Original Article

Free Radical Scavenging and Antioxidant Impact of Indian Medicinal Plant Extracts on H_2O_2 Mediated Oxidative Stress on Human Erythrocytes

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ABSTRACT

In the present study four traditional Indian medicinal plants *Orchis latifolia, Boswellia serrata, Boerhavia diffusa* and *Centratherum anthelminticum* were investigated for their antioxidant potential and their protective effect in preventing haemolysis and lipid peroxidation in human red blood cells. In this study methanolic (MeOH) extracts of these four plants were partitioned with various solvents of different to obtain fractions. The fractions first subjected to phytochemical analysis followed by evaluation of their antioxidant potential by measuring the total phenolic content, total flavonoid content, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging potential and Ferric reducing antioxidant power **(**FRAP) potential. After this the selected fractions were tested *in vitro* to determine their protective effect against H_2O_2 induced haemolysis and lipid peroxidation in human RBCs. The study demonstrated a strong antioxidant potential of the ethyl acetate fraction of *C. anthelminticum* and *O. latifolia* as evident from high phenolic and flavonoid content and strong free radical scavenging activity. Further, the results also show a strong relation between the total phenolics and flavonoid content and antioxidant activity as demonstrated by the selected plant fractions. The fractions also protected membrane integrity resulting in a reduction of RBC haemolysis and lipid peroxidation during artificially induced oxidative stress. The results also provide scientific evidence to support the folk medicinal utilization of these plants for the treatment of various ailments and may offer new possibilities in the therapy of pathological conditions related to generation of free radicals.

Keywords: Medicinal plants, Antioxidant potential, Phenolics, Flavonoid haemolysis, Lipid peroxidation.

INTRODUCTION

Oxidative stress (OS) plays a major role in the development of chronic and degenerative ailments such as arthritis, aging, autoimmune disorders, neuro-degenerative and cardiovascular disorders and cancer[1]. Although cells are equipped with an impressive repertoire of antioxidant enzymes as well as small antioxidant molecules, these agents may not be enough to normalize the redox status under oxidative stress caused under adverse physicochemical, environmental or pathological conditions, when either the generation of free radicals is enhanced or their scavenging is inhibited. Under these circumstances supplementation with exogenous antioxidants is required to restore the redox homeostasis in cells[2].

Many synthetic compounds like butylated hydroxytoluene (BHT) and butylated hydroxy anisole (BHA) are commercially available as antioxidants. However, they are not recommended for usage due to the toxicity associated with them[3]. Therefore, there is a need to substitute these with naturally occurring antioxidants. Many plant species have been investigated in search of novel antioxidants. Plants synthesize several antioxidants to them against damage caused by active, reactive oxygen species (ROS) [4,5]. These compounds include chlorophyll derivatives, alkaloids, essential oils, phytosterols, phenolics and polyphenolics[6,7]. Some of the antioxidants that have been isolated from plants include curcumin, eugenol, flavonoids, coumarins, carotenoids, tannins, gallic acid, limonene, terpenoids, β-sitosterol etc[8,9].

Use of plants as a source of medicine has been an ancient practice and is an important component of the health care system in India. Various plant extracts and oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties. Many such plant extracts are being widely used by local medical practitioners. However, in most of the cases systematic inclusive investigations involving chemists and biologists have not been organized. Though bioactivity guided fractionation is the most widely used approach, but wiser approach could be to investigate each fraction methodically. Moreover, these bio-active fractions have hardly been evaluated against target specific study or *in vivo* studies. Hence, these lacuna need to be cared urgently to develop new drugs that are safe and effective antioxidants.

Erythrocytes have been the subject of a wide range of investigation involving oxidative stress due to high content of polyunsaturated fatty acids (PUFA). Oxidative damage to erythrocytes is manifested as increased haemolysis and lipid peroxidation. It has been suggested by various researchers that phytocompounds can protect erythrocytes or can even increase their resistance towards artificially generated oxidative stress as in exposure to H_2O_2 or exposure to chemotherapeutic drugs used during treatment of cancer[10].

In the present study four traditional Indian medicinal plants were investigated for their antioxidant potential and their protective effect in preventing haemolysis and lipid peroxidation in human red blood cells.

Orchis latifolia grows in wet meadows and marshes in rich soils. It is found in Europe, including Britain, north of Norway and east to W. Asia at a height of about 8-12 thousand feet. Traditionally, this plant is used to cure dysentery, diarrhea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures and general weakness, particularly in debilitated women after delivery and to increase regenerative fluids. Tubers of this plant are found to be rich in

starch, mucilage, sugar, phosphate, chloride and a glucoside-loroglossin[11]. Antibacterial and anticandidal properties of *n*-hexane (*n*-Hex), dichloromethane (DCM) and ethyl acetate (EtOAc) fractions against multi drug resistant bacteria have previously been reported by us[12]. Antihypertensive and antidyslipidemic properties of *O. mascula* have also been reported by Aziz *et al*[13].

Boswellia serrata is a deciduous middle sized tree, which is mostly concentrated in tropical parts of Asia and Africa. *B. serrata* gum resin has been reported to have analgesic, anti-inflammatory, antiarthritic and anti-pyretic activity. The gum resin comprising of mainly β-boswellic acids along with 11-keto-β-boswellic acids and their acetates has been reported to have antibacterial activity[14]. The gum resin has been shown to have a definite role in the treatment of rheumatoid arthritis[15] and boswellic acid has been shown as most potent inhibitor of 5-lipoxygenase, a key enzyme involved in inflammation[16]. Animal studies and pilot clinical trials support the potential of B. serrata gum resin extract (BSE) for the treatment of a variety of inflammatory diseases like inflammatory bowel disease, rheumatoid arthritis, osteoarthritis and asthma[17]. Methanol and aqueous extracts of *B. dalzielii* stem bark have been found to possess wide spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. The extracts of *Boswellia* species have been reported to exert anti-carcinogenic, antiproliferative, antitumor, apoptotic, and cytostatic activities[18,19].

Boerhaavia diffusa is a herbaceous plant widely distributed in the tropics and subtropics. The whole plant or its specific parts (leaves, stem, and roots) are reported to have medicinal properties including antibacterial, cytotoxic and antioxidant properties[20]. In another study, crude methanolic extract of *B. diffusa* and its liriodendrin-rich fraction has shown a dose-dependent protection against PTZ-induced convulsions have been reported[21]. Antibacterial activity against gram-positive bacteria like *S. aureus, B. subtilis, S. faecalis* and all gram-negative bacteria have been demonstrated by Mahesh *et al*. in a recent study[22].

Centratherum anthelminticum commonly known as 'black cumin' is used in Indian medicine to cure common ailments such as fever, cough, and diarrhea. Many secondary metabolites such as aliphatic fatty acids, flavones, saponins, steroids and glycosides have been reported from the C. *anthelminticum*. The plant extract is also reported to have analgesic, antibacterial, antifungal, antidiuretic, antifilarial, anthelmintic, antihyperglycemic, antimicrobial, antimalarial and antipyretic properties[23]. Phenolic extracts of *C. anthelminticum* also exhibit antioxidant property and inhibit free radical induced DNA damage in prokaryotes[24]. Chloroform fraction of *C. anthelminticum* seeds have been known to induce apoptosis in human melanoma cells[25].

In the present study methanolic (MeOH) extracts of these four plants were partitioned with various solvents to obtain fractions. The fractions were first subjected to phytochemical analysis followed by evaluation of their antioxidant potential by measuring the total phenolic content, total flavonoid content, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging potential and Ferric reducing antioxidant power (FRAP) potential. After this the selected fractions were tested *in vitro* to determine their protective effect against H_2O_2 induced haemolysis and lipid peroxidation in human RBCs.

MATERIALS AND METHODS

Plant material collection and extraction

Plant material was procured from an authorized vendor from Delhi. The identity was confirmed and voucher specimen was deposited in the herbarium of Amity Institute of Biotechnology, Amity University, Uttar Pradesh, Noida, India. 500 g of each plant material was extracted with MeOH: Water (9:1) at room temperature. The concentrated methanol extract of the plant was then partitioned with *n*-hexane (*n*-Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and aqueous (Aq) fractions as described by Priyanka *et al*[26]. Concentrated fraction of each plant was subjected to phytochemical investigations and evaluated for antioxidant activity.

Phytochemical analysis of the fractions

Detailed phytochemical analysis was performed with *n*-Hex, DCM, EtOAc and Aq fractions of methanolic extract of the tested plants for the presence of various phytochemicals as described by Rajesh *et al*[27]. Flavonoids, steroids, alkaloids and tannins were detected by the NaOH / HCl test, Salkowski's reaction, Dragendroff's reaction and ferric chloride test respectively. Additional tests were carried out to check the presence of reducing sugars, cardiac glycosides, anthraquinones, triterpenoids and phlobatannins.

Determination of total phenolic content (TPC)

Total phenolic content was determined by modified Folin - Ciocalteu method as described by Celiktas *et al*[28]. Briefly 200µL of 1mg/mL of extract prepared in methanol was diluted with 1800 µL of Milli Q water. To this reaction mixture 2000 µL of Folin- Ciocalteu reagent and 2.0 mL of saturated sodium carbonate was added and mixed well. This was left to stand at room temperature for 30 minutes and the absorbance was determined spectrophotometrically at 760 nm using UV-Visible double beam spectrophotometer. The TPC was expressed as gallic acid equivalent (GAE) in the mg / g extract and obtained from the standard curve of gallic acid with the equation ($y = 0.0026x + 0.1898$; $R^2 = 0.9939$), where y is absorbance at 760nm and x is concentration in GAE ($n = 3$).

Determination of flavonoid content of the extracts

The total flavonoid concentration was measured by the aluminium chloride colorimetric assay as described by Adnan[29]. 500µL of 0.5mg/ mL methanolic solution of each fraction was diluted with 2 mL of distilled water. To the above mixture, 150µL of 5% $NaNO_2$ was added. After 5 min, 150µL of 10% $AlCl_3$ was added, followed by the addition of 1 mL of 1 M NaOH after 6 min. The solution was mixed well and the absorbance was measured at 510 nm. Total flavonoid contents were calculated as quercetin equivalent (mg/g) using the following equation based on the calibration curve: $y = 0.001x + 0.013$, $R^2 = 0.998$, where x was the absorbance and y was the quercetin equivalent (mg/g).

DPPH free radical scavenging assay

The antioxidant activity of the selected extracts was measured in terms of hydrogen donating ability 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical assay[28] in a reaction mixture containing different concentrations of extracts (2, 10, 20 and 50 µg/ml) and 1 mM methanolic solution of DPPH. Subsequently, the mixture was shaken vigorously and left to stand for 30 min in the dark. Disappearance of the purple colour was monitored at 517 nm using a spectrophotometer. Test samples and positive control ascorbic acid were tested in triplicate over the same range of sample concentrations.

The radical scavenging activity (RSA) was calculated as-

$$\% RSA = 100 (1-A_E/A_D),$$

Where A_E is the absorbance of the solution containing antioxidant extract and A_D is the absorbance of the methanolic DPPH solution.

The antioxidant activity of each test sample and ascorbic acid was expressed in terms of concentration required to inhibit 50% methanolic DPPH radical formation (IC_{50} µg/ml) and calculated from the graph of % RSA and plant extract concentrations.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the modified protocol of Benzie and Strain as adapted by Krishnaraju *et al*[30]. The assay was based on the ability of the antioxidant to reduce ferric to ferrous ions in the presence of 2, 4, 6-tri (2-pyridyl)-S- triazine (TPTZ), forming an intense blue ferrous–TPTZ complex with absorption maxima at 593nm. An assay mixture containing 2.5 ml of 30mM acetate buffer (pH 3.6), 0.25 ml of 10 mM TPTZ in HCl, 0.25 ml of 20 mM ferric chloride and different concentration of plant extract was incubated for 30 min at room temperature and the absorbance of the coloured product (ferrous tripyridyltriazine complex) was read at 593 nm. A standard graph for ferrous sulphate in methanol at different concentrations was prepared. FRAP values of the fractions were expressed as mM of Fe (II)/g of extract.

Inhibition of erythrocyte haemolysis

Erythrocytes were obtained from healthy donors and processed by protocol as described by Battistelli *et al*[31]. After the removal of plasma and buffy coat, the erythrocytes were washed thrice with phosphate buffer saline (PBS) and used for subsequent analysis. The erythrocytes were resuspended in the same buffer and used for performing haemolysis and lipid peroxidation experiments.

For haemolysis modified protocol as described by Okoko *et al*[10], was used. The reaction mixture contained 200 µL of erythrocyte suspension and 10 µL of the test plant fraction. The mixture was incubated for 30 min at 37^0 C. Haemolysis was induced by the addition of 100 µL of 100 µM of H_2O_2 followed by incubation at 37^0C for 3 hours. 200 µL of supernatant was diluted with 1.4mL of PBS and the samples were centrifuged at 3000 rpm for 10 min and absorbance of the content was measured at 540nm. For this experiment, the absorbance obtained from H_2O_2 alone without the plant extract was taken as 100% haemolysis. Hence, the absorbance values obtained at 540 nm were expressed as % haemolysis inhibition.

Inhibition of lipid peroxidation

The most widely used method for measurement of lipid peroxidation is thiobarbituric acid reactive substances (TBARS) method. The TBARS method is based on the principle that malondialdehyde forms a 1:2 red colored adduct with thiobarbituric acid, which can be quantitatively estimated either spectrophotometrically (532 nm) or by fluorimetry. RBCs were processed as described in section 2.7 and after incubation for 3h, the proteins were precipitated by the addition of 10% TCA followed by centrifugation at 3000 rpm for 5 min. To 1 mL of the obtained supernatant 0.67% of TBA reagent was added. The reaction mixture was boiled for 20 min and the absorbance was read at 532 nm. The results were expressed as % decrease in lipid peroxidation[32].

Statistical analysis

All experiments were carried out in triplicates and the results were expressed as mean ± SD values wherever applicable.

RESULTS AND DISCUSSION

Phytochemical analysis

The qualitative analysis of the methanolic extract and fractions of the plant revealed extensive presence of alkaloids in all the fractions of all the four plants. In contrast, the DCM and EtOAc fractions of *O. latifolia, B. serrata* and *C. anthelminticum* showed the presence of flavonoids (Table 1). Presence of steroids was detected in a DCM fraction of *O. latifolia*, DCM and EtOAc fraction of *B. serrata* and *n*-hex fraction of *C. anthelminticum*. Triterpenoids were found in all the fractions of *B. serrata* and *C. anthelminticum*. Tannins were also detected in *B. diffusa*. Various plant secondary metabolites like flavonoids, saponins, cardiac glycosides, tannins, triterpenes and alkaloids have been reported to possess antioxidant activities and observed a wide range of antioxidant properties of the methanol extract and fractions can be explained by the presence of various groups of potentially active classes of these secondary metabolites.

Total phenol content

Methanolic extracts of all the four plants selected for the present study showed high phenolic content as determined by the Folin - Ciocalteu method and expressed as mg GAE/g of sample (Fig.1). EtOAc fraction of *C. anthelminticum* showed highest phenolic content (237.54 ± 7.73 mg GAE/g) followed by EtOAc fractions of *B. diffusa* and DCM fraction of *O. latifolia* (192.67 ± 2.52 and 153.46 ± 6.39 mg GAE/g respectively). It is interesting to note that maximum phenolic content was found in the EtOAc fraction as compared to other fractions in three out of four plants under present investigation. Phenolics are widely distributed in the tissues of plants and play an important role as antioxidants[33]. The results of the present study strongly suggest that the high phenolic contents of these plants may be responsible for their antioxidant and antihemolytic activities.

Total flavonoid content

Flavonoids are polyphenolic substances present in most plants and act as hydrophilic antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and as UV-light filters and substrate for polyphenol oxidases protecting tissue after physical damage to plants. It is reported that flavonoids might account for at least part of the health benefits associated with vegetable and fruit consumption[34]. Flavonoids and other polyphenols due to their redox properties play an active role in quenching of free radicals[35].The total flavonoid content of various fractions of the four plants under the present study is presented in Fig.2. Highest flavonoid content was found in EtOAc fraction of *C. anthelminticum* (691.87 ± 10.91 mg QE/g) followed by EtOAc fraction of *O. latifolia* (350.65 ± 4.12 mg QE/g). DCM fractions of *O. latifolia* and *C. anthelminticum* similar flavonoid contents (160.25 ± 7.34 and 161.08 ± 10.91 mg QE/g respectively). *B. serrata* and *B. diffusa* in general had lower flavonoid content in all the fractions as compared to *O. latifolia* and *C. anthelminticum*. Since phenolics and flavonoids are important constituents of these plants, the observed antioxidant activities of these plants may be accounted for by their high flavonoid contents.

DPPH free radical scavenging activity

DPPH, a highly stable free radical has been widely used to assess the antioxidant potential of many natural products[32]. The effect of antioxidants is considered to be due to their hydrogen donating ability to the DPPH free radical. IC_{50} in µg/mL was calculated for each fraction as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by

50%. The lower the IC_{50} value, the higher is the antioxidant activity. The data for IC_{50} for each fraction is presented in Table 2 and Fig.3. Lowest IC_{50} was found to be for EtOAc fraction of *B. diffusa* and *O. latifolia* (20.19 and 20.17 µg/mL, respectively). Notably, none of fractions had IC_{50} values more than 69.24 µg/mL signifying the high proton donating and free radical scavenging potential of these fractions. Hence, the present study indicates that these extracts may be useful for treating free radical related pathological damage.

FRAP assay

The FRAP assay is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. The method measures the reducing ability of antioxidants against the oxidative effects of ROS. The higher the absorbance, the higher is the antioxidant activity which is indicated by the high FRAP value. Among all the tested fractions, EtOAc fraction of *C. anthelminticum* exhibited highest FRAP Value (606.95 ± 0.95 mM of Fe(II) /g) followed by *n*-Hex and DCM fractions of *O. latifolia* (114.32 ± 0.95 and 107.57 ± 0.82 mM of Fe(II)/g respectively) . All the fractions of *B. serrata* and *B. diffusa* showed lower FRAP values. The results of FRAP assay are depicted in Table 1 and Fig.4.

Relationship between antioxidant potential and total phenol and flavonoid content

Recent studies have reported a highly positive relationship between total phenols, flavonoids and antioxidant activity appears in many plant species[36]. Awika *et al*[37] in a study on *Sorghum bicolor* found positive correlations between the phenolic content and antioxidant activity using the oxygen radical absorbance capacities (ORAC), ABTS, and DPPH assay. In a similar study a high correlation between total phenolic content and antioxidant capacity was found in all the cultivars and fruit tissues of apple cultivars analyzed, except in the apple pulp was reported[38]. To understand the nature of active principles involved in antioxidant mechanisms, correlation graphs were plotted for various pairs of antioxidant activities for different plant fractions. For each graph the correlation equation was determined and correlation coefficient, R^2 was calculated. The results are depicted in Table 3.

The results of correlation studies indicated a high degree of correlation between phenol / flavonoid content and antioxidant activity measured by the DPPH / FRAP method for the four plants under investigation. The highest degree of correlation was found between phenol content and DPPH/ FRAPS activities of *B. diffusa* (R^2 = 0.996 and R^2 = 0.985 respectively) (Fig. 5 and 6). Similarly a high degree of correlation was also found between flavonoid content and DPPH of *O. latifolia*, *C. anthelminticum* and *B. serrata* (Fig. 7, 8 and 9). Highest R^2 was found for *O. latifolia* (R^2 = 0.995) followed by *B. serrata* (R^2 = 0.8774) and *C. anthelminticum* (R^2 = 0.8397). Similarly DPPH activity and FRAP activity of *B. diffusa* was also found to be highly correlated (R^2 =0.996) (Fig.10). These results suggest that a higher percentage of the antioxidant capacity of these plants may be a result of the higher phenolic content. Also, it can be concluded that the antioxidant activity of plant extracts is not limited to phenolic content, but also comes from the presence of other antioxidant secondary metabolites, such as flavonoids. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers[39]. The antioxidant action of phenolic compounds may also be due to their high tendency to chelate metals. Since phenolics have hydroxyl and carboxyl groups, they are able to bind to iron and copper, thus preventing this generation of highly reactive

hydroxyl radical thus preventing oxidative damage to biomolecules. Phenolic antioxidants also inhibit lipid peroxidation by trapping the lipid alkoxyl radical[39]. Hence, the presence of phenolic compounds in plant extracts may contribute significantly to their antioxidant potential. Antioxidant properties of phenolic compounds have been directly linked to their structure as they are composed of one (or more) aromatic rings bearing one or more hydroxyl groups and hence are therefore potentially an agent to quench free radicals from forming resonance-stabilized phenoxyl radicals[40]. Flavonoids, on the other hand, are thermodynamically stable due to their low redox potential and hence can reduce most of the oxidizing free radicals. The stability of the flavonoid free radical is further extended by extensive conjugation[41].

Inhibition of H_2O_2 induced erythrocyte haemolysis and lipid peroxidation

Selected fractions which exhibited maximum antioxidant potential was further selected for *in vitro* determination of antioxidant activity on H_2O_2 induced erythrocyte haemolysis and lipid peroxidation. The ability of EtOAc fractions of the four medicinal plants under present investigation to inhibit H_2O_2 induced erythrocyte haemolysis and lipid peroxidation are presented in Table 4. Hydrogen peroxide is one of the most important ROS formed from the superoxide. It has the ability to form potentially toxic hydroxyl radical which can react with many macromolecules including proteins and DNA. Hydrogen peroxide can also damage cells via direct oxidation of cellular components ultimately leading to cell death via mitochondrial driven apoptosis[32]. High concentrations of polyunsaturated fatty acids coupled to inhibition of active oxygen transport contribute towards haemolysis of RBCs. Various plant extracts with antioxidant activity have been found to protect the erythrocytes from oxidative stress or increase their resistance to damage caused by oxidizing agents like H_2O_2[42]. In our present study, we found that the plant derived fractions could restore or reduce the effects of oxidative stress induced by exposure to H_2O_2 as manifested by a decrease in % haemolysis and lipid peroxidation. The fractions were first tested to determine the safe concentrations that did not show any harmful effect on the erythrocytes which were optimized to10 µg/mL and used for our set of experiments. At concentrations higher than this, haemolysis was induced by the extract itself.

Maximum inhibition of haemolysis and lipid peroxidation was observed for EtOAc fraction of *C. anthelminticum* (99.3 and 81.2 %, respectively). Even EtOAc fraction of *O. latifolia* also exhibited significant inhibition of haemolysis and lipid peroxidation (71.4 and 64.3 % respectively). Other fractions exhibited a comparatively lower inhibitory activity as compared to *C. anthelminticum* and *O. latifolia.* A similar study conducted by Ajila and Rao[43] has reported up to 85% inhibition in H_2O_2 induced haemolysis in rats by mango peel extracts. Another study by Battistelli *et al*[23] the authors have reported the protective effect of *Rhodiola rosea* (a high altitude plant of Europe and Asia) extract on oxidative haemolysis generated by hypochlorous acid on erythrocytes. Hence, we can conclude that EtOAc fraction of *C. anthelminticum* and *O. latifolia* exhibited a protective effect against oxidative stress induced erythrocyte damage.

CONCLUSION

The strong antioxidant activity of EtOAc fraction of *C. anthelminticum* and *O. latifolia* has been demonstrated by high phenolic and flavonoid content and strong free radical scavenging activity in our study. The results of the present study also show a strong relation between the total phenolics and flavonoid content and antioxidant activity

as demonstrated by the selected plant fractions. The fractions also protected membrane integrity resulting in a reduction of RBC haemolysis and lipid peroxidation during osmotic and oxidative stress. The antioxidant activity may be attributed to chelation of metal ions by phenolics or by quenching of free radicals or a combination of both the mechanisms. The results also provide scientific evidence to support the folk medicinal utilization of these plants for the treatment of various ailments. These results may offer new possibilities in the therapy of pathological conditions related to generation of free radicals. Further phytochemical studies are required to isolate these potent natural antioxidants.

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Table 1. Phytochemical screening of methanol extract and fractions of the plants *Orchis latifolia, Boswellia serrata, Boerhavia diffusa and Centratherum anthelminticum*

Phytoconstituents	*Orchis latifolia*			*Boswellia serrata*			*Boerhavia diffusa*			*Centratherum anthelminticum*		
	n-Hex	DCM	Eto AC	*n*-Hex	DCM	Eto AC	*n*-Hex	DCM	Eto AC	*n*-Hex	DCM	Eto AC
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	-	+	+	-	+	+	-	-	-	-	+	+
Steroids	-	+	-	-	+	+	-	+	+	+	-	-
Reducing sugars	-	-	-	-	-	-	-	+	+	-	-	-
Cardiac glycosides	-	-	-	-	-	+	-	+	+	-	-	+
Triterpenoids	-	-	-	+	+	+	-	-	-	+	+	+
Anthraquinones	-	-	-	-	-	-	-	-	+	-	-	-
Tannins	-	-	-	-	-	-	-	+	+	-	-	+
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	+	-	+	-	-	-

\+ indicates Present
\- indicates Absent

Table 2. Total phenol and flavonoid content and antioxidant activity of various plant fractions of *O. latifolia, B. serrata, B. diffusa and C. anthelminticum*

Plant Extracts	Total phenol content (mg GAE/g dry wt. of sample)*	Total Flavonoid content (mg QE/g dry wt. of sample)*	DPPH IC_{50} (µg/mL)	FRAP value ± S.D.(mM Fe (II)/g of extract)
Orchis latifolia				
n-Hexane	21.69 ± 0.98	73.59 ± 4.12	50.66	114.32 ± 0.95
DCM	163.50 ± 0	160.25 ± 7.34	39.37	107.57 ± 0.82
Ethyl acetate	153.46 ± 6.39	350.65 ± 4.12	20.17	56.96 ± 0.82
Boswellia serrata				
n-Hexane	17.93 ± 1.04	8.70 ± 0.85	69.24	11.75 ± 0.33
DCM	1.61 ± 0	20.26 ± 0	39.17	5.00 ± 0.19
Ethyl acetate	22.95 ± 1.39	47.34 ± 1.97	20.19	9.05 ± 0.04
Boerhavia diffusa				
n-Hexane	37.32 ± 1.54	21.10 ± 1.06	41.81	6.37 ± 0.08
DCM	65.23 ± 1.72	44.43 ± 1.73	41.53	7.35 ± 0.00
Ethyl acetate	192.67 ± 2.52	47.34 ± 2.41	40.61	9.72 ± 0.04
Centratherum anthelminticum				
n-Hexane	88.20 ± 3.82	76.17 ± 2.24	47.83	2.64 ± 0.11
DCM	56.83 ± 2.87	161.08 ± 10.91	38.89	2.30 ± 0.07
Ethyl acetate	237.54 ± 7.73	691.87 ± 10.91	30.43	606.95 ± 0.95

Total phenol content expressed as mg GAE/g dry wt. of extract.
Total Flavonoid content expressed as mg QE/g dry wt. of extract.
DPPH radical scavenging activity expressed as IC_{50} in µg/mL extract required to scavenge 50 % of free radicals.
FRAP value expressed as mM of Fe (II) formed /g of extract.

Table 3. Correlation between antioxidant activities and phenol and flavonoid content of the fractions

Samples	Flavonoid content (mg QE/g) and DPPH IC_{50} (µg/mL) of *O. latifolia*	Flavonoid content (mg QE/g) and DPPH IC_{50} (µg/mL) of *C. anthelminticum*	DPPH IC_{50} (µg/mL) and FRAP value (mM Fe (II)/g of *B. diffusa*	Phenol content (mg GAE/g) and DPPH IC_{50} (µg/mL) of *B. diffusa*	Phenol content (mg GAE/g) and FRAP value(mM Fe (II)/g of *B. diffusa*	Flavonoid content (mg QE/g) and DPPH IC_{50} (µg/mL) of *B. serrata*
Equation of correlation	$y = -0.108x + 57.88$	$y = -0.0239x + 46.45$	$y = -2.738x + 120.9$	$y = -0.007x + 42.06$	$y = 0.020x + 5.781$	$y = -1.16820x + 72.577$
Correlation coefficient	$R^2 = 0.995$	$R^2 = 0.8397$	$R^2 = 0.996$	$R^2 = 0.996$	$R^2 = 0.985$	$R^2 = 0.8774$

Table 4. Effect of selected plant fractions on prevention of H_2O_2 induced haemolysis/lipid peroxidation of erythrocytes

Sample	Inhibition of haemolysis (%)	Inhibition of lipid Peroxidation (%)
EtOAc fraction of *O. latifolia*	71.4	64.3
EtOAc fraction of *B. serrata*	36.2	24.8
EtOAc fraction of *B. diffusa*	66.8	53.8
EtOAc fraction of *C. anthelminticum*	99.3	81.2

Inhibition expressed as percentage decrease in released haemoglobin/MDA

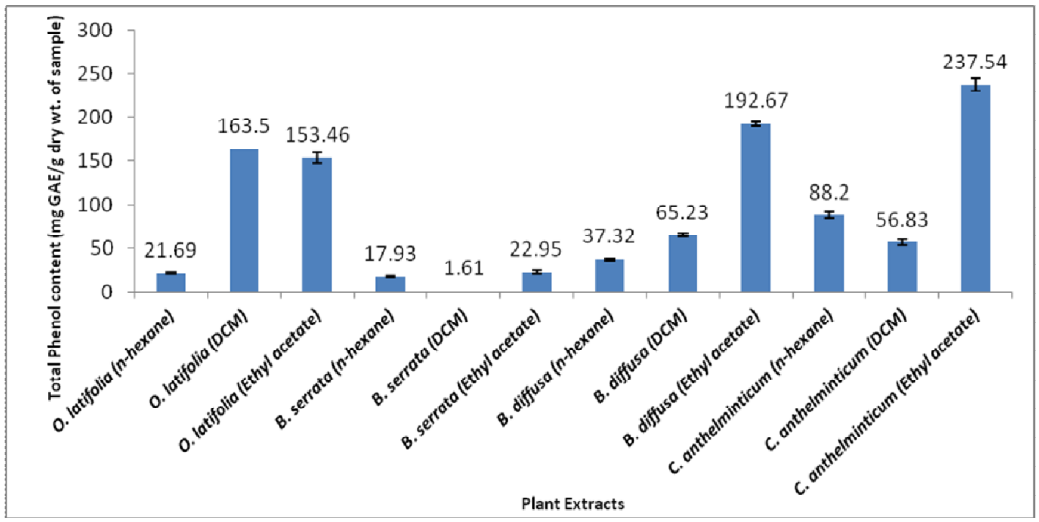

Figure 1. Total Phenol content of various fractions of *O. latifolia, B. serrata, B. diffusa and C. anthelminticum*. The phenolic contents are expressed as mg Gallic Acid Equivalent/g dry wt. of extract

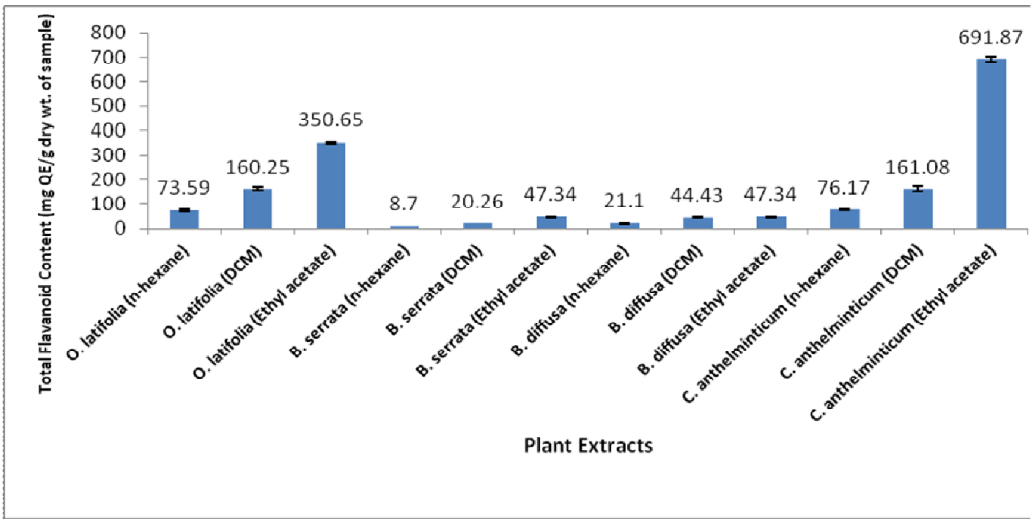

Figure 2. Total Flavonoid content of various fractions of *O. latifolia, B. serrata, B. diffusa and C. anthelminticum*. The flavonoid content is expressed as mg quercetin equivalent/g dry wt. of extract

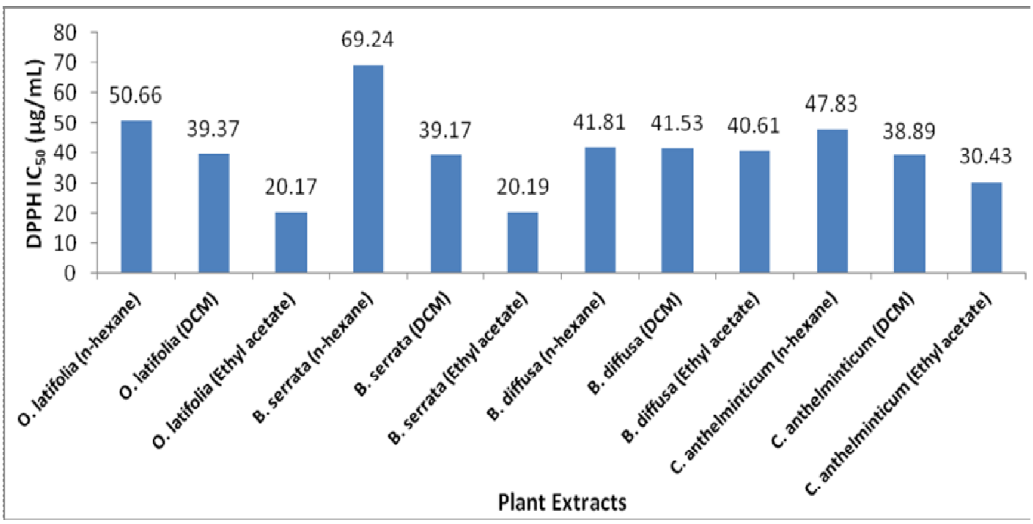

Figure 3. IC_{50} values for DPPH free radical scavenging of various fractions of *O. latifolia, B. serrata, B. diffusa and C. anthelminticum* expressed as µg/ml required to scavenge 50% of free radicals

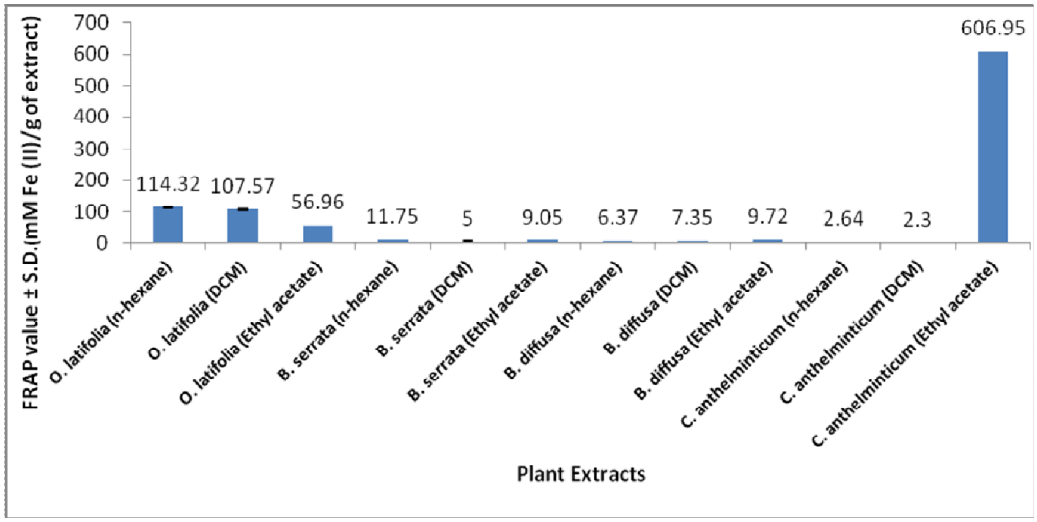

Figure 4. FRAP antioxidant capacity of various fractions of *O. latifolia, B. serrata, B. diffusa and C. anthelminticum* expressed as mM of Fe (II) formed /g of extract

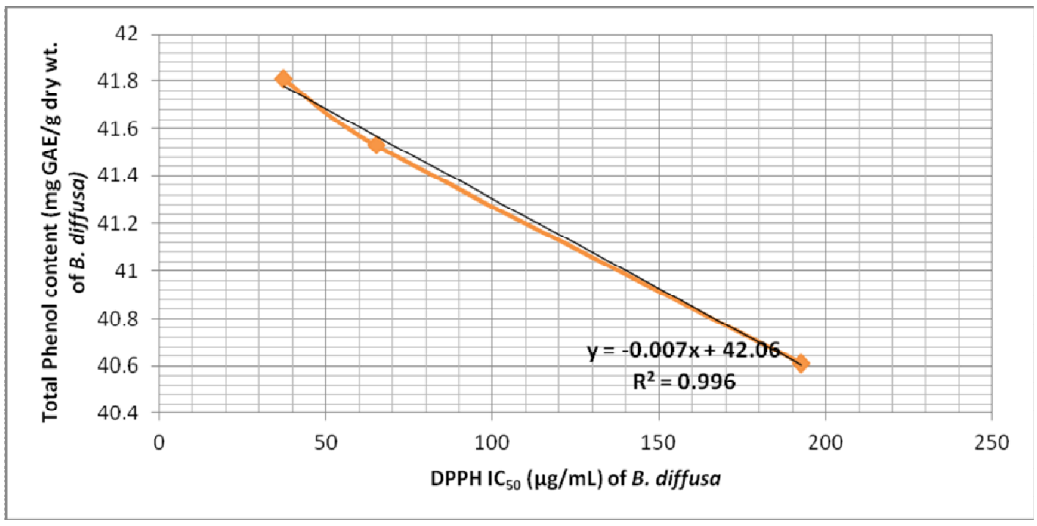

Figure 5. Correlation between Total Phenol content and IC_{50} values for DPPH free radical scavenging of *B. diffusa*

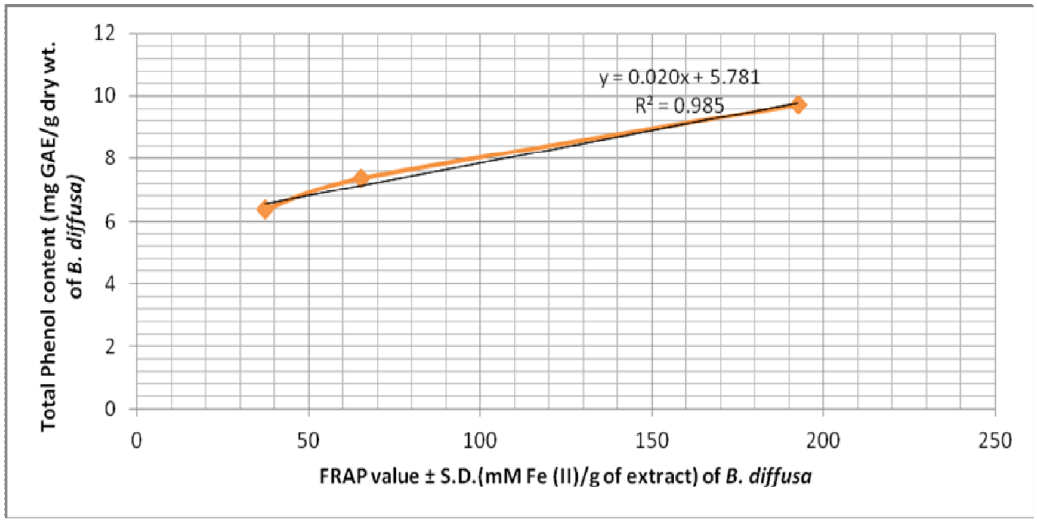

Figure 6. Correlation between Total Phenol content and FRAP value of *B. diffusa*

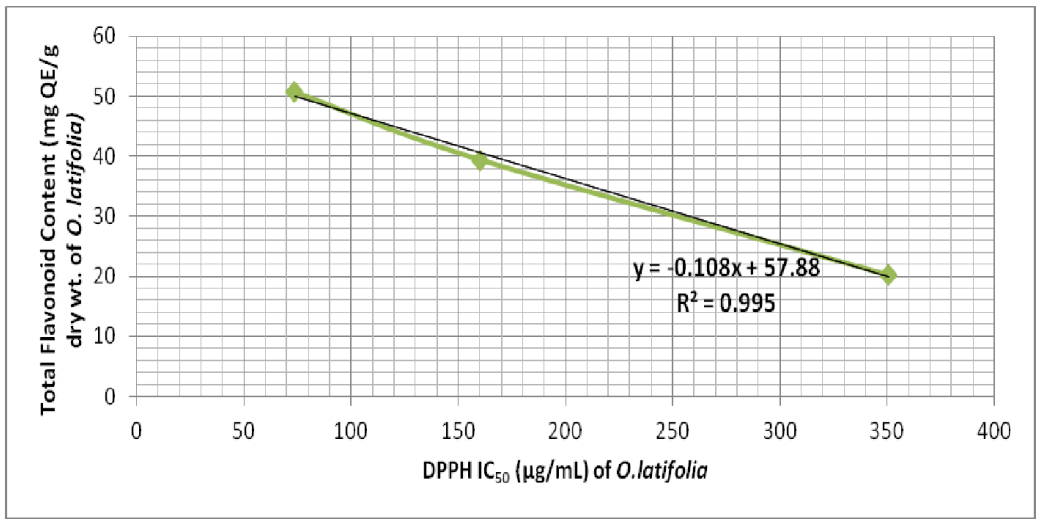

Figure 7. Correlation between Total Flavonoid content and IC_{50} values for DPPH free radical scavenging of *O. latifolia*

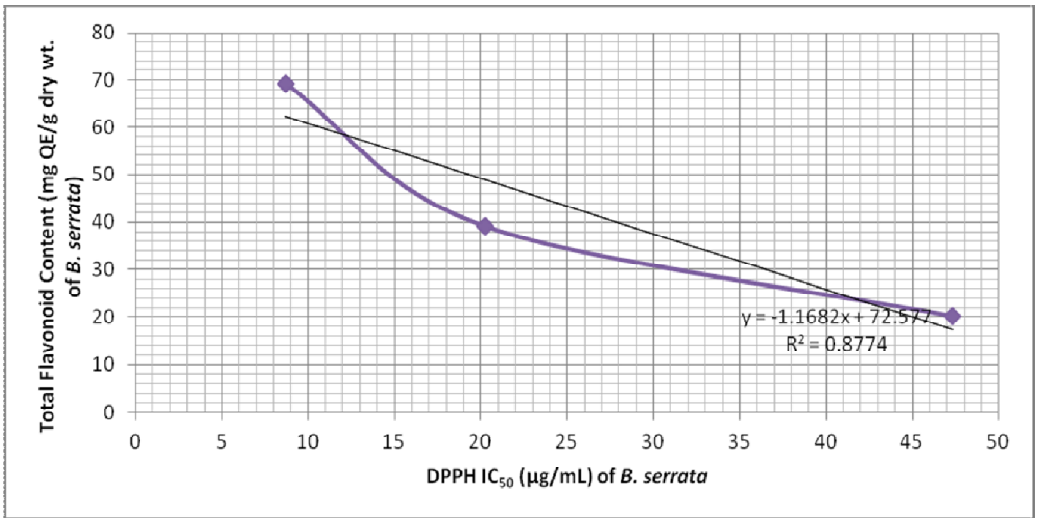

Figure 8. Correlation between Total Flavonoid content and IC_{50} values for DPPH free radical scavenging of *B. serrata*

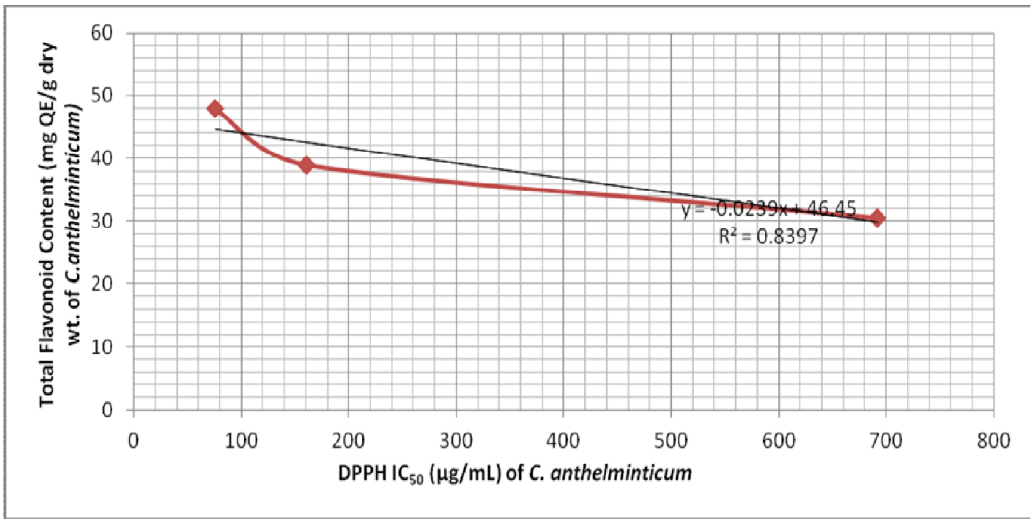

Figure 9. Correlation between Total Flavonoid content and IC_{50} values for DPPH free radical scavenging of *C. anthelminticum*

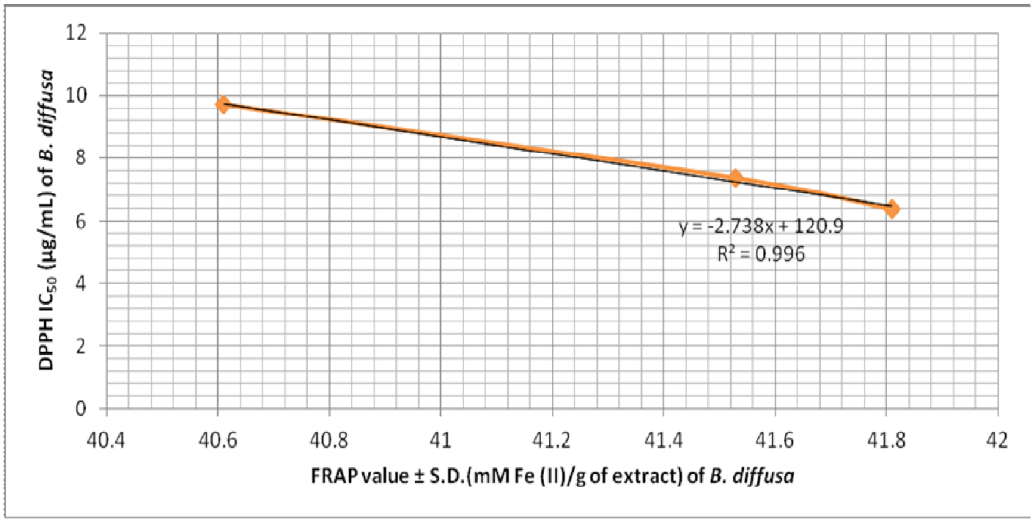

Figure 10. Correlation between DPPH free radical scavenging and FRAP value of *B. diffusa*

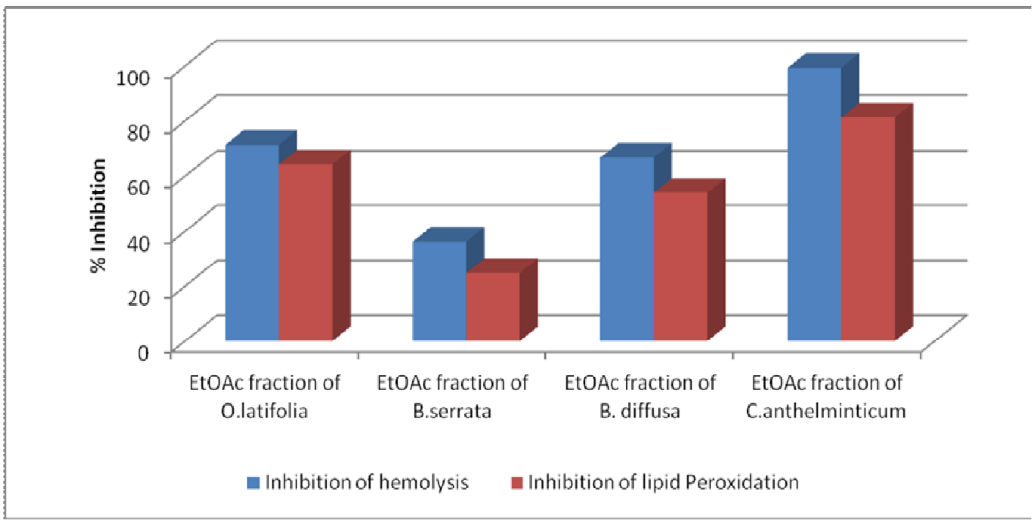

Figure 11. Effect of selected plant fractions on prevention of H_2O_2 induced haemolysis/lipid peroxidation of erythrocytes

Inhibition expressed as percentage decrease in released haemoglobin/MDA