidant activity and both glabridin and glabrene possess estrogen-like activity [26]. Glycyrrhizin and glabridin inhibit the generation of reactive oxygen species (ROS) by neutrophils at the site of inflammation [27]. Thus the presence of these compounds in the methanolic extract account for the antioxidant properties of liquorice.

Liquorice extract was evaluated for malondialdehyde production, catalase activity and glutathione reductase activities in HepG2 cells in treated (liquorice, H_2O_2 , ascorbic acid, H_2O_2 +liquorice and H_2O_2 +ascorbic acid) and control groups. The results indicated that the H_2O_2 treated cells exhibited maximum MDA production, lower catalase and glutathione reductase level as compared to other groups, indicating that H_2O_2 treated group undergoes lipid peroxidation. Catalase activity was found to be similar in liquorice treated group and ascorbic acid-treated group.

Glutathione is an essential water-soluble antioxidant it is synthesized from the amino acids glycine, glutamate and cysteine. Glutathione can directly neutralize ROS such as lipid peroxides. Glutathione system includes glutathione, glutathione reductase, glutathione peroxidase and glutathione S-transferases [21]. We observed increased levels of glutathione reductase in liquorice treated groups as compared to other groups. Thus it is evident from our results that liquorice exhibits high antioxidant activity which is due to the presence of several polyphenolic compounds as well as high glutathione reductase and catalase activity.

CONCLUSION

In conclusion, our study has reported that liquorice extract has comparable antioxidant activity with standard ascorbic acid. These results are further validated by LC-MS analysis which confirms the presence of eight different polyphenolic compounds. Thus, liquorice can be used as a natural antioxidant source, which can be used as

natural supplements to combat the oxidative stress in various diseases post it's validation at the *in vivo* level.

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CONFLICTS OF INTERESTS

Declared none

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