Asian Journal of Chemistry; *Vol. 23, No. 8 (2011), 3461-3470*

ASIAN JOURNAL OF CHEMISTRY

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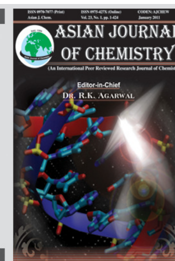

Antioxidant Activity of Gemmo Therapeutically Treated Indiginous Medicinal Plants

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(*Received*: 10 September 2010; *Accepted*: 22 April 2011) AJC-9826

The aim of this study is to investigate and compare the antioxidant potential of gemmomodified and commonly used parts of medicinal plants. Four medicinally important plants including *Terminalia arjuna*, *Euphorbia tirucalli*, *Trigonella foenum* and *Cyperus rotundus* were selected. Methanolic extracts of commonly used parts like bark of *Terminalia arjuna* seed of *Trigonella foenum*, rhizomes of *Cyperus rotundus* and whole plant of *Euphorbia tirucalli* were used for evaluation of antioxidant potential. In addition to the conventional use of medicinal plants, a newly emerging way of therapy, the gemmotherapy (gemmomodificaion) has also been included in this study. Gemmotherapy is a therapeutic method, where plant bud extracts and other young tissues freshly harvested from living growing plants are used. The determination of total polyphenols of both native and gemmomodified extracts was carried out by Folin-Ciocalteu method. The ability of extracts to neutralize the free radicals was measured using standard DPPH$^•$, ABTS$^{•+}$, NO$^•$ and O_2^- radical scavenging assays. The ability of extracts to inhibit the lipid per-oxidation was evaluated through ammonium thiocyanate assay. The reducing potential of medicinal plants was also evaluated. The total polyphenolic contents in gemmomodified extract *Terminalia arjuna* was comparable to its bark extracts. Non-significant difference was found in amount of polyphenols in both gemmomodified and whole plant extracts of *Euphorbia tirucalli*. Gemmomodified extract of *Trigonella foenum* found higher polyphenolic contents than seed extracts. Whereas in case of *Cyperus rotundus* less polyphenolics was identified in gemmomodified extract as compared to rhizome extract. Gemmomodified extracts of *Terminalia arjuna*, *Trigonella foenum* and plant *Euphorbia tirucalli* showed higher antioxidant potential than their commonly used parts.

Key Words: Antioxidant, Medicinal plants.

INTRODUCTION

Gemmotherapy is a new and less studied field. Gemmotherapy is super active form of medicine, made from embryonic tissues of various trees and shrubs (buds and young shoots), reproductive parts and newly grown tissues. The powerful medicinal potential of fresh germinating parts of plants is getting attention of scientists working in various biological disciplines. These parts of plants are rich in hormones like gibberellins, various nutrients vitamins and enzymes, present only at this stage of plant growth. Fast metabolism in growing tissues causes oxidative stress, so a large number of antioxidants are also produced to attenuate this oxidative stress. Gemmo therapeutically treated plants act on cellular level and has strong power of cleansing cells by removing toxic metabolites. Synthetic pharmaceuticals and other herbal medicines (prepared from whole plant) may not have many key element (growth factor, phyto hormones, nutrients vitamins antioxidants), because these principles compounds disappear during the process of plant maturation and development. Moreover gemmotherapeutically treated remedies are safe, abundance in nutrients and impart positive effect on cure and recovery of human health.

Free radicals are reactive species generated in the body as a result of many metabolic pathways like respiration and cell mediated immune functions. Free radicals are also introduced through exogenous sources such as environmental pollution, pesticides and exposure to radiations[1]. They are categorized as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Reactive oxygen species include free radicals like super oxide anion (O_2^-), hydroxyl radical (OH$^•$) and non-radical species like hydrogen peroxide (H_2O_2) and singlet oxygen. Reactive nitrogen species include NO$^•$, $NO_2^•$ as free radicals and HNO_2, N_2O_4 as non-radicals. Different environmental factors and aging elevate the level of free radicals and cells become unable to work efficiently against the free radicals leading to accumulation of radicals and oxidative stress which results in cellular damage[2]. Reactive oxygen species and RNS deteriorate many biological molecules like fatty acid, lipids, proteins and DNA and become major cause of heart diseases, diabetes, cancer, inflammations and weak immune system[3-8].

Nature has gifted the defense system to protect the body from injurious effects of free radicals. Which include enzymatic defense system like superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), *etc*. and non-enzymatic are vitamins (A, C, E) and polyphenols[9,10]. All of these acts as antioxidants and maintain the level of free radicals in the body. Their mode of action varies from one and other and the most common are, reduction, scavenging of free radicals and singlet oxygen, formation of complexes with prooxidant metals[11-13]. The balance between antioxidants and oxidation is believed to be essential for healthy biological system[14].

From last few years interest in studying and quantifying the antioxidant components of fruits, vegetable and medicinal plants has been increased due to their potential health benefits. Phenolic antioxidants stop oxidation in food system as well as in human body and defend from the detrimental effects of free radicals. Polyphenols are the bounteous antioxidants in the diet. Their total consumption as diet could be much higher than other groups of phytochemicals and recognized nutritional antioxidants like vitamin C, E and carotenoids[15,16]. Polyphenols as source of antioxidants play incredibly imperative role to the prevention of cardiovascular diseases, cancers, osteoporosis and neurodegenerative diseases and diabetes mellitus[17].

There is a great need for effective antioxidants from natural sources in order to prevent the free radicals associated diseases. Recent interest in phenolic compounds and their antioxidant properties prompted this research work to explore the beneficial health properties of many indigenous medicinal plants. Four indigenous medicinal plans were selected for this research project. The brief description of these four plants is given in following paragraphs.

Terminalia arjuna (arjun) is very important medicinal plants. Its bark is mostly used as medicines; most important use is in cardiovascular diseases and showed significant antioxidant activity[18-20]. It improves the blood circulation and used as anticholesteremic agent[21,22]. It is nephro and hepato protective[23] and stomach tonic[24]. It shows antimutagenic[25], antibiotic[26] and anthelmintic activities[27]. Arjuna immuno modulatory potential is still controversial and limited.

Trigonella foenum (methi) is commonly used herb and favorite vegetable in Pakistan. It contains flavonoids and phenolics. Its seed extract possess strong antidiabetic[28-30] as well as anticholesteremic activities[31-33]. It also possess antiinflammatory, antipyretic[34-36], hepatoprotective[37,38] and antimicrobial potential[39].

Euphorbia tirucalli (milk bush) showed antioxidant[40] hepatoprotective[41] and antimicrobial potential[42].

Cyprus rotundus (motha) is reported as antioxidants[43-45], wound healer[46] antidiabatic[47], antidiarrheal[48] and antimicrobial agent[49].

EXPERIMENTAL

Following analytical grade, Merck and Sigma chemicals and reagents were used during this research plan. DPPH (1-1-diphenyl 2-picryl hydrazyl), 2,2-azino*bis*-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), nitro blue tetrazolium (NBT), Folin-Ciocalteu reagent, butylated hydroxyl toluene (BHT), sulphanilamide, phosphoric acid, N-1-napthyl ethylenediamne dihydrochloride, hydroxylamine hydrochloride, potassium ferricyanide, ammonium thiocyanate, linoleic acid, ferrous chloride, ferric chloride, methanol, acetone, *n*-hexane and ethyl acetate.

Collection of plants: Medicinal plants were collected from Botanical garden, University of Agriculture, Faisalabad and Ayub Agriculture Research Center, Faisalabad and got identified from plant taxonomist, Department of Botany University of Agriculture, Faisalabad. Four medicinally important and locally available plants were selected for this study.

Name of plant	Common name	Parts used
1. *Terminalia arjuna*	Arjun	Bark, yound buds and shots
2. *Euphorbia tirucalli*	Milk bush	Whole plant, fresh buds
3. *Cyprus rotundus*	Motha	Rhizome, buds
4. *Trigonella foenum*	Methi	Seed, young leaves, shots

Fresh parts (buds and shoots) of plants were washed thoroughly and ground into paste for the preparation of gemmo modified extract. Other parts of plants were dried under shade and changed into fine powder form and kept into air tight containers.

Extract preparation

Solvent extracts: The plant material (30 g) used to prepare extract containing active compounds, was subjected to 12 h extraction with methanol. The extract was then filtered and solvent was completely evaporated with rotary evaporator under reduced pressure approximately at 40 ºC. Yield of extract was noted and stored in refrigerator at 4 ºC for further use in investigations.

Preparation of gemmo modified extract: Embryonic tissues (buds, shoots and young leaves) of plants were procured in spring season. Remedies formed from buds and shoots are very active form of medicines, as in spring season growth cycle of the plant is on its peak and blessed with high amount of enzymes, growth factors and defense phytochemicals (polyphenols). These chemicals exert strong medicinal potential than whole plant extracts.

Paste of plant material (100 g), which is freshly harvested from plants during their growing stage was macerated with 1 L mixture of glycerin and methanol in a ratio of 1:2 and shake vigorously. After 1 month macerate was filtered and solvent was removed with rotary evaporator and crude extract was stored in refrigerator till further analysis.

Determination of total polyphenolics: The total polyphenolic contents of all extracts of plants were carried out colorimetrically by means of Folin-Ciocalteu method[50,51].

One mL of plant extract solution (10 mg/mL) was mixed with 5 mL Folin-Ciocalteu reagent (10 times diluted) and sodium carbonate (4 mL, 20 %) in a test tube and shaken thoroughly. This mixture was kept for 2 h at room temperature for the development of blue colour. Then absorbance was noted at 765 nm with spectrophotometer. Quantification of total polyphenolics was done with respect to calibration curve prepared from different concentrations (10-100 µg/mL) of gallic acid. The results of total polyphenolic contents were expressed in terms of mg gallic acid equivalent (GAE)/g of

dry plant material and per gram of fresh plant material in case of gemmo modified extract. All extracts were assayed in triplicate.

Evaluation of antioxidant activity: Assessment of antioxidant activity of different extracts of medicinal plants was proceeded by following six assays.

DPPH radical scavenging assay: Radical stock solution of DPPH (0.1 mM) was prepared in methanol. Different concentrations (10-100 μg/mL) of test samples (3 mL) were added in DPPH radical stock solution (1 mL) and incubated at ambient temperature in dark for 20 min. After incubation absorbance was recorded[52] against blank at 517 nm. Butylated hydroxy toluene (BHT) was standard of choice for antioxidant activity. All analysis was performed in triplicate. Inhibition of DPPH radical in term of percentage (%) was calculated by following formula:

$$\text{Inhibition of DPPH radical (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

A_c = Absorbance of control, A_s = absorbance of sample.

ABTS radical scavenging assay: Experiment was performed according to the method of Re *et al.*[53]. Briefly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by the oxidation reaction of ABTS with potassium per sulphate. Radical cation solution was prepared by mixing the equal quantities of aqueous solutions of ABTS (7 mM) and potassium per sulphate (2.4 mM). Then, this mixture was kept in dark to react completely for 14 h at 37 ºC. After reaction period the methanol was added into dark coloured $ABTS^{\bullet+}$ radical solution to adjusted the absorbance value equal to 0.700 at 734 nm. Then, 2 mL of diluted ABTS solution was mixed with various concentrations (10-100 μg/mL) of plant extracts (0.2 mL). Absorbance was noted after 6 min of the mixing against blank. All analysis was performed in triplicate. ABTS radical scavenging ability of all extracts was compared with BHT. ABTS radical scavenging activity was calculated according to following formula:

$$\text{Inhibition of ABTS radical (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

A_c = Absorbance of control, A_s = absorbance of sample.

Super oxide radical scavenging assay: Experiment was conducted according to the method of Vaidya *et al.*[25]. Plant extracts (1 mL), sodium carbonate (1 mL, 10 %), NBT (0.4 mL 150 μM) and EDTA (0.2 mL, 1 %) were taken in a test tube. Absorbance was recorded immediately after the mixing of above reagents. The reaction was commenced with the addition of hydroxylamine hydrochloride (0.4 mL) into above solution. After the incubation period of 5 min at 25 ºC, the reduction of NBT in terms of absorbance was noted at 560 nm. Control and standard BHT (synthetic antioxidant) were also run in the same way. All analysis was performed in triplicate. Super oxide radical scavenging activity was calculates as:

$$\text{Inhibition of super oxide (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

A_c = Absorbance of control, A_s = absorbance of sample.

Nitric oxide scavenging assay: Nitric oxide scavenging assay was performed according to the procedure of Balakrishnan *et al.*[54]. Different concentrations of extracts (10-100 μg/mL) were prepared in methanol. Solution of sodium nitroprusside (5 mM, 2 mL) prepared in phosphate buffer saline was added into 2 mL of plant extract and this mixture was kept at ambient temperature for 0.5 h. A control without test sample was also performed with similar procedure. After incubation period of 0.5 h, equal quantities (1.5 mL) of above incubated solution and Greiss reagent were mixed and left it stand for 0.5 h. After this period absorbance of colour complex was noted at 546 nm. Standard antioxidant BHT was used as reference. Percentage scavenging of nitric oxide was measured with reference to control as follow:

$$\text{Inhibition of nitric oxide (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Ac = Absorbance of control, A_s = absorbance of sample.

Determination of reducing power: Reducing power was measured according to the method described by Yen and Chen[55]. Potassium ferricyanide $[(Fe^{3+}CN^{6-})]$ was reduced into $[Fe^{2+}(CN)^{6-}]$ by direct donation of electron from antioxidant compound. After the reduction, Fe^{2+} formed the blue complex which was quantified by measuring the absorbance at 700 nm. Increased in absorbance indicates high reducing power.

Equal volumes of (2.5 mL) phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1 %) were added into varied concentrations of plant extracts (2-10 mg/mL) in test tubes and shaken thoroughly. Then, this mixture was kept in oven at 50 ºC for 20 min. After incubation time trichloroacetic acid (2.5 mL, 10 %) was mixed with this mixture and centrifuged for 10 min. In the last 2.5 mL supernatant solution of this centrifuged mixture was mixed with freshly prepared solution of $FeCl_3$ (0.5 mL, 1 %). The blue colour was developed which absorbance was recorded at 700 nm. BHT was best choice as standard.

Linoleic acid per-oxidation inhibition assay: Antioxidant activity of various extracts was determined by ammonium thiocyanate assay[56]. Linoleic acid (0.248 g) was homogenized with 0.248 g of emulsifier (Tween 20) in phosphate buffer to prepared linoleic acid emulsion. Then this emulsion (2.5 mL 0.02 M, pH 7) with phosphate buffer (2 mL, 0.2 M pH 7.0) was mixed with one mL aqueous solution of plant extract (500 μg/mL). This reaction mixture was kept in oven at 37 ºC for 72 h. Incubated sample (0.1 mL) was taken after regular interval of 12 h and mixed with $FeCl_2$ (0.1 mL, 0.02 M in 3.5 % HCl) and ammonium thiocyanate (30 %, 0.1 mL). Absorbance of the solution was noted with in 3 min after mixing. Peroxides value in terms of absorbance was measured. A control without extract was proceeded through similar method. All values are compared with synthetic antioxidant. Percentage inhibitation was calculated with following formula.

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - \left[\frac{A_s}{A_c} \times 100\right]$$

A_c = Absorbance of control; A_s = absorbance of sample.

RESULTS AND DISCUSSION

Determination of total polyphenols by Folin-Ciocalteu method: In recent years interest in plant polyphenols has increased due to their neutraceutical importance. Polyphenols are the stumbling blocks in the occurrence of free radical associated disorders like heart diseases, cancer, infections and immune disorders[2,4,6,7].

Total polyphenolic contents (TPC) were determined by Folin-Ciocalteu method and expressed as mg gallic acid equivalent (GAE)/g of dry plant material. Folin reagent gives blue colour complex with phenols which can be quantified using spectrophotometer[50]. The quantities of polyphenols extracted with methanol and gemmomodified extracts from various plants have been presented in Table-1. Maximum total polyphenolic contents were found in the *Terminalia arjuna*. Methanol extract showed maximum polyphenols from the bark of *Terminalia arjuna* (136.3 ± 1.06 GAE mg/g). Gemmo modified extract of *Terminalia arjuna* showed 133 ± 3.1 GAE mg/g total polyphenolics contents.

TABLE-1
TOTAL POLYPHENOLIC CONTENTS OF GEMMOMODIFIED AND COMMONLY USED PARTS OF MEDICINAL PLANTS IN mg GAE/g, DRY OR FRESH WEIGHT OF PLANTS

Plant extracts	Total polyphenolic contests in mg GAE/g dry or fresh weight of plants
Terminalia arjuna (bark)	136.3 ± 1.06
Terminalia arjuna (gemmomodified)	133.1 ± 3.1
Cyperus rotundus (rhizomes)	91.467 ± 1.5
Cyperus rotundus (gemmomodified)	41.45 ± 1.5
Euphorbia tirucalli (gemmomodified)	75.22 ± 1.30
Euphorbia tirucalli	76.0 ± 2.54
Trigonella foenum (seed)	38.33 ± 0.5
Trigonella foenum (gemmomodified)	50.40 ± 0.38

All values are expressed as mean ± SD (n = 3).

Total polyphenols in terms of GAE mg/g in methanolic extract from *Cyperus rotundus* rhizomes were found 91.46 ± 1.45. Less total polyphenols were detected in gemmo modified extract of *Cyperus rotundus* (41.46 ± 1.26 GAE mg/g) as compared to methanolic extract of rhizomes.

Gemmo modified extract of *Euphorbia tirucalli* contained greater phenolics (76 ± 1.52 GAE mg /g) than dry plant (75.22 ± 1.05 GAE mg/g). However, this difference was non-significant ($p < 0.05$) with the contents of methanolic extract. These values are close to the finding of Sharma *et al.*[40], who reported 52.92 ± 5.62 mg GAE/g of crude extract TPC in another plant (*Euphorbia hirta*) of family Euphorbiaceae. This variation may be due to difference of species and nature of extract.

Gemmo modified extract of *Trigonella foenum* showed considerable high total polyphenolics (50.4 ± 0.37 GAE mg/g) than methanolic extract of seeds (38.33 ± 0.05 GAE mg/g).

Significant difference ($p < 0.05$) was observed in amount of total polyphenols extracted from different plant materials. All under study plants offered appreciable amount of total polyphenols. Among medicinal plants bark and gemmo modified extracts of *Terminalia arjuna* offered highest amount of total polyphenolic contents followed by, *Cyperus rotundus*, *Euphorbia tirucalli* and *Trigonella foenum*. Highest extract yield and total polyphenols was obtained with methanol followed by water. Methanol was also reported as best extracting solvent in many pervious studies[56-59].

Antioxidant activity: In this study, four radical scavenging (DPPH, ABTS, superoxide anion and nitric oxide), reducing power and inhibition of lipid peroxidation assays were performed to determine the antioxidant potential.

DPPH radical scavenging assay: The important property of antioxidant is its ability to scavenge free radicals. Stable radical, DPPH scavenging is most commonly used method for assessment of antiradical activity of medicinal plants. DPPH method is simple and time saving method. DPPH contains an odd electron which gives absorption maximum at 517 nm and is purple in colour. When free radical scavenging antioxidants (phenolics) donates its hydrogen to free radical, it becomes paired with hydrogen and formed reduced form of DPPH[2,60]. After reduction, the colour of DPPH is changed from purple to yellow-this discolouration is stoichiometric with respect to radical scavenging activity.

Free radical scavenging activity of four indigenous medicinal plants, *Terminalia arjuna*, *Euphorbia tirucalli*, *Cyperus rotundus* and *Trigonella foenum* was evaluated. Extracts were assessed for DPPH inhibition at different concentrations (10-100 µg/mL). Percentage inhibition of DPPH radical with selected medicinal plants has been presented in Fig. 1. All these medicinal plants showed good antiradical activity having direct correlation with dose concentrations but with different efficacies.

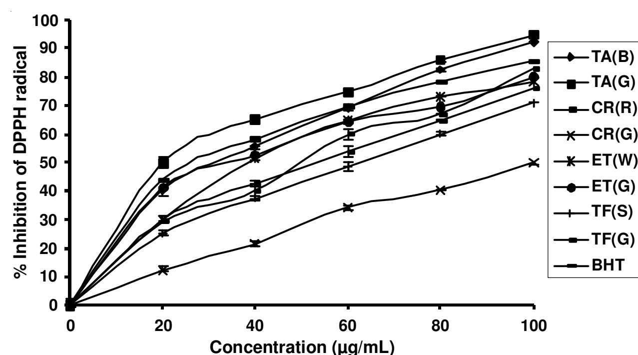


Fig. 1. DPPH radical scavenging activity of various medicinal plants. All values (mean ± SD) are average of triplicate experiments. TA (B); *Terminalia arjuna* bark, TA (G) *Terminalia arjuna* gemmomodified, ET (W) *Euphorbia tirucalli* whole plwnt, ET (W) *Euphorbia tirucalli* gemmomodified, CR (R) *Cyperus rotundus* (rhizome), CR (G) *Cyperus rotundus* gemmomodified, TF (S) *Trigonella foenum* seed, *Trigonella foenum* gemmomodified

Terminalia arjuna extracts exhibited significantly highest ($p < 0.05$) radical scavenging activity among the under investigation plants. Methanol extract of bark exhibited good antioxidant activity (92 ± 0.64 %) which is greater than standard synthetic antioxidant BHT (butylated hydroxyl toluene 85.5 ± 1.00 %). Gemmo modified extract showed 94.96 ± 0.72 % scavenging activity, which is greater than bark extract and standard.

Cyperus rotundus also proved as good free radical scavenger. The methanolic extract of *Cyperus rotundus* inhibited 82.9 ± 0.115 % radical at 100 µg/mL, which is very close to

BHT (85.4 ± 0.2.31 %). Gemmo extract of *Cyperus rotundus* showed less free radical quenching activity (50 ± 1.59 %) in comparison to methanolic extract of rhizome and standard BHT (85.4 ± 0.2.31 %).

Gemmo modified extract of *Euphorbia tirucalli* was most effective DPPH radical scavenger (80.2 ± 0.25 %) than methanolic extract (78.2 ± 0.20 %). However, this difference was statistically non-significant ($p < 0.05$). Antioxidant activity of *Euphorbia tirucalli* extracts was comparable with standard.

Trigonella foenum Gemmo modified extract was proved as more radical inhibitor as compared to seed extracts. Gemmo modified extract exhibited 76 ± 2.00 % radical inhibition activity which is greater than methanolic seed extract (71.0 ± 1.00 %). *Trigonella foenum* extracts showed less free radical quenching activity in comparison of standard.

ABTS radical scavenging assay: 2.2-Azino*bis*-3-ethyl benzo thiazoline 6-sulfonate (ABTS) radical scavenging assay is very important spectrophotometer method for the evaluation of antiradical abilities of plant constituents. The ABTS method is an easy, highly sensitive and rapid method. It involves the electron transfer process for neutralization of free radical cation (ABTS). In the presence of extracts (antioxidant compounds) at different concentrations, absorbance of ABTS radicals at 734 nm is decreased and antioxidant activity is measured in term of decolourization with reference to control[60]. Plant extracts with greater phenolics concentration quickly and strongly inhibited the ABTS radical cation[14]. ABTS radical cation decolourization assay showed similar trends to that obtained by DPPH assay. The under study plants showed good antioxidant activity which is confirmed by inhibiting the ABTS radical cation. Different concentrations of extracts (10-100 μg/mL) and standard BHT (butylated hydroxy toluene, synthetic antioxidant) showed ABTS inhibiting activity in dose dependant manner (Fig. 2).

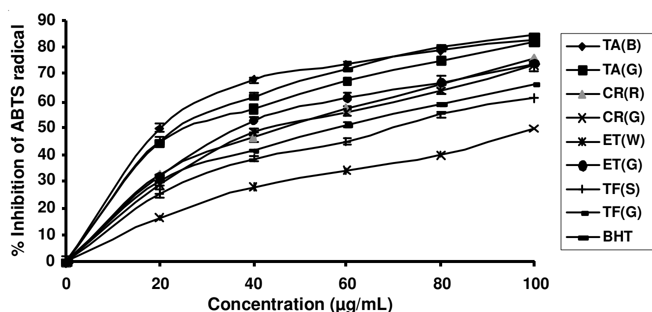


Fig. 2. ABTS radical scavenging activity of various medicinal plants. All values (mean ± SD) are average of triplicate experiments. TA (B); *Terminalia arjuna* bark, TA (G) *Terminalia arjuna* gemmomodified, ET (W) *Euphorbia tirucalli* whole plwnt, ET (W) *Euphorbia tirucalli* gemmomodified, CR (R) *Cyperus rotundus* (rhizome), CR (G) *Cyperus rotundus* gemmomodified, TF (S) *Trigonella foenum* seed, *Trigonella foenum* gemmomodified

Terminalia arjuna showed highest antiradical activity. Gemmo modified extract inhibited 82.2 ± 1.00 % ABTS radical at the concentration of 100 μg/mL, which is higher than BHT (81.16 ± 1.04 %). Methanolic extract of bark and gemmo modified extract (young buds shoots and leaves) showed antiradical activity higher than BHT, however this difference was non-significant ($p < 0.05$).

Methanolic extract of *Cyperus rotundus* rhizome possessed good antiradical activity (76.2 ± 0.34 %). Gemmo modified extract had inhibited 49.8 ± 1.00 % radical, which is significantly ($p < 0.05$) less than rhizomes. *Euphorbia tirucalli* gemmo modified and methanolic extract showed almost similar antiradical activity (74.3 ± 2.54, 73 ± 1.00 %, respectively. Gemmo modified extract of *Trigonella foenum* was proved as good free radical scavenger (66.16 ± 0.44 %) as compared to methanolic extract of seed (61 ± 1.00 %). All other extract of *Trigonella foenum* showed low level of antioxidant activity.

Nitric oxide scavenging assay: Nitric oxide (NO) is an effective pleiotropic mediator of various biochemical processes such as cell signaling, muscle relaxation, inhibition of platelet aggregation. It plays very important role in biological systems and shows its immense potential in vasodilatation, antimicrobial and anticancer activities. Surplus amount of nitric oxide is linked with numerous diseases. Oxygen reacts with the surplus of NO to produce nitrite and peroxynitrite anion, which act as free radicals and causes many inflammatory diseases[44,,54, 61].

All under investigation extracts showed antiradical activity in dose dependant manner (Fig. 3). Gemmo modified extract of *Terminalia arjuna* inhibited 70.8 ± 2.00 % radical at the concentration of 100 μg/mL. Methanolic extract of *Terminalia arjuna* (bark) inhibited 67.3 ± 0.76 % NO, which was comparable to BHT (65.26 ± 0.20 %) Fig. 3).

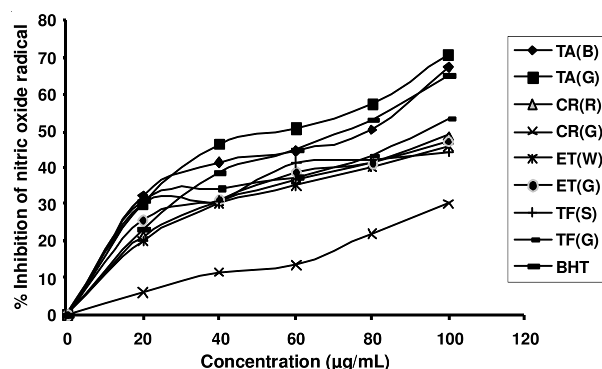


Fig. 3. Nitric oxide radical scavenging activity of various medicinal plants. All values (mean ± SD) are average of triplicate experiments. TA (B); *Terminalia arjuna* bark, TA (G) *Terminalia arjuna* gemmomodified, ET (W) *Euphorbia tirucalli* whole plwnt, ET (W) *Euphorbia tirucalli* gemmomodified, CR (R) *Cyperus rotundus* (rhizome), CR (G) *Cyperus rotundus* gemmomodified, TF (S) *Trigonella foenum* seed, *Trigonella foenum* gemmomodified

Methanolic extract of *Cyperus rotundus* rhizomes possessed 48.7 ± 0.56 % anti nitric oxide radical activity. Gemmo modified extract of *Cyperus rotundus* (30 ± 1.00 %) had no remarkable activity against reactive nitric oxide (Fig. 3).

Gemmo modified and methanolic extract of *Euphorbia tirucalli* exhibited 44 ± 0.99 and 45.40 ± 0.90 % activity, respectively. Nitric oxide scavenging activity of *Euphorbia tirucalli* was significantly ($p < 0.05$) lower than standard BHT (65.28 ± 0.20 %) (Fig. 3).

Gemmo modified extract of *Trigonella foenum* was effective nitric oxide (NO) radical scavenger (53.16 ± 1.04 %) than seed extract (44.3 ± 2.08 %).

Super oxide scavenging assay: Super oxides anion (O_2^-) is generated through many processes and damage biomolecules that may resulted in lipid peroxidation, cardiac diseases and many other disorders[2,44]. This activity was performed by using NBT (nitro blue tetrazolium) reagent. Super oxide radical is generated by autoxidation of hydroxylamine hydrochloride in presence of NBT. Super oxide could reduce NBT resulting in formation of blue formazine, which gives maximum absorbance at 560 nm. Decrease in absorbance in the presence of free radical scavengers (antioxidant compounds) is indicating super oxide anion scavenging activity of the extract. Percentage scavenging of super oxide anion (O_2^-) is increases with increase in concentration of antioxidant extract[7].

Terminalia arjuna gemmo modified extract at concentration of 100 µg/mL inhibited the formation of reduced NBT and percentage inhibition was 74 ± 0.76 % which was greater than BHT (71.16 ± 1.04 %). Methanolic extract of *Terminalia arjuna* bark inhibited 72.3 ± 1.36 % super oxide anion (O_2^-) radical which was greater than BHT and less than gemmo modified extract (Fig. 4).

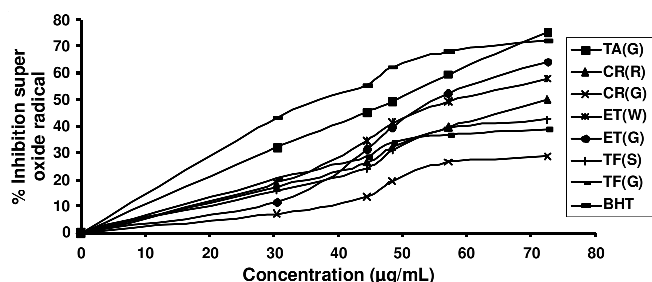


Fig. 4. Super oxide radical scavenging activity of various medicinal plants. All values (mean ± SD) are average of triplicate experiments. TA (B); *Terminalia arjuna* bark, TA (G) *Terminalia arjuna* gemmomodified, ET (W) *Euphorbia tirucalli* whole plwnt, ET (W) *Euphorbia tirucalli* gemmomodified, CR (R) *Cyperus rotundus* (rhizome), CR (G) *Cyperus rotundus* gemmomodified, TF (S) *Trigonella foenum* seed, *Trigonella foenum* gemmomodified

Gemmo modified extract of *Euphorbia tirucalli* had higher inhibition (64.33 ± 1.52 %) effect on super oxide than methanolic extract of dry plant (58.83 ± 3.2 2%). However this effect was lower than BHT (Fig. 4).

Methanolic extract of *Cyperus rotundus* exhibited 50 ± 1.12 % inhibition followed by gemmo modified extract (28.66 ± 1.52 %). Water extract (23.3 ± 1.52 %) had no significant effect on radical scavenging (Fig. 4).

Methanolic extract of *Trigonella foenum* (seed) showed less reactivity toward super oxide inhibition (44.4 ± 0.28 %) while Gemmo modified extract of *Trigonella foenum* had better effect on inhibition (53.4 ± 1.00 %) of anion radical which is comparably very low than BHT (71.16 ± 1.04 %) (Fig. 4).

Lipid peroxidation inhibition assay (ammonium thiocyanate assay): Free radical mediated chain reactions leads to oxidative damage of polyunsaturated lipids. Lipids peroxidation is responsible for oxidative stress in biological system. Many toxic products of lipid peroxidation can deteriorate other biomolecules, including DNA[7]. Ammonium thiocyanate method measures the amount of peroxide produces during the experimental period by air oxidation of linoleic acid (unsaturated fatty acids). Peroxides oxidized Fe^{2+} to Fe^{3+} the later formed complex of red colour with thiocyanate ion, which absorbed at 500 nm. Increase in absorbance indicates high concentration of peroxides. Effect of different extracts of medicinal plants on lipid peroxidation has been shown in Fig. 5.

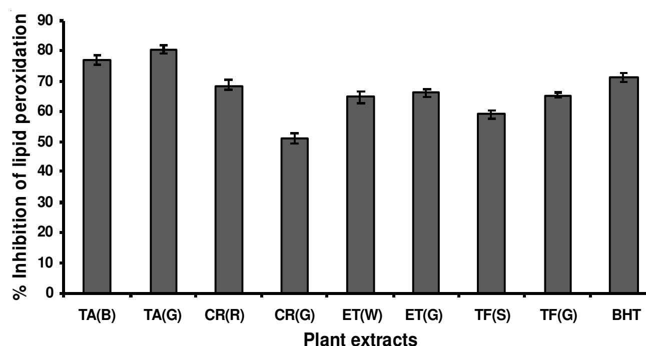


Fig. 5. Lipid peroxidation inhibition potential of various medicinal plants. All values (mean ± SD) are average of triplicate experiments. TA (B); *Terminalia arjuna* bark, TA (G) *Terminalia arjuna* gemmomodified, ET (W) *Euphorbia tirucalli* whole plwnt, ET (W) *Euphorbia tirucalli* gemmomodified, CR (R) *Cyperus rotundus* (rhizome), CR (G) *Cyperus rotundus* gemmomodified, TF (S) *Trigonella foenum* seed, *Trigonella foenum* gemmomodified

Terminalia arjuna both bark and gemmo modified extracts exhibited effective antioxidant activity in linoleic acid system. 100 µg of methanolic bark extract inhibited the lipid peroxidation (77.01 ± 0.055 %) up to 72 h as compared to control. Gemmo modified extract prevented the oxidation of linoleic acid (80.4 ± 0.07 %) higher than BHT (71.38 ± 0.053 %).

Cyperus rotundus (methanolic rhizome extract) was found to be more effective. It inhibited the lipid peroxidation up to 68 ± 0.058 %. Gemmo extract of Cyperus was less inhibitor of lipid peroxidation (50.7 ± 3.36 %).

Gemmo modified and methanolic extract of *Euphorbia tirucalli* both were effective to delay lipid peroxidation up to 72 h and inhibited the formation of peroxides up to 66 ± 0.09 and 64.6 ± 0.08 %, respectively.

On the other hand gemmo modified extracts of *Trigonella foenum* was powerful than seed extract. It inhibited 65.23 ± 0.06 % oxidation. While seed extract (methanolic) exhibited 59.07 ± 0.07 % activity.

Determination of reducing power: Antioxidants exert their action by breaking free radical chains and reacting with many peroxides intermediates by direct donation of electron or hydrogen atom (reduction). This is because the reducing capability of a compound may serve as a significant marker of its potential antioxidant activity. In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron (Fe^{3+}) in ferric chloride to ferrous (Fe^{2+}). Prussian blue colour complex formed between reduced form[2,7] of iron (Fe^{2+}) and ferricyanide, which absorbed at 700 nm. In the presence of extracts increase in the absorbance, indicates reducing potential of extracts.

In this study, the reducing power of different extracts of medicinal plants found to increase in direct proportion to the increasing concentration of extracts from 2-10 mg/mL). The

reducing power of gemmo modified extract of *Terminalia arjuna* was found to be (3.15 ± 0.02) at the concentration of 10 mg/mL. Reducing potential of gemmo modified extract of *Terminalia arjuna* was more pronounced than BHT (2.84 ± 0.03). Methanolic extract of *Terminalia arjuna* (bark) showed greater reducing power (3.2 ± 0.06) than standard (Fig 6).

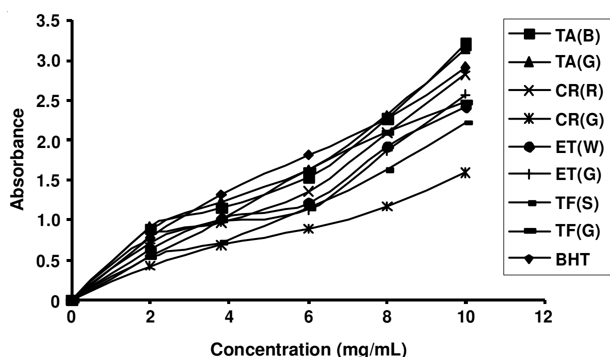


Fig. 6. Reducing potential of various medicinal plants. All values are average of triplicate experiments and represented as mean ± SDTA (B); *Terminalia arjuna* bark, TA (G) *Terminalia arjuna* gemmomodified, ET (W) *Euphorbia tirucalli* whole plant, ET (W) *Euphorbia tirucalli* gemmomodified, CR (R) *Cyperus rotundus* (rhizome), CR (G) Cyperus rotundus gemmomodified, TF (S) *Trigonella foenum* seed, *Trigonella foenum* gemmomodified

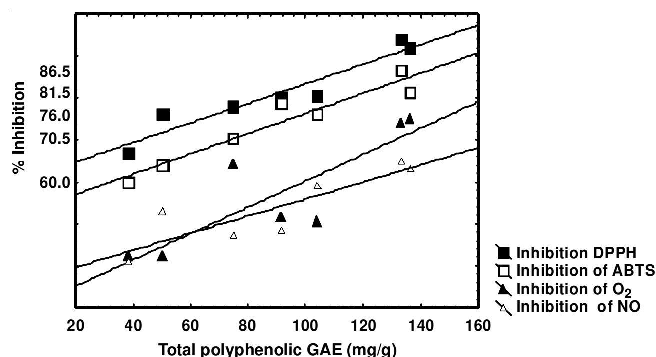
Gemmo modified extract of *Euphorbia tirucalli* has good reducing power (2.58 ± 0.056) which was higher than methanolic extract of whole plant (2.42 ± 0.09) and lower than BHT (2.79 ± 0.036) (Fig. 6).

Methanolic extract of *Cyperus rotundus* rhizomes had good reducing power (2.86 ± 0.057). Gemmo modified extract of *Cyperus rotundus* had shown reducing potential (1.54 ± 1.05) but its activity was significantly lower ($p < 0.05$) than BHT (2.89 ± 0.05) and methanolic extract of rhizome (Fig. 6).

Trigonella foenum gemmo modified extract demonstrated powerful reducing ability (2.48 ± 0.048) than methanolic seed extract (2.23 ± 0.056). However, this activity was less than standard (2.9 ± 0.09).

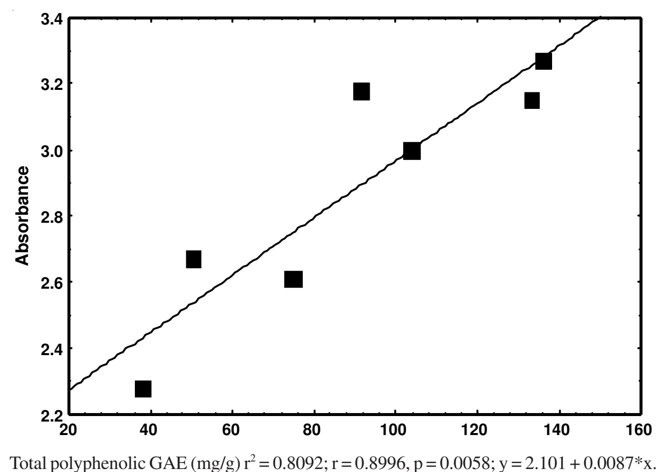
Correlation analysis of polyphenolic contents with antioxidant activity: Calculated coefficients of correlations between antioxidant activity, scavenging effects on radical and contents of phenolic compounds of different medicinal plants are shown in Figs. 7(a-b). The antioxidant activity of all medicinal plant extracts was significantly correlated with their scavenging effects on DPPH, ABTS, O_2^- NO radicals and reducing potential. Therefore, the antioxidant activity of plant extracts may be due to their scavenging effects on radicals, lipid peroxidation and reducing potential. The scavenging effect of medicinal plants on DPPH (r = 0.9537), ABTS (r = 0.9610), super oxide anion (r = 0.8508), nitric oxide (r = 0.8750) and reducing potential (r = 0.899) were well correlated with their contents of total polyphenolic compounds. The results are in agreement with previous reports that have been reported about highly positive relationship between total phenolics and antioxidant activity in many plant species[14,62-66].

Over all the results revealed that all studied plants showed antioxidant behaviour but different in potential and varying polyphenolic contents.



Correlation between total polyphenolics and inhibition of DPPH: $r^2 = 0.9095$; r = 0.9537, p = 0.0009; y = 60.0578 + 0.2337*x. Correlation between total polyphenolics and inhibition of ABTS: $r^2 = 0.9235$; r = 0.9610, p = 0.0006; y = 52.2103 + 0.2414*x. Correlation between total polyphenolics and inhibition of O_2^-: $r^2 = 0.7239$; r = 0.8508, p = 0.0152; y = 28.8108 + 0.3145*x. Correlation between total polyphenolics and inhibition of NO: $r^2 = 0.7656$; r = 0.8750, p = 0.0099; y = 35.5616 + 0.2038*x.

Fig. 7. (a) Correlation between total polyphenolic contents and antiradical potential of medicinal plants



Total polyphenolic GAE (mg/g) $r^2 = 0.8092$; r = 0.8996, p = 0.0058; y = 2.101 + 0.0087*x.

Fig. 7. (b) Correlation between total polyphenolic contents and reducing potential of medicinal plants

Results obtained from six different antioxidant assays were varying in values. Complexity of extract which include different functional group polarity and chemical behaviour could give variation in the results, due to difference in mechanism of antioxidant assays[67].

Owing to different chemical nature and complexity of antioxidant compounds present in extract of medicinal plants, there is variation in their mode of actions, so more than one assay are advised for evaluation of antioxidant activity. There are various methods to evaluate the antioxidant potential of medicinal plants. These methods are different from each other on the basis of assay principles and reaction conditions. In this work antioxidant activity was carried out through six assays. Antioxidant can show their mechanisms through different ways, due to complex nature of antioxidant compounds, it is suggested that *in vitro* antioxidant analysis were carried out essentially with more than one assay. Analysis through multiple assays would be more informative and compulsory to cover a wide range of possible applications of antioxidants[1,8,12,52,56,67-70].

The results of this study showed that extracts were very powerful towards all antiradical assays (DPPH, ABTS, NO,

O_2^-). Extracts gave strong effect in DPPH assay followed by ABTS assay, lipid peroxidation inhibition assays and weaker in nitric oxide, super oxide scavenging. The variations in response of different extracts towards all *in vitro* antioxidant assays were due to difference in polyphenolic contents and composition. Results of analysis also depend upon the characteristic of a particular test reaction.

The solubility of the test compounds in the assay environment is another important factor. Some tests are used for hydrophilic or some are used for hydrophobic compounds[67,68,71]. DPPH and ABTS were used for hydrophilic and hydrophobic compounds[10,72]. Lipid peroxidation and reducing power assays have also been applied for both hydrophilic as well as hydrophobic compounds. Nitric oxide (NO) assay required an aqueous buffered, hence more compatible with those compounds which can react in buffer with free radicals[71]. Stereo selectivity of a radical or solubility of extract in different testing systems have been also effect the ability of extract to neutralize different radicals[1,73].

Powerful antioxidant activity of methanolic extract is due to presence of hydroxyl groups in antioxidant components[58,74]. Antioxidant activity is mainly due to collective antioxidant potential of polyphenols. The results are in agreements with previous reports that phenolic compounds significantly contribute to antioxidant potential of plants[6,12,57,63-65,75,76]. Among the gemmo modified extracts all tested extracts except gemmo modified extract of *Cyperus rotundus* showed powerful antioxidant potential.

In present investigations amongst the understudied plants, the significantly highest polyphenolic contents and paramount antioxidant potential has been recorded in *Terminalia arjuna*. The amount of total polyphenols found in bark extract of arjun was higher than earlier reported by Sultana *et al.*[77], (12 g/100 g) and lower than reported by Bajpai *et al.*[78], (Bark 145 mg/g, leaves 143 mg/g fruit 133 mg/g). In another study, Shridhar and Gopal[59] reported just 5.98 mg/g polyphenols in arjun. Variation in total polyphenols quantity may be due to difference in extraction technique, experimental conditions, nature of solvents and climatic and varietal variations. Glycerin and methanol mercerized extract (gemmo modified) of young shoot and buds of *Terminalia arjuna* showed considerable high TPC value, which is comparable to TPC of bark. As gemmotherapy is new field no considerable previous work has been reported by any one.

Gemmo modified extract (fresh leaves, young shoots, buds, mercerized in glycerin and methanol) showed higher antioxidant activity toward all tested assays, than bark extract of *Terminalia arjuna*. Many studies[25,59] have been reported about antioxidant activity of bark extracts, but the antioxidant activity of gemmo extract is performed first time in Pakistan. Gemmotherapy; is the less studied research field, which is based on the use of embryonic and germinating parts of plants. The fact is that at this stage metabolic activity is on peak and activity of enzymes and hormones is high. Some other studies have been suggested that phenolics are natural defense compound for plants. During early stage of germination when biological activity is on peak free radical are generated frequently and increased oxidation stress. The variety of secondary metabolites are synthesized due to photosynthesis, the phenolic contents are higher in young leaves[79-90]. The results of this study supports the high antioxidant activity of gemmo modified extract however total polyphenols were less than bark but this difference was non-significant ($p < 0.05$). Gemmo modified extract of *Terminalia arjuna* could be an additional and superior source of natural antioxidants.

Gemmo modified extract of *Cyperus rotundus* showed significantly ($p < 0.05$) fewer amount of total phenolics than methanolic extract of rhizome. According to the results of this study amount of TPC found in *Cyperus rotundus* rhizome was different than earlier reported (73.27 ± 4.26 g/100g extract) by Nagulendran *et al.*[44].

Euphorbia tirucalli has good quantity of antioxidant polyphenolics and demonstrated strong action towards free radicals. In some previous reports plants of Euphorbiaceae family such as *Euphorbia hirta*[40] and *Euphorbia tirucalli*[41] showed high phenolic contents and antioxidant activity. No significant ($p < 0.05$) difference in polyphenolic contents and antioxidant activity was observed between gemmomodified and methanolic extracts of dry plant. Both showed almost similar performance towards free radicals.

Trigonella foenum is commonly used herb having a lot of medicinally important properties. The value of total polyphenolic contents in seed are higher than the earlier reported values, investigated by Souri *et al.*[82]. (1.94.53 mg/g), Wojdylo *et al.*[72], (7.0 GAE mg/100 g) and lower than Kaviarasan *et al.*[38], (78.6 GAE mg/g). Gupta and Prakash[83] reported 6.37 GAE mg/g of polyphenolic contents in leaves of *Trigonella foenum*. Variation in total polyphenols quantity may be due to difference in extraction techniques, experimental conditions and nature of solvents, climatic and varietal variations. No pervious studies are available about polyphenolic contents of gemmomodified extracts of under study plants.

Both gemmomodified and seed extract of *Trigonella foenum* showed better antioxidant potential towards ABTS and DPPH antioxidant assays. They showed weak effect towards nitric oxide and O_2^- scavenging assays. Among the both extracts gemmo modified extract offered a high antioxidant activity than seed extracts. Fresh geminating leaves contained high polyphenolic contents and antioxidant enzymes activity which contributes in its improved antioxidant activity than seed extracts. These findings are in agreement with the results of Randhir *et al.*[79], who reported greater antioxidant activity in germinating parts. The antioxidant activity of *Trigonella foenum* seed has been earlier reported by kaviarasan *et al.*[38] and Souri *et al.*[82], but limited studies have been available on antioxidant potential of leaves and gemmomodified extract.

The strong antioxidant potential of gemmo-modified and commonly used parts of understudy medicinal plants are attributed to their flavonoids and phenolic acid contents. All types of polyphenols have shown their potential in antioxidant activity. For examples, *p*-coumaric acid has ability to stop oxidative damage and found to inhibit the lipid peroxidation[83,84]. Important phenolic acids like caffeic acid[2], ferulic acid[85,86], gallic acids and chlorogenic acid[87,89] showed strong antioxidant potential[89]. Flavonoids are widely distributed polyphenolic compounds, acts as free radical scavengers by fast donation of hydrogen atoms to free radicals. Antioxidant activity of medicinal plant is manly attributed to flavonoids content of

medicinal plants. Catechin[90-93], quercetin[91,94], myricetin and kaempferol are also well reported antioxidants and exhibit strong pharmacological actions[91,95-97] .

Conclusion

It is concluded that all studied medicinal plants showed good antioxidant activity but different in efficacy. Gemmo modified extracts of *Terminalia arjuna* and *Trigonella foenum* showed improved antioxidant potential as compare to their natively used parts. A linear correlation is present between polyphenolic contents and antioxidant activity. Plant with higher phenolic contents showed superior antioxidant capability.

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EUROANALYSIS 16 CHALLENGES IN MODERN ANALYTICAL CHEMISTRY

11 — 15 SEPTEMBER, 2011

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