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EVALUATION OF DIFFERENT METHODS FOR THE EXTRACTION OF ANTIOXIDANT PHENOLIC COMPOUNDS FROM GLYCYRRHIZA GLABRA ROOTS.

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ABSTRACT

Glycyrrhiza glabra is a medicinal herb of Fabaceae family that contains wide range of biologically active substances including phenols. Several medicinal properties of the plant such as antioxidant and anti inflammatory activity are due to the presence of phenolic compounds. The aim of the current research was to determine a suitable method for efficient extraction of antioxidant phenolic compounds from *Glycyrrhiza glabra* roots. Root extracts were prepared with 70% eathanol using four different extraction methods - Heated Stirred extraction (HSE), Microwave assisted extraction (MAE), Ultrasonication assisted extraction (UAE) and Reflux condensation (RFC). The extracts were tested for the free radical (DPPH˙) scavenging activity and reduction potential. Total phenolic contents of all the extracts were determined. Among the extracts obtained, HSE extracts exhibited highest anti radical activity (84%), highest reduction potential (OD=0.84) and maximum total phenolic content (127 mg gallic acid equivalent per gram dry weight). The GC-MS analysis of the extracts revealed the presence of pharmaceutically acive phenolic compounds like Licochalcone A, Licoisoflavone B, Liquirtigenin, Glabridin, and Hymechromone. Correlation analysis showed that there was a positive correlation between TPC and DPPH antiradical activity (Pearson's coefficient R = 0.671), as well as TPC and reduction potential(R=0.641). The studies led to the conclusion that considerable quantity of wide array of phenolic compounds were obtained by extraction of *G. glabra* roots by Heated Stirred method and that these compounds contribute significantly to the antioxidant activity of the plant.

KEY WORDS: *Glycyrrhiza glabra*; Extraction Methods; Total phenolic content; GC-MS; Reducing power.

INTRODUCTION

Plants contain a number of phenolic compounds which are effective free radical scavengers and used at antioxidants to reduce the oxidative damages during pathological conditions. Free radicals like superoxide anion radicals (O2•-) and hydroxyl radicals (OH•) are generated under oxidative stress which may lead to oxidative modification in cellular membranes or intracellular molecules like proteins, DNA, lipids etc. These radicals degrade proteins and enzymes essential for the human growth and severly affect body metabolism. Synthetic antioxidants and non-steroidal inflammatory drugs (NSAIDs) are commercially available and frequently used to prevent and cure oxidative damages. However, these chemicals are toxic and their risk to health has increased the demand for natural antioxidants.[1] Antioxidant drugs lacking ill effects are being searched all over the world as alternatives to synthetic drugs. Several studies have reported that phenolic compounds in plants significantly contributed to their antioxidant.[2,3] and pharmaceutical properties.[4] In recent years, phenolic compounds from natural sources have been the subject of interest due to their positive effects on human health, attributed mainly to their free radical scavenging activity.

Glycyrrhiza glabra Linn commonly known as Licorice is one of the most recognized plant from the ancient medical history of Ayurveda, both as a medicine and as a flavoring herb. It is popularly used worldwide in food, confectionery and pharmaceutical products, such as cough syrups, herbal supplements, chewing gums, drinks, and candy. Licorice roots and rhizomes are extensively used in herbal medicines for their emollient, anti-inflammatory, anti-viral, anti-allergic, gastro-protective, and anticancerous properties. Various antioxidant properties of *Glycyrrhiza glabra* roots has also been reported.[5-7]

Earlier reports have revealed the presence of phenolic antioxidants such as Glabridin, Glabranin, and Liquirtigenin.[8] But, there is not much scientific documentation on the optimization of extraction conditions of phenolic antioxidants from *Glcyrrhiza glabra* except some phenolic profile and chemistry of the plant. In many cases, prediction of extraction conditions for plant metabolites are subject to several factors and are certainly not straight forward.[9] Therefore, good experimental design and optimization of extraction conditions is necessary for efficient extraction of metabolites. Extraction is infact an important step in the isolation and later in the identification and quantifiication of phenolic compounds. Since the

phenolic compounds of different plants differ structurally, it is very difficult to develop a standardized extraction method that would simultaneously extract all inherent phenolic compounds.[10] The extractability of the phenolic compounds depends mainly on extraction method.

Therefore, the objectives of this study were to examine the influence of different extraction methods on extractability of phenolic compounds from Licorice roots, to measure the antioxidant capacity of resultant extracts and to determine the phenolic profile present in the extracts through GC-MS analysis.

MATERIAL AND METHODS

Plant material and Extraction

The roots of the plant *Glycyrrhiza glabra* were obtained from Herbal garden, Department of Botany, Jamia Hamdard University, New Delhi and shade dried for 3 days. The dried root were pulverized to powder in a mechanical grinder and subjected to different types of extraction methods. Phenolic compounds were extracted from *Glycyrrhiza glabra* roots using 70% ethanol as solvent.

Heated Stirred Solvent Extraction

The extraction was carried out as described by Vijayalakshmi et al.[11] with some modifications.One gram of dried and powdered *Glycyrrhiza glabra* roots were taken into a flask and extracted with three volumes of 70% ethanol. The mixture was heated at 850C for 4 hours with constant agitation. The extract was filtered and re-extracted two times under same conditions. Each time the filtrate was collected in the same flask, filtered and used for further analysis.

Microwave assisted extraction

The extraction was carried out as described by Laghari *et al.*[12] with some modification.The sample was placed into the extraction vessel in addition with solvent up to volume of 50 ml and subjected to extraction in a microwave apparatus, setting the microwave power at 1000W at set temperatures for predefined irradiation time.

Ultrasonication

An extractor equipped with an ultrasonic horn transducer (Model 750W, Sonics &material Inc., USA) working at 20 kHz frequency and 750W input power with amplitude range was used for the extraction. The extraction was carried out as described by Laghari *et al*.[12] with some modification. One gram of sample was extracted with 10ml of 70% ethanol was kept in the ultrasound bath for 30 minutes. Sonication was carried out at room temperature and the extracts were filtered and concentrated in-vacuo.

Reflux condensation

One gram of the powdered root sample was extracted with 50 ml of 70% ethanol in a reflux condensation unit and heated at 45^{o}C for 4 hours with constant condensation. The condensed ethanol was regularly transferred to the extractor and final extract was concentrated in-vacuo.

Determination of Total Phenolics

The quantitative estimation of total phenolics (TP) in extracts was done by the Folin–Ciocalteau colorimetric method taking gallic acid as standard.[13] Extract solution (0.2 ml) was taken in a test tube and equivolume Folin–Ciocalteau reagent was added to it and the contents were mixed thoroughly. After 4 minutes one ml of 15% sodium carbonate (Na_2CO_3) was added. The mixture was allowed to stand for 2 hours at room temperature in dark before the absorbance was measured at 760 nm spectrophotometrically. The concentration of the total phenolics was determined as mg of gallic acid equivalents and the values were presented as the mean ± SE of three replicates.

DPPH radical scavenging activity

Antioxidant activity of the plant extracts was measured on the basis of scavenging activities of the stable 2,2-diphenyl-1-picrylhydraziyl (DPPH˙) radical as outlined by Yu et al.[14] The antioxidant reaction was initiated by transferring 0.5 ml of plant extract into a sample cavity containing 3.5 ml of freshly prepared DPPH˙ methanol solution (0.004 g DPPH˙ to 100 ml methanol). After of incubation in the dark and at room temperature, the absorbance was measured at 517 nm at different time intervals 10, 20, 30 and 40 minutes using a spectrophotometer.

Inhibition of DPPH˙ in percent (I%) of each extract sample was calculated from the decrease of absorbance according to the formula:

$$\mathbf{I\ \% = (A_{blank} - A_{sample}) / A_{blank} \times 100.}$$

where,

A_{blank} is the absorbance of blank consisting of $DPPH^{\cdot}$ in aqueous methanol;

A_{sample} is the absorbance of different extracts.

Reducing power assay

The reducing power of different extracts or fractions were measured according the method used by Hinneburg et al.[15] One ml of sample extracts with different concentrations were mixed with 2.5 ml of phosphate buffer (200 mM; pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and incubated at 50^0C for 20 minutes. The mixture was added with 2.5 ml of 10% TCA and centrifuged at 3000 rpm for 10 minutes. An aliquot of supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%) and the absorbance was measured spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated higher reductive potential.

Chromatographic analysis

The analysis of phenolic compounds in the extracts were performed using GC SHIMADZU QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS).The GC-MS conditions were adjusted as stated further. The sample (2 µl) was injected into a RTX-5 column (60 m x 0.25 mm internal diameter, film thickness 0.25 µm) of GC-MS. Helium was used as carrier gas at a constant column flow of 1.21 ml/min at 85.4 kpa inlet pressure. Temperature programming was maintained from 80°C to 250°C with constant rise of 5°C/minute and then held isothermal at 250°C for 10 min; further the temperature was increased by 30°C/minute up to 310°C and again held isothermal at 320°C for 22 min. The injector and ion source temperatures were 270°C and 230°C, respectively. The crude extract dissolved in methanol (Chromatography grade, Merck, India) was injected with a split ratio of 1:20. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and the total GC/MS running time was 35 minutes.

Identification of phenolic compounds

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns and WILEY 8. The mass spectrum of unknown components were compared with the spectrum of the known components stored in these libraries. The name, molecular weight and structure of the phenolic compounds of the test materials were ascertained.

Statistical methods

Results were expressed as mean value ± SE (n=3). Linear regression analysis was performed to find out the correlation coefficient and to determine the relationship between total phenolic content and antioxidant activity.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

Glycyrrhiza glabra roots are rich source of various phenolic antioxidants. The DPPH· scavenging activity of different extracts was estimated as a function of time at 10, 20, 30 and 40 minutes and was recorded as percentage inhibition of DPPH radical. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 515 nm. This purple colour generally fades in presence of an antioxidant in the medium. Antioxidant molecules quench DPPH free radicals by providing hydrogen atoms or by electron donation, conceivably *via* a free-radical attack on the DPPH molecule, and convert them to a colourless or bleached product (2,2 diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 515 nm.[16] The scavenging activity of DPPH· radical was used as a direct measure of antioxidant potential of G. *glabra* roots due to its simple, rapid, sensitive and reproducible procedure.

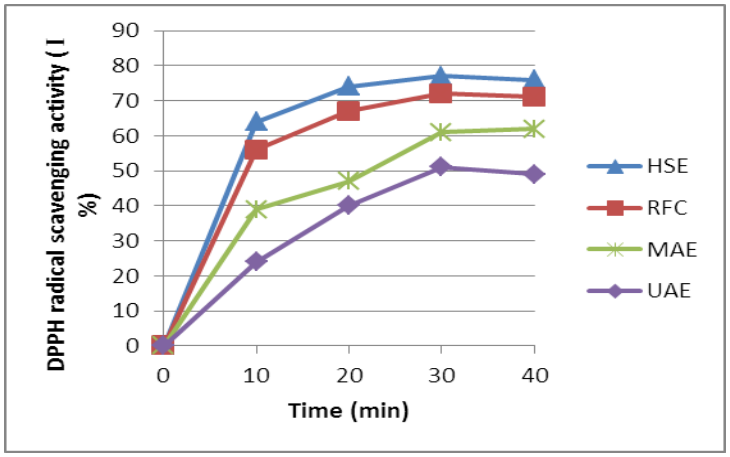

Figure. 1. Time response curve for DPPH radical scavenging activity of *Glycyrrhiza glabra* root extracts obtained from different extraction methods. (HSE-Heated stirred extraction; RFC-Reflux condensation; MAE-Microwave assisted extraction; UAE-Ultrasonication assisted extraction).

The time-response curve for radical-scavenging activity is shown in figure-1. The results of our experiments demonstrated that all of the extracts possessed effective free radical-

scavenging activity. The percentage of inhibition (I %) of DPPH· initially increased with increase in reaction time. Maximum I % was achieved by all extracts at reaction time of approximately 30 minutes culminating into a steady state. The extent of inhibition differed considerably for different extraction methods, which was indicative of the DPPH· scavenging capacity of the extracts. HSE and RFC extracts showed highest DPPH radical scavenging activity upto 77% and 72% respectively, while MAE and UAE were found to be less active in quenching the free radical (I%= 61 and 51 respectively).

Reducing power Assay

Extracts from different extraction methods were tested for their reducing power by assessing the amount of reduction of the Fe^{3+} into Fe^{2+}.[17] Figure- 2 shows the reducing activities of various extracts. The absorbance of the reaction mixture was considered as a direct measure of reducing power. Among all the extracts, HSE extracts showed a highest reducing power as in the case of DPPH activity. MAE extracts were also found to be efficient as reducing agents. The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron which is an important mechanism of phenolic antioxidant action.[18] Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts.[19, 20]

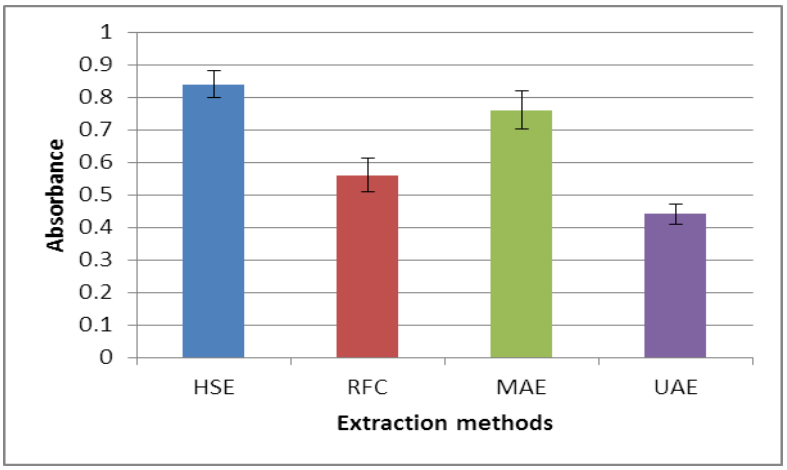

Figure 2. Effect of different extraction methods (HSE-Heated stirred extraction; RFC-Reflux condensation; MAE-Microwave assisted extraction; UAE- Ultrasonication assisted extraction) on reducing power. Values are presented as the mean ± SE of each three replicates.

The high reducing power of HSE and MAE extracts was attributed to higher amount of phytochemicals contained by the two, that could probably act as electron donors and could

react with free radicals to convert them into more stable products and then terminate the free radical chain reactions.

Total Phenolic Content (TPC)

It has been well documented that the phenolic content of plants is strongly associated with their antioxidant capacity.[21] Plant-derived phenolics are well-known natural antioxidants and can contribute directly to the antioxidative action.[22] Phenolic composition of plants extracts is affected by different factors such as plant variety, climate, storage, processing etc. In order to study the effect of different extraction methods on phenolic composition, total phenolic contents (TPC) of each extract were determined using Folin-Ciocalteu reagent. The TPC in root extracts of *Glycyrrhiza glabra* was quantified using gallic acid as standard. A calibration curve was prepared and the total phenolic content of the extract was expressed as mg of gallic acid equivalent per gram of dried extract (mg GAE/ gDW).

Since the recovery of phenolic compounds from plant material is greately influenced by the solubility of the phenolic compounds in the solvent used for the extraction process, we carried out preliminary extractions using different concentrations of ethanol ranging from 50% to 100%. Most suitable solvent was found to be the 70% ethanol which dissolved wide range of phenolic compounds and therefore was chosen for extraction using different methods.

As shown in figure- 3, different extraction methods had a considerable influence on the total phenolic content. TPC determined in extracts obtained from these extraction methods ranged from 20 to 127 mg GAE/ g DW. Results showed that highest TPC was extracted using HSE followed by MAE, RFC and UAE extraction methods. As a result, it could be suggested that HSE is a suitable option to extract phenols in higher amounts using 70% ethanol as a solvent.

Our findings are in agreement with the results of Claudia et al.[11] who reported the heat stirred reactor method to be the best method for obtaining higher yields of phenols and flavonoids in *Bauhinia forficata* Link as compared to UAE and other methods.

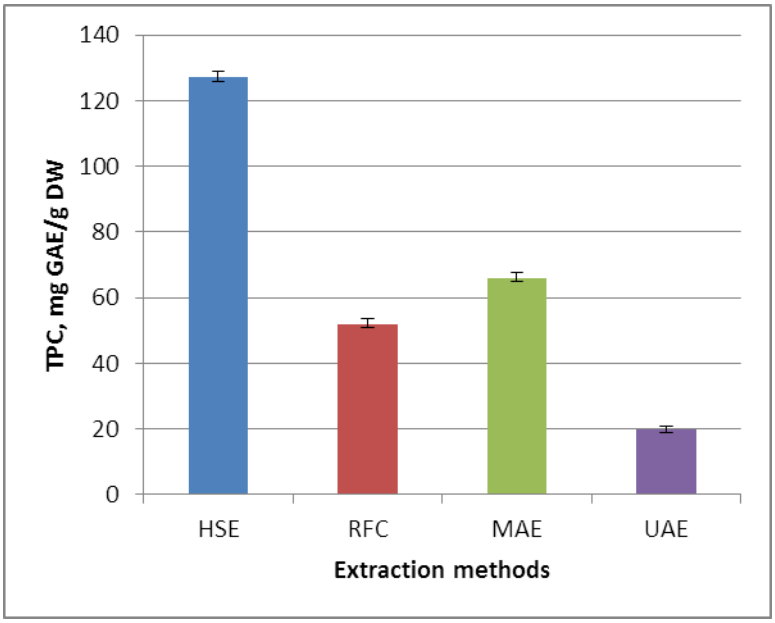

Figure 3. Total Phenolic Content (TPC) in *Glycyrrhiza glabra* roots depending on different methods of extraction (HSE-Heated stirred extraction; RFC-Reflux condensation; MAE-Microave assiated extraction and UAE- Ultrasonication assisted extraction).Values are presented as the mean ± SE of each three replicates.

Correlation analysis

A number of scientific reports signify that certain plant phenolics such as tannins, coumarins, and flavonoids have protective effects against oxidative stress owing to their antioxidant capacities.[23] Taking this into consideration linear correlation analysis was performed to determine relationship between TPC and DPPH· Scavenging activity (Figure. 4) as well as TPC and Reducing power (Figure. 5) of all the extracts.

The results of correlation analysis showed that there was a positive correlation among the antioxidant capacities and Total phenolic contents in the extracts. Our findings are in agreement with the results of some other reports suggesting positive correlation between antioxidant activity and phenolic content in different plant extracts.[24-28] In this study the phenolic content is found to contribute significantly to the antioxidant activity of this plant.

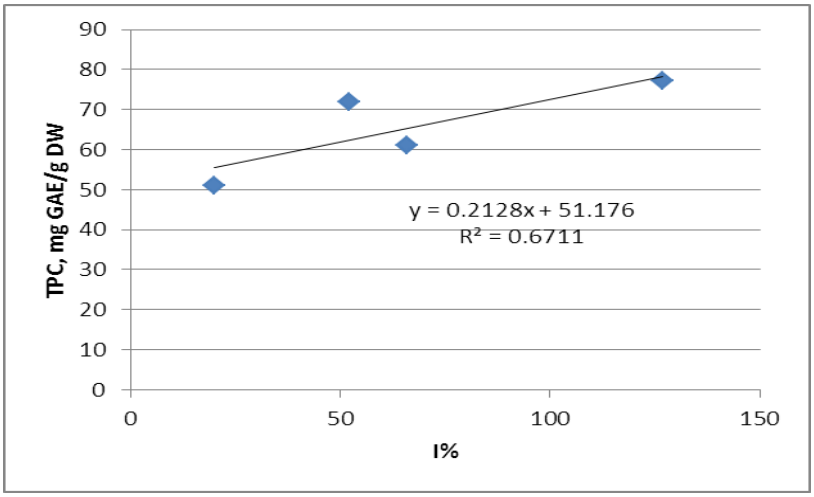

Figure 4. Correlation between TPC and DPPH˙ scavenging activity of Glycyrrhiza glabra roots extracted with different extraction methods. (HSE-Heated stirred extraction; RFC-Reflux condensation; MAE-Microwave assisted extraction and UAE-Ultrasonication assisted extraction).

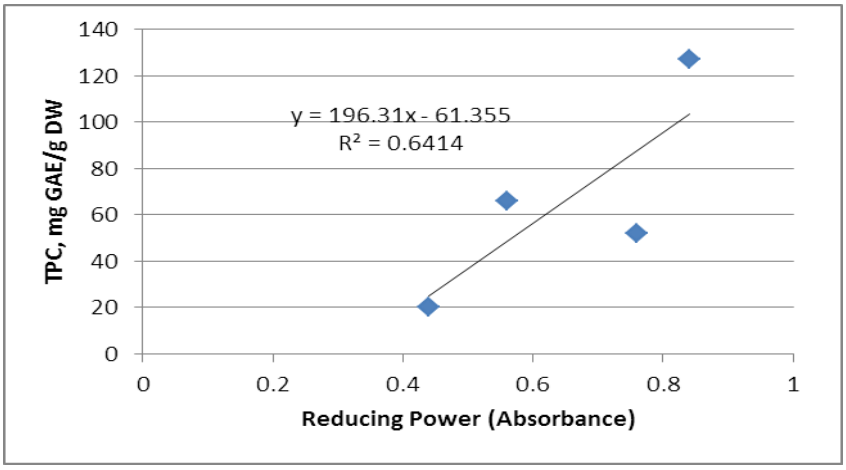

Figure 5. Correlation between TPC and Reducing Power of *Glycyrrhiza glabra* roots extracted with different extraction methods. (HSE-Heated stirred extraction; RFC-Reflux condensation; MAE-Microwave assisted extraction and UAE- Ultrasonication assisted extraction).

GC-MS Analysis

All the ethanolic extracts were further analysed by GC/MS to ascertain their phenolic composition in order to determine the basis of their antioxidant activity. The chromatogram of GC/MS analysis of the HSE, MAE, UAE and RFC extracts are shown in fig. 6-9. Phenolic compounds present in these extracts were identified by comparing the mass fragmentation pattern, molecular weight and molecular formula of compounds to that of

NIST and WILEY-8 library. The GC-MS analysis of the extracts revealed the presence of large number of secondary metabolites including some of the major phenolic compounds of *Glycyrrhiza glabra* root like Licochalcone A, Licoisoflavone B, Liquirtigenin, Glabridin, Hymechromone, (Table 1).

Table 1. Phenolic compounds Identified through the GC-MS analysis of extracts obtained from different procedures (HSE-Heated stirred extraction; RFC-Reflux condensation; MAE-Microave assiated extraction and UAE- Ultrasonication assisted extraction) and their relative peak area%.

COMPOUNDS	Molecular weight	Retention time (RT)	Peak area % at different extraction methods			
			RFC	HSE	MAE	UAE
Hymechromone	176	23.04	0.06	0.2	0.1	-
7-acetoxy 4-methyl coumarine	218	26.2	-	0.1	0.05	-
Glabridin	324	25.03	0.03	1.52	-	0.68
7-Hydroxy-8-(γ-γ-dimethyllalyl flavonaone	308	23.3	-	0.62	-	-
Liquirtigenin	256	30.7	0.09	0.09	0.1	0.78
Licochalcone A	338	30.3	0.03	0.43	-	0.27
Licoisoflavone B	352	27.1	-	0.49	-	-
5,7,8, tri methyl di-hydro coumarine	190	28.1	0.1	0.68	-	-

Pharmaceutically important phenolic compounds were identified from the GC-MS profiles and compared in all extracts. HSE yielded eight phenolic compounds, RFC yielded five compounds and UAE and MAE extracts revealed only three phenolic compounds each. Molecular weight, retention time and peak area percentage of all these compounds are given in Table-1. Glabridin (Peak Area%= 1.52) and Licochalcone A (Peak area% = 0.43) were extracted in good amount by HSE method as compared to other methods.

These phytocomponents are well recognized for their antioxidative action and we assume that these components could also be the contributing factor for antioxidant capacity of these extracts .

CONCLUSION

Selection of an appropriate extraction method is critical for yielding optimum productivity of phenolic compounds from plant extracts. On overall analysis it can be said that heat stirred extraction is the most suitable method for yielding diverse antioxidant phenolic compounds

of pharmaceutical importance in considerable quantity from *Glycyrrhiza glabra* roots and can be preffered over other tested methods. However, methods like UAE and MAE have advantage of less time consumption compared to HSE, the efficiency of extraction can be improved by standardizing the conditions leading to efficient and cost effective production of such compounds.

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