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Original Research

Effects of Extraction Media and Techniques on the Antioxidant Properties and Recovery of Phenolics from Roots of *Glycyrrhiza Glabra*

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ABSTRACT

Objective: Medicinal herbs have been widely explored due to their ability to scavenge free radicals and protect from the reactive oxygen species those are involved in several kinds of degenerative diseases. *Glycyrrhiza glabra* is a perennial herb, its extensive soft and fibrous root system is good source of nutrients and antioxidants applicable in different types of diseases and wide range of pharmacological activities including expectorant, antitussive, emollient, anti-inflammatory, antipyretic activities. This study aimed to determine the best combination of extraction media and techniques for the extraction of phenolic compounds from its roots for best utilization in medicinal applications. **Methods:** The extractions were carried out in methanol, chloroform and ether by using soxhlet, ambient and orbital shaker extraction techniques. The antioxidant activity of these extracts was evaluated by determining crucial parameters, such as total phenolic contents, total flavonoid contents, DPPH radical scavenging activity and estimation of antioxidant activity in linoliec acid system. **Results:** We have found that all extraction techniques exhibited a wide range of total phenolic, 9.689 ± 1.015 to 56.7 ± 5.935 mg/g of gallic acid equivalents and total flavonoid content, 9.6153 ± 0.985 to 53.77 ± 4.29 mg/g of quercitin equivalents. In addition, these extraction techniques also inhibited oxidation of linoleic acid by 45.113 ± 2.567 % to 88.516 ± 2.188 % , while DPPH radical scavenging activity in the range of 7.706 ± 0.258 % to 92.627 ± 3.0 %. Moreover, soxhlet extraction technique showed the highest recovery of TPC and maximum AA content, while orbital shaker extraction technique showed the highest DPPH scavenging activity. The extraction efficiency of antioxidant compounds was highest in methanol. **Conclusions:** Analysis of the TPC, TFC and free radical scavenging activity of *Glycyrrhiza glabra* extracts showed differences depending on extraction method and solvent used. Among the tested parameters, more polar solvent with soxhlet extraction is recommended for the extraction of antioxidants from roots of *Glycyrrhiza glabra*.

KEY WORDS: Antioxidant properties; DPPH free radical scavenging activity; Extraction media; Extraction techniques; *Glycyrrhiza glabra*; Phenolics.

INTRODUCTION

For food processors, lipid oxidation is of great economic concern. It is seen that product shelf life is seriously reduced by lipid oxidation. Many food products can be spoiled by oxidation processes, which results in the decrease of their shelf life [1]. In order to protect the food from lipid oxidation, many food industries uses a variety of synthetic oxidants (e.g., BHT and BHA). But, recently these additives have been banned due to health concerns [2]. However, the demand of consumers for natural, healthy products has been increased in recent years. So, researchers are now trying to explore the new sources of natural antioxidants, mainly from medicinal herbs as an alternate to synthetic antioxidants [3, 4].

It is proved by research that natural antioxidants possess nutritional, health promoting and disease preventing properties. Crude extracts of numerous plant species including herbs, spices, cereals, legumes, vegetables and fruits are reported to be rich in polyphenolics and are of increasing interest for the food industry, as they have the capacity to retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The phenolic compounds are also reported to exhibit anti-carcinogenic, anti-inflammatory, anti-artherogenic, immune modulating and analgesic activities [5].

Though numbers of plants have been explored as potential sources of antioxidants, but proportionally growing demands for antioxidants demonstrate exploration of Further newer sources of antioxidants and development of highly sensitive methodologies for the extraction of antioxidants with improved yields. The extraction of phenolic compounds is one of the critical steps in sample preparation. In this regard, different methods have been employed for the extraction of phenolics as well as other antioxidative compounds and significant effects of extraction method on their recovery/yield have been reported.

The selection of extraction method depends upon many factors such as nature of plant material and type of compounds present in the plant [6]. Therefore, researchers have tried various methods such as ambient, soxhlet, microwave and ultrasound-assisted extractions for the highest recovery of compounds of interest [7]. Besides extraction technique, yield of phenolics is also significantly influenced by extraction medium [8]. But choice of

extraction technique and medium is dependent on the chemical nature of antioxidative compounds, which varies from plant to plant. Therefore, proper extraction technique and medium is required to be chosen for individual plant material.

Glycyrrhiza glabra is a perennial herb, belongs to family Fabaceae. The plant is widely distributed in the subtropical and warm temperate regions of the world *Glycyrrhiza glabra* has extensive root system and its soft, fibrous taproot with bright yellow interior is used for medicinal purposes [9]. Furthermore, plant has shown a wide range of pharmacological activities including expectorant, antitussive, emollient, anti-inflammatory, antipyretic activities [10].

Although, a couple of researchers have explored the antioxidant activity of roots of *Glycyrrhiza glabra* [11, 12], but their focus was restricted to either single extraction technique or single extraction media. Therefore, due to prime importance of *Glycerrhiza glabra* a detailed study was required. In this study, we have employed different extraction media and techniques and explored their effects on the antioxidant properties and recovery of phenolics. We have successfully found out the best combination of extraction media and technique and optimized the conditions for the highest recovery of phenolics.

MATERIALS AND METHODS

Samples collection

The roots of *Glycyrrhiza glabra* were purchased from local market of Sargodha and identified by the Dr. Shahid Iqbal Rana, Assistant Professor, Faculty of Chemsitry, University of Sargodha, Sargodha.

Chemicals and Reagents

Methanol, chloroform, pet ether, folin-Ciocalteau reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium carbonate, chromogen reagent, concentrated HCl, Catechin, gallic acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ascorbic acid (Vitamin C) and other chemicals were purchased from E Merck or Sigma-Aldrich. All reagents purchased were of analytical grade.

Drying and Grinding

The roots of *Glycyrrhiza glabra* were washed, oven dried at 40°C and then ground to make coarse powder in an electric grinder. This powder was then passed through sieve number 50 and stored at 25°C in a well-closed container for Further analysis.

Preparation of Extracts

1 kg finely grinded powdered material of *Glycyrrhiza glabra* roots was used for extraction process. Extraction process was performed by using different techniques (ambient, orbital shaker and soxhlet extraction) with three solvents, varying in polarity i.e. methanol, chloroform and ether. In case of ambient extraction, 10g of sample was extracted sequentially with 100 and 200 ml of methanol, chloroform and ether for 7 days. In soxhlet extraction 25 g of powder sample was extracted in 300 and 500 ml of all selected solvents for 3 hours. On the other hand, orbital shaker extraction was performed with ethanol, chloroform and ether for 6 hours at 150 rpm at room temperature. All of these 18 extracts were filtered with Whatmann filter paper. These extracts were concentrated by using rotary evaporator and stored at 0°C for more analysis to study in vitro antioxidant and free radical scavenging activity of *Glycyrrhiza glabra* root extracts.

Total Phenolic Contents (TPC)

Folin-Ciocalteu reagent was used for the determination of total phenolic content from roots of *Glycyrrhiza glabra* [13]. Double distilled water was used to make concentration up to 3 ml from 0.1 ml *Glycyrrhiza glabra* root extract. Folin-Ciocalteau reagent (0.5 ml) was added to it and permitted the solution to stand at room temperature for almost 10 minutes. Additionally, 2 ml of 7% sodium carbonate solution was added and kept in boiling water bath for 1 minute and then cooled. A blue color was developed in each tube because the phenols undergo a complex redox reaction with phosphomolibdic acid in folin ciocalteau reagent in alkaline medium which resulted in a blue colored complex, molybdenum blue. After that, the absorbance was read at 650 nm against blank. The concentration of TPC was calculated by using catechol as standard, and the results were expressed as milligram gallic acid equivalents per gram extract.

Total Flavonoid Contents (TFC)

TFC was measured using colorimetric assay with slight modifications [14]. Briefly, 1 ml of appropriately diluted sample was added to 10 ml volumetric flask, containing 4 ml of doubly distilled water, followed by immediate addition of 0.6 ml of freshly prepared 5 % $NaNO_2$, 5 ml of 10 % $AlCl_3$ after 5 minutes and 2 ml of 1 M NaOH after 1 min. Further; contents of each reaction flask was diluted with 2.4 ml of doubly distilled water and mixed immediately. The absorbance of resulted pink colored solution was noted at 510 nm. TFCs were expressed as (mg/g) quercitin equivalents. All the samples were analyzed thrice and results were averaged.

DPPH Scavenging Assay

The method reported by Brand-Williams et al., (1995) was applied to determine the free radical scavenging activity of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) [15]. During determinations, each extract (10.0 mg) was dissolved in 5 ml of methanol with the help of sonication. Then the serial dilution technique was used to obtain various concentrations such as 2000, 1000, 500, 250, 125 and 50 μg/ml from the stock solution of each extract. From each concentration (2.5 ml) of the

methanolic solution extract was taken out and mixed with 3.75 ml of a DPPH-methanol solution (20μg/ml). After that, it was allowed to stand for half an hour in order to allow the reaction to proceed. Then the absorbance was determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

% inhibition = [1- (ABS sample / ABS control)] x 100

Then % inhibitions were plotted against respective concentrations used and from the graph IC50 was calculated by using ascorbic acid, used as positive control.

Antioxidant activity determination in linoleic acid system

In order to determine the antioxidant activity of *Glycerrhiza* glabra root extract in terms of % inhibition measurement of per oxidation in linoleic acid system, we followed a reported method by Iqbal and Bhanger (2005) [16]. After preparation of solution mixture containing linoleic acid (0.13 ml) and 99.8% ethanol (10 ml), it was stabilized by 0.2 M sodium phosphate buffer (pH 7). Extracts (5 mg) of each treatment were added to a solution mixture and total mixture was diluted up to 25 ml with ultra distilled water. After that, the solution was incubated at 40°C and its degree of oxidation was determined with the help of thiocyanate method. The following reagents were added sequentially; 10 ml of ethanol (75 %), an aqueous solution of ammonium thiocyanate 0.2 ml (30 %), 0.2 ml of sample solution and 0.2 ml of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5 % HCl). The above prepared solution was homogenized by constant stirring for 5-7 minutes and the absorption maximum at 500 nm was measured as peroxide contents. A control experiment was performed with linoleic acid system without utilizing other extracts. Ascorbic acid (200 ppm) and butylated hydroxytoluene (BHT) were used as a positive control. The maximum value of peroxidation was calculated at 360 h, the sample that showed no antioxidant component was used as a test point. % inhibition of linoleic acid per oxidation was calculated to by using following formula:

100 – [(Abs. increase of sample at 360 h/Abs. increase of control at 360 h) 100]

RESULTS

Total Phenolic Content (TPC)

The TPC determined in different *Glycyrrhiza glabra* extracts are shown in Figure 1. The amount of TPC determined in these extracts depends upon the combination of solvent and technique employed. In case of soxhlet, extraction was performed in three solvents, varying in polarity appreciably. Likewise ambient and orbital shaker extraction was also performed by following the same design. In *Glycyrrhiza glabra* root extract TPC ranged from 11.5343 ± 1.933 to 56.7 ± 5.935 mg GAE/ g by using soxhlet extraction. While, TPC ranged from 10.25 ± 2.012 to 41.495 ± and 9.689 ± 1.015 to 20.9 ± 1.958 mg GAE/ g by applying orbital shaker and ambient extraction respectively.

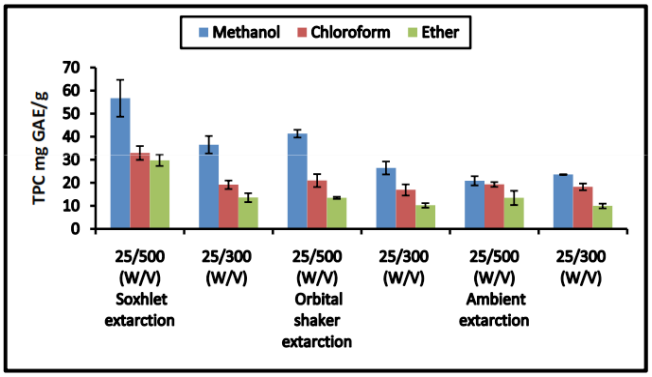

Figure 1. A comprehensive comparison demonstrating amount of TPC in *Glycyrrhiza glabra* root extracts by varying extraction techniques along with extraction media.

Total Flavonoid Content (TFC)

Figure 2 shows the quantity of TFC determined by applying various commonly used techniques with different solvents. In *Glycyrrhiza glabra* root extract TFC ranged from 24.726 ± 0.187 to 53.77 ± 4.29 mg GAE/ g by using soxhlet extraction. While, the amount of TFC ranged from 15.428 ± 1.397 to 49.4 ± 3.0 and 9.6153 ± 0.985 to 29.47 ± 0.60 mg GAE/ g by applying orbital shaker and ambient extraction respectively.

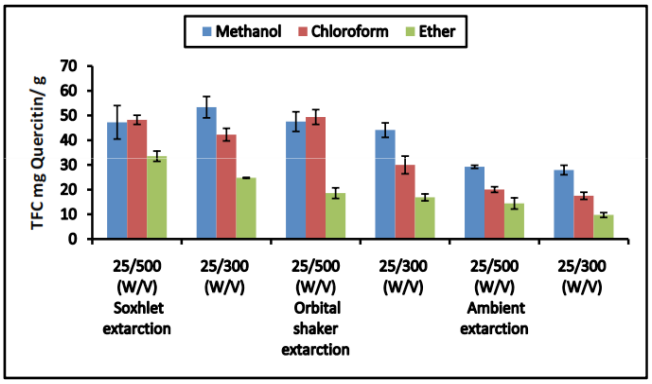

Figure 2. A comprehensive comparison demonstrating amount of TFC in *Glycyrrhiza glabra* root extracts by varying extraction techniques along with extraction media.

DPPH Radical Scavenging Assay

Radical scavenging activity of roots of *Glycyrrhiza glabra* has been determined by using DPPH (a stable radical). DPPH contains the nitrogen as central radical which shows the maximum absorbance at 517 nm. These extracts were prepared in different solvents by applying different techniques. The radical scavenging effect of these extracts

depends upon the combination of extraction media and technique employed. In soxhlet extraction, the maximum effect shown by *Glycyrrhiza glabra* extract against different solvents ranged from 13.597 ± 1.10 to 92.627 ± 3.0 % (Fig. 3). On contrary, the inhibitory effect shown by orbital shaker and ambient extractions were ranged from 10.597 ± 1.003 to 90.45 ± 4.105% and 7.706 ± 0.258 to 70.93 ± 2.75 % respectively (Figure 4, 5). With respect to all of the techniques and solvents, DPPH radical activity was determined at four different concentrations of 2000, 1000, 500, 250, 125 and 50 µg/ ml.

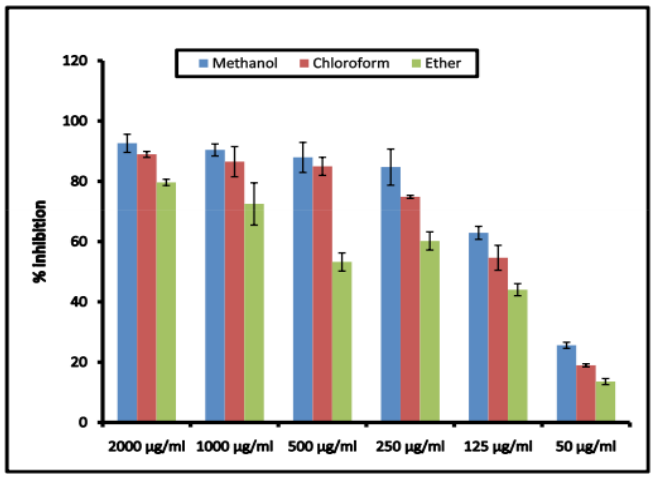

Figure 3. A comparison of radical scavenging activity of *Glycyrrhiza glabra* root extracts (25/500 w/v) under different concentrations (2000µg/ml, 1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, and 50µg/ ml) in different solvents using soxhlet extraction technique.

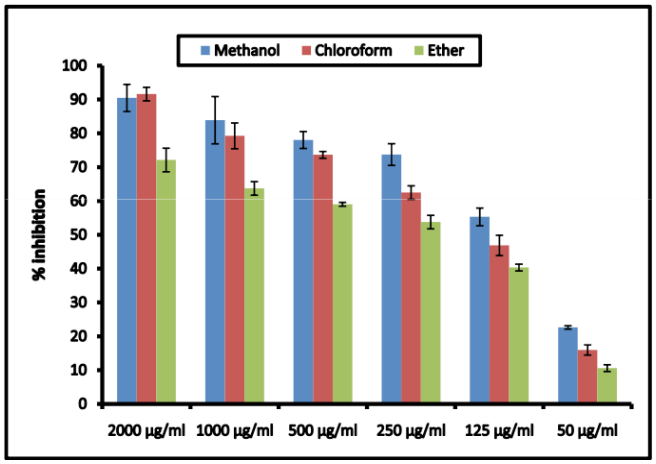

Figure 4. A comparison of radical scavenging activity of *Glycyrrhiza glabra* root extracts (25/500 w/v) under different concentrations (2000µg/ml, 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, and 50µg/ ml) in different solvents using orbital shaker extraction technique.

Inhibition of linoleic acid peroxidation

All the *Glycyrrhiza glabra* root extracts exhibited appreciable inhibition of per oxidation ranging from 45.113 ± 2.567 % to 88.516 ± 2.188 % and were compared with BHT having inhibition of per oxidation 82.7 %, respectively (Figure 6).

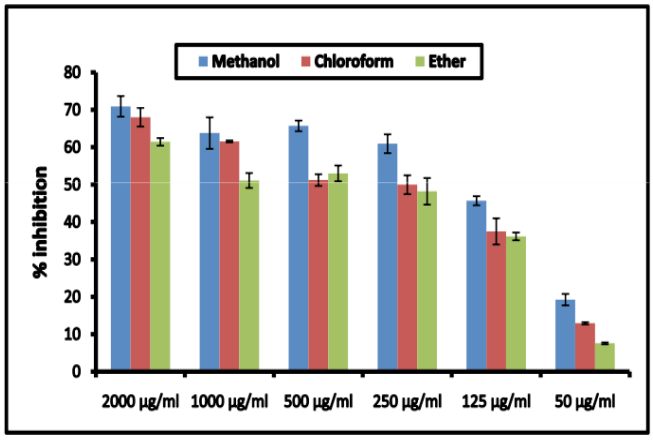

Figure 5. A comparison of radical scavenging activity of *Glycyrrhiza glabra* root extracts (25/500 w/v) at four different concentrations (2000µg/ml, 1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, and 50µg/ ml) in different solvents using ambient extraction technique.

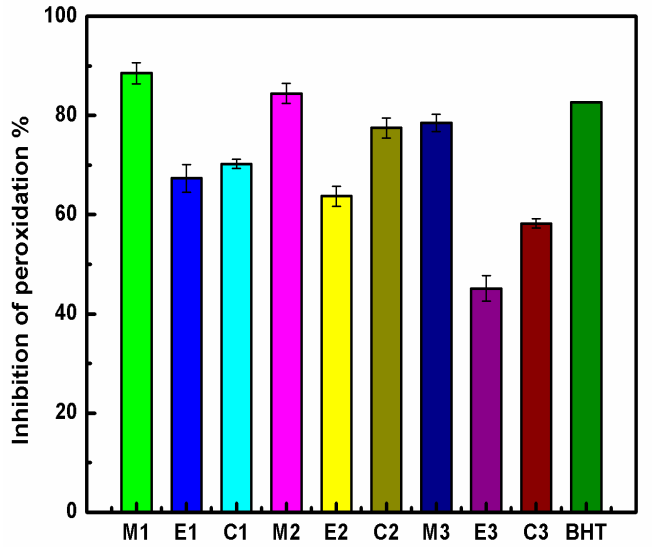

Figure 6. Antioxidant activity in various solvents (5 mg/25 ml) of different *Glycyrrhiza* glabra extracts: M (methanolic extract) E (ether extract) C (chloroform extract). 1, 2, 3 are representing soxhlet extraction, orbital shaker extraction, ambient extraction, respectively and BHT for Butylated hydroxyl toluene.

DISCUSSION

Phenolics are well known antioxidants. These compounds have the ability to chelate redox-active metal ions and they also possess the ability to retard free radical reactions. TPC were determined by using most widely used follin Ciocalteu reagent. Figure 1 shows that TPC is highly affected by extraction technique and solvent effects. Mainly the results obtained by using soxhlet extraction are higher as compared to both other techniques.

In this study, the quantity of TPC from *Glycyrrhiza glabra* root extract is comparable or slightly higher than other plants [17, 18] which resembles to some previous reports [19, 20]. The Recovery of phenolics from plants was highly dependent on their solubility in the solvent used. So, we

have used three solvents of different polarity (Methanol, Chloroform, and Ether) and they showed significantly different quantities of polyphenols extracted from *Glycyrrhiza glabra* plant.

Obtained results showed that TPC generally increased by increasing a polarity of solvents, and a tendency is more pronounced in the soxhlet extraction. The extraction efficiency of solvents can be arranged as follows (starting from non polar solvents) Ether < Chloroform < Methanol. The analyses showed that the highest TPC of *Glycyrrhiza glabra* was extracted by using methanol by all of three extraction methods. Mostly, alcohol and water mixtures are used for the extraction of phenols from plant materials [21]. So, the aqueous methanol possessed the better ability to dissolve a variety of phenols, which was confirmed by our results for *Glycyrrhiza glabra*.

Flavonoids are "high level" antioxidants, including flavones, flavanols, isoflavonoles, anthocyanins and condensed tannins. There are many reports presented on the quantification and identification of flavonoids from liguminacae family. Results of this research are also supporting the presence of flavonoids from the root extract of *Glycyrrhiza glabra* plant. This extraction tendency is seen more in soxhlet extraction followed by orbital shaker and ambient extraction. Figure 2 revealed that TFC amount increased with increasing the polarity of solvents. This study also confirmed that *Glycyrrhiza glabra* root extract possessed higher TFC as compared to other plants [22].

DPPH radical possesses the great ability for hydrogen donation that leads towards retardation of lipid per oxidation. DPPH free radical shows acceptance for electron to become a diamagnetic molecule. The activity of free radical is related with change in color from purple to yellow through radical scavenging in various root extracts of *Glycyrrhiza glabra* plant. The ability of various root extracts of *Glycyrrhiza glabra* to scavenge free radical formation when compared with the quercitin is used as a standard.

All of the sample extracts were analyzed for DPPH assays. By using soxhlet extraction, it did not increase the radical scavenging activity of DPPH considerably because more compounds that are not effective antioxidants, react with Folin–Ciocalteu reagent, are extracted. But overall, use of soxhlet extraction has an edge over the remaining two techniques.

Herein, a detailed study was also conducted for the comparison of better extraction media with all of the three employed techniques. Interestingly it was seen that methanol and chloroform showed very similar radical scavenging activity at all concentrations in case of soxhlet and orbital extraction (Figure 3, 4), while chloroform showed a considerable less activity in case of ambient extraction technique (Figure 5). Ether, being a non-polar solvent showed comparatively less antioxidant activity. Figure 3, 4, 5 also shows a trend that free radical scavenging activity increases in dose dependant manner. Higher the concentration, higher will be antioxidant activity.

Glycyrrhiza glabra root extracts were used to determine the antioxidant activity in terms of % inhibition of linoleic acid oxidation [23]. Linoleic acid itself is a fatty acid (polyunsaturated), which can form peroxides during oxidation process and can oxidize Fe^{2+} to Fe^{3+}, the Fe^{3+} can form complex with SCN^{-1}. The resultant concentration is measured by spectrophotometric method (absorbance at 500 nm). The higher value of absorbance indicated the higher concentration of peroxides and lower level of antioxidant activity.

Methanolic extract of soxhlet and orbital extraction were found to be more effective towards inhibition of peroxidation than BHT. In case of chloroform extraction, both of the extracts exhibited comparable activity with BHT except its extract prepared by using ambient. The effectiveness of methanol and chloroform extract towards inhibition of per oxidation was found to be greater than ether extracts. Furthermore, the results also indicate that ambient extraction is not as effective as the remaining two techniques in the inhibition of per oxidation. It is concluded that the effectiveness of techniques and solvent media are in the following order: soxhlet > orbital shaker > ambient and methanol > chloroform > ether. Considerable inhibition of peroxidation of *Glycyrrhiza glabra* roots could be attributed to presence of established antioxidants, such as xanthones, flavans, flavonols and di-antr-aquinones potentially responsible for the considerable activity of the bark extracts.

CONCLUSIONS

Analysis of the TPC, TFC and free radical scavenging activity of *Glycyrrhiza glabra* extracts showed differences depending on extraction method and solvent used. Soxhlet extraction proved to be a better technique for determination of TPC and TFC but it did not increase the radical scavenging activity of DPPH considerably. It can be concluded that using Soxhlet extraction method more compounds that are not effective antioxidants, react with Folin–Ciocalteu reagent, are extracted. Overall more polar solvent with soxhlet extraction is recommended for extraction of antioxidant from root of *Glycyrrhiza glabra*.

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