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Full Length Research Paper

Antioxidant activity and GC-MS analysis of *Grewia optiva*

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In this study, total antioxidant capacity (TAC) of extracts of *Grewia optiva* Leaves (*GOL*) was investigated. The antioxidant components were initially extracted in methanol and subjected to partitioning in solvents of different polarity. Using ABTS$^{\cdot+}$ decolorization assay and FRAP assay *GOL* extracts showed a wide range of antioxidant activity. Trolox equivalent antioxidant capacity (TEAC) values for different fractions ranged from 0.09-0.915 mM of Trolox equivalents for *GOL* extracts. FRAP values for different fractions ranged from 22.64-43.28 mg/L $FeSO_4$ equivalents. Using TPC Assay the amount of total phenolics for different fractions of *GOL* ranged from 10.57-78.37 mg/L Gallic acid equivalents. Employing inhibition of lipid peroxidation assay by ferric thiocyanate method, the extracts showed inhibition of lipid peroxidation comparable to Trolox. The Superoxide percentage scavenging activity for different fractions of *GOL* ranged from 4%-12%. Percentage bound iron for metal chelating activity varied from 3.69%-11.96% for *GOL*. On the basis of these results obtained here *GOL* may be considered as a rich source of antioxidant.

Key words: Antioxidants, ABTS radical cation, Free radicals, TEAC, Reactive oxygen species

INTRODUCTION

Oxidation and reduction reactions of molecules occur in every cell. As a result of these reactions free radicals are generated in the cells. A free radical is any Chemical species which is capable of independent existence and possess one or more unpaired electrons. The free radicals are highly unstable molecules and are result of naturally occurring processes such as oxygen metabolism and inflammatory processes. They cause oxidation of bio molecules such as lipids, proteins, and DNA, disturbing normal body functions and may contribute to a variety of diseases. Oxygen centered free radicals, also known as Reactive Oxygen Species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH), peroxyl (ROO^-) and alkoxy (RO^-) are produced in vitro during oxidation (Halliwell, 1992). ROS cause lipid peroxidation that leads to food deterioration and development of the variety of diseases, including cellular ageing, mutagenesis, carcinogenesis, coronary heart disease, diabetes and neuro degeneration (Halliwell, 1990; Larsen, 1993). Although all the living organisms possess antioxidant defense and repair systems to protect against oxidative damage, these systems are insufficient to prevent the damage entirely (Helfand and Rogina, 2004; Sohal , 2002, Zia-Ul-Haq et al., 2012; 2013 a,b,c).

Vegetables and Fruits are sources of different antioxidants, such as, tocopherol, glutathione, carotenoids, ascorbic acid and anthocyanins which protect against oxidative damage. Many plants have been identified as having potential antioxidant activities and their consumption is recommended due to their flavonoides and anthocyanin contents (Gutteridge and Quinlan, 1992; Halliwell et al., 1992; Hu et al., 1992; Reznick et al., 1992; Wiseman and Halliwell, 1993). Bioactive phenols including bio falconoid are good source of antioxidants because of their natural origin and their ability to act as efficient free radical scavengers (Scott, 1988; Daniels, 1988; Burton and Ingold, 1984). *Grewia optiva* is used in medicine for treating indigestion and gastric problems. Fresh leaves are boiled in water to

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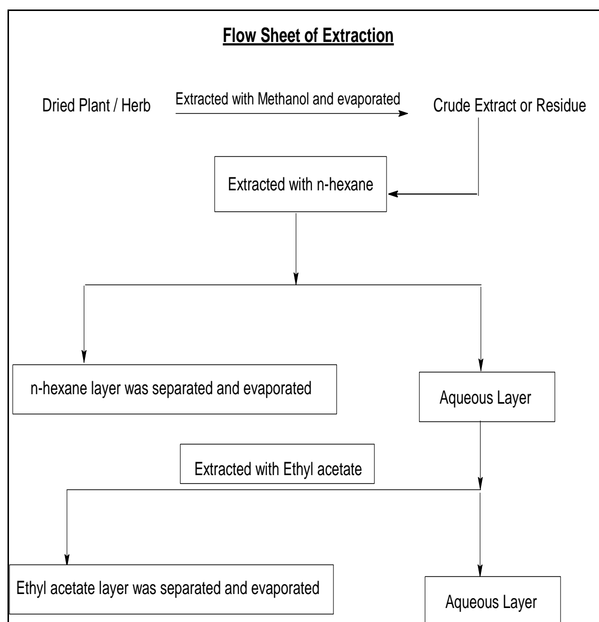

Figure 1. Extraction scheme of *GOL* in different solvents.

prepare decoction which is further concentrated at low temperatures.

The concentrated paste is applied to cure joint pains. Flavonoids from different species of *Grewia* are reported by GC-MS. (Gornell et al., 1979; Gornall et al., 2008; Harborne and Turner, 1984). The current study is performed to evaluate the antioxidant potential of *Grewia Optiva* leaves (*GOL*).

METHODOLOGY

All of the following chemicals which were used during experimental work were of analytical grade. Methanol, n-hexane, Ethyl acetate, Distilled water, Glacial acetic acid, Potassium persulphate, Potassium dihydrogen phosphate, Di potassium hydrogen phosphate, Iron(III) chloride, Hydrochloric acid, Sodium chloride, Sodium acetate were purchased from Merck.

The following chemicals were obtained from Fluka (Switzerland) and were of highest purity grade available. Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (diphenyl picryl hydrazyl chloride), Butylated hydroxy anisole (BHA), ABTS (2,2'-azinobis-(3-ethylbenzo-thiazoline-6-sulphonic) diammonium salt), Butylated hydroxy toluene (BHT), Gallic acid, Ascorbic acid, Uric acid, Nitrotetrazolium Blue Chloride (NBT), Nicotinamide Adenine Dinucleotiode (NADH), Phenozinemetho Sulphate (PMS), and Ferrozine were obtained from Aldrich Chemical Co., Gillingham, Dorset, UK.

Plant

Leaves *(GOL)* sample of *Grewia optiva* were obtained from Kashmir and dried in shade. A finely ground amount (*GOL* 200 gms) of these medicinal plants was soaked in methanol in 10:250 (w/v) in cork-fitted flasks for 24 hours at 30°C and 240 rpm. The next day, extract obtained was filtered and stored at 4°C, while the residue was re-soaked in methanol in the same proportion at 240 rpm and 30°C for 24 hours. The filtrate obtained the second day was mixed with the filtrate of first day. The methanolic residue was obtained by evaporating methanol at 30°C using rotary evaporator. The residue thus obtained was weighed and dissolved in appropriate volume of distilled water. Subsequently different fractions of aqueous extract were obtained using organic solvents (25X3mL) of different polarity according to the scheme shown in Figure 1.

The residue obtained at each extraction step was weighed and subsequently dissolved in appropriate volume of respective solvent to obtain stock solution to be used in antioxidant assays.

GC-MS Analysis

GC-MS analysis of *Grewia* was carried out. GC-MS spectra were recorded on Shimadzu GCMS-QP2010A system in EI mode (70eV) equipped with a split/splitless injector (280°C), at a split ratio of 50/50 using DB-5MS column (30 m×0.25 mm i.d., film thickness: 0.25 µm, J & W Scientific, Fulsom, CA, USA). Helium was used as a carrier gas at the rate of 1mL/min. 1 µL of sample was injected keeping ion source temperature 200°C and interface temperature at 250°C. The column temperature was kept at 100°C for 1 min after injection and then increased at the rate of 10°C min-1 to 275°C which was held for 20 min.

Antioxidant Activity

ABTS$^{•+}$ Assay protocol

ABTS$^{•+}$ Assay, which was followed, was developed by Re *et al.* (1999). 7mM stock solution of ABTS was prepared by dissolving ABTS in doubly distilled water... ABTS radical cation (ABTS$^{•+}$) was generated by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 hrs before use. For the evaluation of antioxidant potential of standard antioxidant and plant samples, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 0.70 (±0.02) at 734 and equilibrated at 30°C. Plant extracts were prepared in the respective solvents. After addition of 10 *µl* of sample to 2.99 ml of diluted ABTS$^{•+}$ solution (A= 0.700 ± 0.020), the absorbance was taken at 25°C exactly, 1 min after initial mixing up to 8 minutes. Appropriate solvent blanks were also run. All determinations were carried out at least three times, and in triplicate at each separate concentration level of the standards. The percentage inhibition of absorbance was calculated by the following formula and was plotted as a function of concentration of antioxidants and of Trolox for the standard reference data.

$$\%\ inhibition_{(at\ 734\ nm)} = (1- A_f / A_o) * 100$$

Where A_o is the absorbance of radical cation solution before addition of sample/standard antioxidants while A_f is the absorbance after addition of the sample/standard antioxidants. Each measurement was made in triplicate and at least three times at each concentration level of standards and sample.

Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay (DPPH Assay)

3 ml solution of DPPH (25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions. The reaction is allowed to proceed at 515 nm over a time period of 30 minutes. Upon reduction, the color of the solution faded. The percentage of the DPPH remaining was calculated as:

$\%DPPH_{rem} = [DPPH]_{rem}/[DPPH]_{t=0} * 100$

A graph showing the scavenging of DPPH radical in terms of decrease in absorbance at 515 nm as a function of time (min) was plotted for each fraction of the samples. EC_{50}, the concentration that causes a decrease in the initial DPPH concentration by 50% was calculated from the kinetic curve (Shimda et al., 1992).

Total Phenolic Content Assay

Total Phenolic contents (TPC), of the extracts were determined by method as developed by Slinkard and Singleton (1997). Gallic acid is used as a standard. Stock solution of gallic acid was prepared by dissolving 0.5 g gallic acid in 10 ml of ethanol in a 100 ml volumetric flask and diluting to volume with double distilled water. 20% Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 ml of double distilled water. After boiling and subsequent cooling of the solution, a few crystals of sodium carbonate were added. The solution was let to stand for 24 hours, filtered and volume was raised to 1L with double distilled water. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 ml of gallic acid stock solution were added into 100 ml volumetric flask separately and then diluted to volume with double distilled water. The resultant solutions contained concentrations of 0, 50, 100, 150, 250 and 500 mg/L gallic acid.

40 µl of sample or blank was pipetted into separate cuvette, and to each 3.16 mL of double distilled water was added. 200µl Folin–Ciocalteu's reagent was added, and mixed well. After 8 minutes, 600 µL of sodium carbonate solution was mixed thoroughly in the solution. The solution was allowed to stand at 40 ^{o}C for 30 min and absorbance of each solution was noted at 765 nm against the blank (without phenolic solution). A concentration versus absorbance linear plot was thus obtained. The concentration of total phenolic compounds of each plant extract in milligram of gallic acid equivalent (GAE), was determined by using the following standard equation.

Absorbance = 0.004x + 0.0723 (Gallic acid (mg/L)

Total Antioxidant Activity Determination

The method developed by Mitsuda et al. (1996) was employed for the determination of total antioxidant activity of aqueous and organic extracts of plant. 100µL each of neat or diluted plant extract the plant was added in 2.4mL of potassium phosphate buffer (0.04M, pH 7.0) and 2.5mL of linoleic acid emulsion in potassium phosphate buffer (0.04M, pH 7.0). Each solution was then incubated at 37°C in sealed bottles in dark for 24 hours. The solution without extract was used as blank, and the solution containing 100µL (50µg/20µL) of Trolox was used as positive control. At intervals of 24 hours during incubation, 0.1mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1mL each of $FeCl_2$ (20mM in 3.5% HCl) and thiocyanate solution (30%) to the ethanolic sample, the solution was stirred for one minute. The absorption values of the solutions measured at 500nm were taken as lipid peroxidation values.

Total Flavonoid Contents

A colorimetric method described by Dewanto et al. (2002) was used for the determination of total flavonoid contents. 250 µL of plant extract or quercetin standard solutions was mixed with 1.25mL of distilled water in a test tube followed by addition of 75 µL of 5% $NaNO_2$ solution. After 5 min, 150 µL of 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for 6 min. 0.5 mL of 1M NaOH was added to above solution and the volume of the mixture was raised to 2.5 mL with distilled water and mixed well. Absorbance was measured at 510 nm and compared to a standard curve of quercetin. The flavonoid contents were expressed as mg of quercetin equivalent per gram of residue.

Ferric Reducing Antioxidant Power

The ferric reducing antioxidant power of plant extract was measured according to the method developed by Benzie and Strain (1996). FRAP solution was freshly prepared by mixing 25 mL of acetate buffer (pH 3.6, 300 mM), 2.5 mL of 10 mM TPTZ solution in 40 mM HCl solution and 2.5 mL ferric chloride (20mM) solution. The mixture was incubated at 37 °C throughout the experiment. 3 mL of FRAP reagent was mixed with 100 µl of sample and 300 µL of distilled water. Absorbance readings were taken at 593 nm after every minute for 6 minutes. Results were compared with standard curve of ferrous sulphate.

Superoxide Anion Radical Scavenging Activity

Superoxide anion radical scavenging activity of GOL was determined using the method employed by Nikishimi et al. (1972). Superoxide radicals were generated by using PMS-NADH system. The reaction mixture contained 100 µL plant extract, 1.5 ml of NBT (200µM), 1.5 ml of NADH (624 µM), and 150µL PMS (80 µM) in 3 ml of phosphate buffer (pH 7.4,0.1 M).The solution is incubated for 2 minutes After the completion of incubation time absorbance was measured by spectrophotometer at 560 nm. The percentage scavenging was calculated using the following formula:

Percent scavenging=[1 – A_s/A_b × 100]

Where A_s and A_b are the absorbance of sample and blank solutions at 560 nm, respectively.

Metal Chelating Activity

Chelating activity of Ferrous ion (Fe^{++}) by plant extracts was evaluated according to the method employed by Dinis et al. (1994). 100 µL of the aqueous and organic extract of plant was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and total volume was raised to 4 mL with ethanol. The mixture was

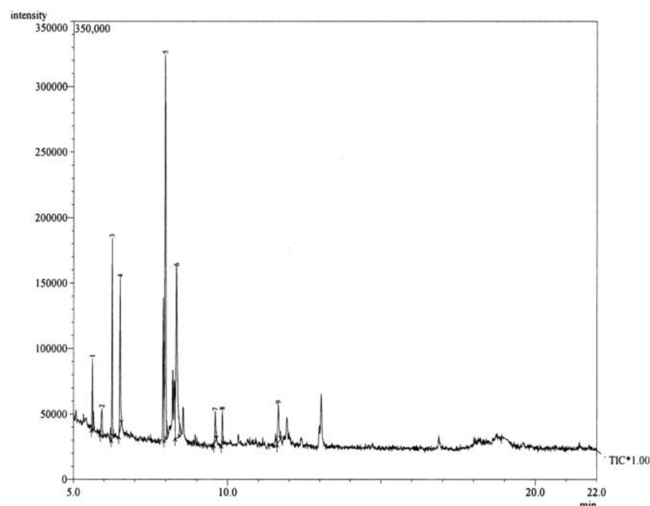

Figure 2. GC-MS Analysis of GOL.

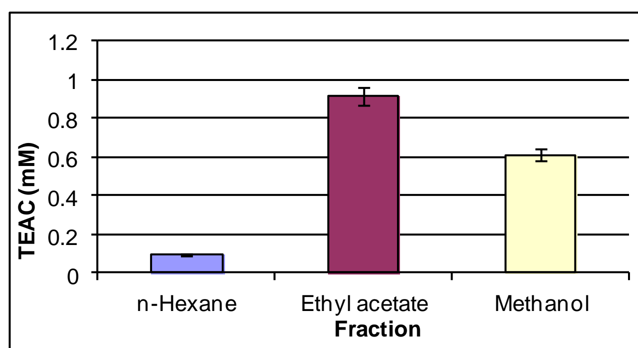

Figure 3. A comparative analysis of TEAC values (mM) of different fractions of GOL using ABTS Assay (pH 7.4)

shaken vigorously and allowed to stand at room temperature for ten minutes. After ten minutes, the absorbance of the solution was measured spectrophotometrically at 562 nm. Te results were expressed as percentage chelating activity of ferrozine-Fe^2. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

% Chelating Activity = [(AControl ASample)/AControl] ×100

Where $A_{Control}$ is the absorbance of the control, and A_{Sample} is the absorbance in the presence of the sample.

RESULTS AND DISCUSSION

GC-MS analysis

The presence of following compounds in GOL was confirmed by GC-MS (Figure 2). The compounds with m/z values are Oxirane, decyl-odecane, 1,2-epoxy-184(5.618), 1-(2,cyano-2-ethyl butyl)3-isopropyl urea 225(5.935), hexadecanoic acid 270(6.260), pentadecanoic acid 242(6.518), cyclopropane octanoic acid 334(7.977), 9,12,15-octadecatrienoic acid, 352(8.343), diazene,butyl[1-(2,2-dimethyl hydrazine)ethyl] 172(9.593), 2-propane amide, N-(1,methyl ethyl)- acryl amide 113(9.827).

ABTS Assay

The basic principle underlying the ABTS decolorization assay is that ABTS on reaction with $K_2S_2O_8$ forms a greenish blue radical cation. Standard and sample antioxidants that are able to transfer an electron to ABTS radical cation scavenge the color of the solution proportionate to their amount. The extent of scavenging depends both upon the concentration of antioxidant and time duration for the reaction.

The method was used to measure the antioxidant activity of aqueous and organic extract of GOL and standard antioxidants by measuring the extent of scavenging of ABTS radical cation on addition of the sample solution at 734 nm for a time period of 8 minutes. Dose-response curves were obtained by plotting the %inhibition of the original absorbance value (fixed at 0.7 ± 0.02 absorbance units) for a range of concentrations of different standard antioxidant compounds. TEAC (Trolox equivalent antioxidant capacity) values of the sample solutions were obtained by comparing the %inhibition with standard curve of Trolox.

The TEAC assay which was originally described by Miller et al. (1993) and later modified by Re et al. (1999) is based upon scavenging of 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^-$) radical anion on addition of sample antioxidants. The extent of scavenging of radical anion, which is detected spectrophotometrically at 734 nm, can be related to the amount of antioxidants present in the sample. TEAC value can be determined for all the compounds/samples which are able to scavenge ABTS radical anion by comparing their scavenging activity with that of Trolox, a water soluble analogue of vitamin E.

A comparative analysis of TEAC values of different fractions of the plant is shown in Figure 3. Ethyl acetate fraction has the highest TEAC value and *n* hexane fraction has lowest TEAC value.

Total Phenolic Content Assay

Phenolic compounds have been reported to be very powerful antioxidants due to presence of hydroxyl groups in their structures (Villano et al., 2007). For *GOL*, the highest phenolic contents were found in methanol while n-hexane has lowest phenolic contents (Figure 4). These results are also depictive of a correlation between the polyphenolic content and the antioxidant activity of the

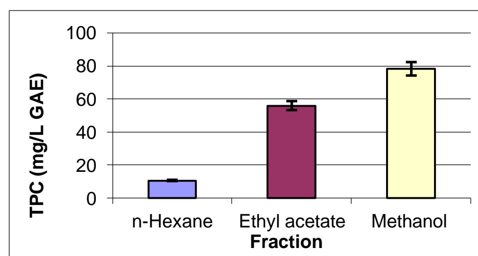

Figure 4. Comparison of TPC analysis of different fractions of GOL

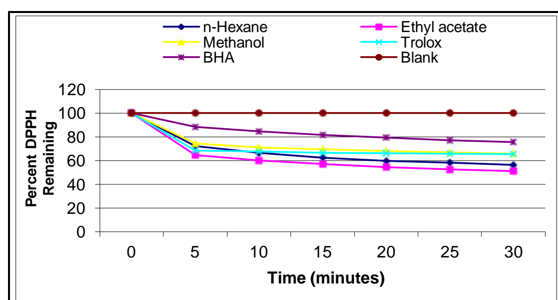

Figure 5. DPPH free radical scavenging activity of Leaves of GOL. Data are mean $\pm$ SD (n=3)

respective fraction, which is in agreement with the already reported results regarding other plants.

DPPH Free Radical Scavenging Activity

DPPH is one of the stable free radicals which are commercially available and widely been used for evaluating scavenging activity of antioxidant standards and herbs/plants extracts (Helfand and Rogina, 2004; Sohal et al., 2002). DPPH radical which is of violet color, accepts an electron or hydrogen atom from the antioxidant compounds and is converted into a colorless or somewhat yellow diamagnetic DPPH molecule (Sohal, 2002).The free radical scavenging activity of aqueous and organic extracts of leaves of *GOL* was estimated. The radical scavenging activity was observed in the decreasing order, Ethyl acetate>n-hexane>Methanol for GOL (Figure 5).

EC_{50} values for each extract of *GOL* was also determined. The data showed n-hexane, methanol, Ethyl acetate, as the best extracting solvents for the GOL (Kitts et al., 2000; Oktay et al., 2003; Huang et al., 2005).

Metal Chelating Activity

Ferrous ion (Fe^{+2}) chelation by plants extracts was

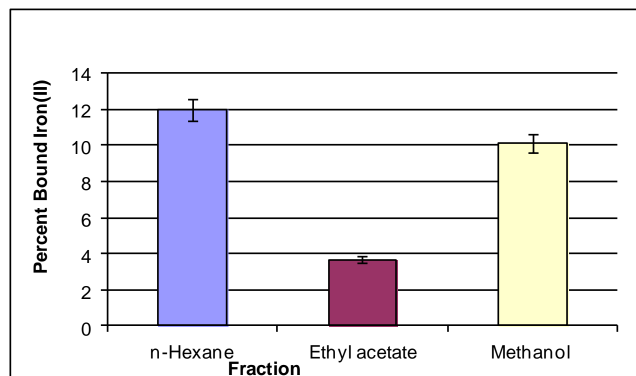

Figure 6. A Comparative study of Metal Chelating activity of *GOL.*

estimated by the Ferrozine assay. Ferrozine makes complex with Iron (II) which can spectrphotometrically be measured at 562 nm. Polyphenolic compounds compete with ferrozine for the formation of this complex and cause a decrease in the absorbance. The results are expressed as percentage of inhibition of ferrozine-Fe^{2+} complex formation. The percentage inhibition of ferrozine-Fe^{2+} complex formation in terms of % bound iron is calculated and graphed for all the fractions of *GOL.* The % bound iron for different fractions ranged from for *GOL* 3.69-11.96 % Higher value of chelation is observed for methanol, ethyl acetate and n-hexane fractions. Transition metals especially Iron (II) found in the biological systems may act as pro-oxidants. A pro-oxidant does not act as harmful agent for bio molecules directly but facilitates production of such species which may cause damage to bio molecules.

Polyphenolic compounds, thus by binding with Fe (II), do not allow them to act as pro-oxidant and thus add to an indirect defense mechanism against potentially harmful radical species. The data shows that *GOL (*Figure 6) extracts contain good amount of chelating agents which are able to engage Fe (II) in the formation of complex with them.

Total Antioxidant Activity in Linoleic Acid Emulsion Systems by Ferric Thiocyanate Method

Alkyl peroxyl radical scavenging activity was determined by ferric thiocyanate method. The Alkyl peroxyl radical formed ($ROO^{\cdot}$) as a result of oxidation of linoleic acid has the ability to oxidize Fe^{2+} to Fe^{3+}. The peroxidation value obtained by spectrophotometer as increase in the absorbance at 500 nm which is due to formation of a complex of Iron (III) with thiocyanate ions (SCN^{-}). In case of standard or sample solutions, the antioxidants will try to inhibit or slow down oxidation of linoleic acid and will therefore, result into low peroxidation value. Thus a low

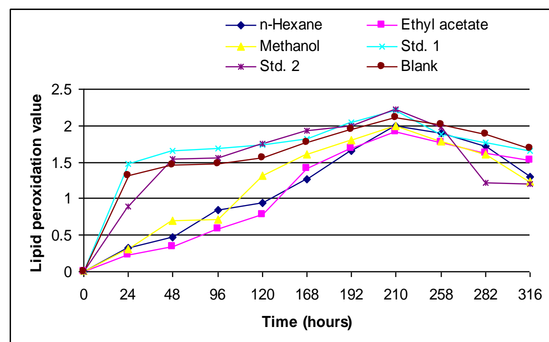

Figure 7. Antioxidant activity (in terms of peroxidation value) of different fraction of *GOL* in the linoleic acid system.

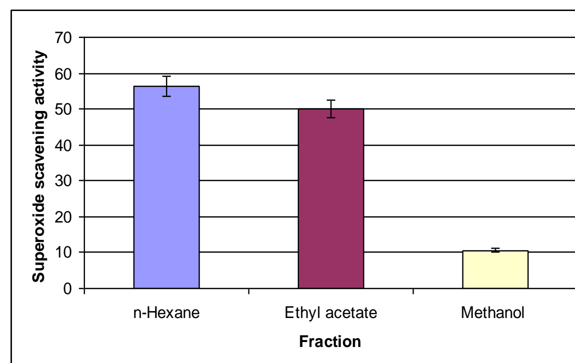

Figure 9. Percentage Scavenging Activity different fractions of *GOL.*

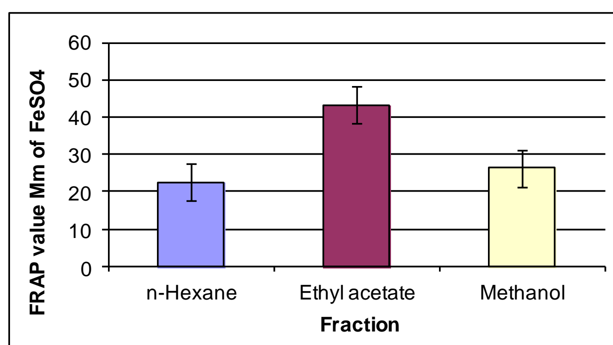

Figure 8. A Comparative file of FRAP assay of *GOL.*

peroxidation value indicates its capacity to inhibit peroxidation of linoleic acid and vice versa. Lipid peroxidation values of different fractions of *GOL* were determined as a function of time. Trolox was used as positive control. Extract of *GOL* has been shown to inhibit the lipid peroxidation and superoxide and hydroxyl radicals *in vitro* (Figure 7).

Our results support the already reported findings as all the fractions of the plants exhibited peroxyl radicals scavenging activity comparable to trolox.

Ferric Reducing Antioxidant Power (FRAP)

The Ferric reducing antioxidant power involves a single electron reduction of the Fe(TPTZ)$_2$(III) complex (pale yellow) to the Fe(TPTZ)$_2$(II) complex (blue) by standards and sample antioxidants at acidic pH. Any antioxidant species with lower reduction potential than that of Fe(III)TPTZ salt (0.7 V) can reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ contributing to FRAP value. The reduction is monitored at 593 nm spectrophotometrically. Appearance of intense blue coloration indicates reducing components in the sample. The original method of Benzie and Strain (1996) uses a 4 minute interval but we noted that the reaction/color change is in progress even after 4-minute interval. Absorbance readings, therefore, were taken at a 6 minute interval after addition of sample to TPTZ reagent allowing the reaction to reach a steady state. The FRAP values of the fractions of *GOL* were measured by way of comparison with a calibration curve obtained using Iron ferrous sulfate as the standard reductant (Figure 8). FRAP values for different fractions of GOL in the range of were 26.24-46.28mg/L. Higher FRAP values were obtained for the samples extracted in more ethyl acetate. It is evident from *(*Figure 8) that the polarity of the extractive solvent has great influence on the extraction of antioxidant compounds.

Superoxide Anion Radical Scavenging Activity

G. optiva extracts inhibited generation of superoxide radical. Superoxide radical is the most representative free radical (Acharya et al., 2011) .In cellular oxidation reactions, superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. Superoxide anion radical actively participate in the initiation of lipid peroxidation. Oxidation of unsaturated fatty acids in biological membranes leads to formation and propagation of lipid radicals, uptake of oxygen, rearrangement of the double bonds in unsaturated lipids, and eventual destruction of membrane lipids, which produce breakdown products (Gardner et al., 1975). For GOL, the percentage scavenging activity ranges from 10.63-56.46 (Figure 9)

Total Flavonoid Content

It has been reported that have Flavonoid compounds are very powerful antioxidants which is because of the presence of phenolic hydroxyl groups in their structures

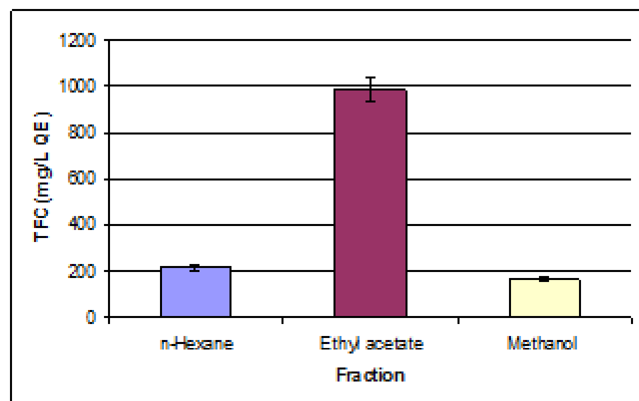

Figure 10. Comparison of TFC analysis of different fractions of leaves of GOL.

(Pahar et al., 2012). An already reported method was used (Dewanto et al., 2002). For *GOL*, the highest flavonoid contents were found in Ethyl Acetate while methanol has lowest flavonoid contents (Figure 10).

Conclusion

The obtained results showed that the ethyl acetate fraction has highest Trolox equivalant antioxidant activity (TEAC value), total flavonoid contents, percent inhibition of DPPH and FRAP value while the n-hexane fraction showed highest metal chelating activity total antioxidant activity and superoxide scavenging activity while methanol fraction showed highest value of total phenolic contents.

Due to the presence of such compounds the ethyl acetate fraction showed good antioxidant activity, the n-hexane fraction showed moderate activity and methanol fraction has lowest values of antioxidant activity due to lower amounts of such compounds. Hence, it was concluded that ethyl acetate fraction and n-hexane fraction are rich in strong antioxidants. These fractions are potentially valuable sources of natural antioxidants and bioactive materials, which would be expected to increase shelf life of foods and fortify against peroxidative damage in living systems in relation to aging and carcinogenesis.

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