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In vitro evaluation of antioxidant properties and their capacity of *Alternanthera sessilis* and *Tephrosia purpurea*

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ABSTRACT

Currently, there is a great interest in functional components of food ingredients. This is mainly due to the fact that many studies have shown that reactive oxygen species (ROS), including oxygen free radicals, are a part of etiology of degenerative disorders including some hepatopathies and other serious organ damage. Aqueous extracts of *Alternanthera sessilis and Tephrosia purpurea* were screened for potential antioxidant properties, which includes various enzymatic antioxidants like superoxide dismutase, catalase, peroxidase, glutathione peroxidase and ascorbate oxidase and various non-enzymatic antioxidants such as reduced glutathione, vitamin C and total phenolics. These plants extracts also analysed for in vitro antioxidant capacity assay, which includes the total antioxidant power, reducing power and the radical scavenging assays. The results clearly indicate that both the plants of the present study are beneficial as an antioxidant sources since both the plants possesses significant levels of enzymatic antioxidants, non-enzymatic antioxidants and also exhibits antioxidant capacity.

Key words: Free radicals, antioxidants, *Alternanthera sessilis, Tephrosia purpurea* and radical scavenging activity.

INTRODUCTION

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals[1]. Free radicals are fundamental to many biochemical processes and represent an essential part of aerobic life and metabolism[2]. The reactive oxygen species (ROS) have been implicated in over a hundreds of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome[3]. In treatment of these diseases, antioxidant therapy has gained an immense importance because dietary plants contain several hundred different antioxidants, it would be useful to know the total concentration of antioxidants in individual items. Such data might be useful in the identification of the most beneficial dietary plants. Antioxidants have been reported to prevent oxidative damage by free radical and ROS and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as oxygen scavengers[4]. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties[5].

Tephrosia purpurea belongs to family Fabaceae. The plant is antihelmintic, alexiteric, cures disease of liver, spleen, heart, blood, tumours, ulcers, leprosy, asthma, poisoning, piles syphilis and gonorrhea. The flavonoid fraction has the ability of modulate both the cell-mediated and the humoral

immune system[6]. The ethanolic extract may inhibit degranulation of mast cells[7]. It was reported that it acts as a skin antioxidant and also be a potent chemo preventive agent against renal oxidative stress and carcinogenesis[8]. Three novel flavonoids were isolated and exhibits potential cancer chemo preventive properties.

Alternanthera sessilis belongs to family Amaranthaceae. The common medicinal properties includes accredited with galactagogue properties. It increases the flow of milk in the cattle and also used for night blindness. The chloroform extract exhibits antimicrobial tests against *Pseudomonas aeruginosa* and *Trichophyton mentagrophytes*[10].

MATERIAL AND METHODS

Thiobarbituric acid and 2,2-diphenyl-3-picryl hydrazyl (DPPH) are obtained from Himedia laboratories Pvt. Ltd, Mumbai, India. 2,4,6-tri- pyridyl-s-triazine, Nitro blue tetrazolium (NBT), reduced glutathione, butylated hydroxy toluene (BHT) are obtained from Sisco research laboratories Pvt. Ltd. Mumbai, India. All other chemicals used are of analytical grade obtained from commercial sources.

Plant collection

The plants are collected from the regional agricultural research station, Anakapalli, Andhra Pradesh, India. Authenticated in the Department of Botany, Andhra University Vishakhapthanam, Andhra Pradesh, India.

Preparation of plant extract

Fresh leaves were collected, cleaned with distilled water and cut into small pieces, separately mashed in pre-cooled mortar and pestle and 10 ml of ice cold 0.1M phosphate buffer, pH 7.6, containing 0.1 mM EDTA to get different concentrations of 25,50 and 100 mg/ml. This extract was filtered through a muscline cloth and centrifuged at 10,000 rpm for 15min. The supernatant obtained was used for the determination of enzymatic, non-enzymatic antioxidants and antioxidants potential of the individual plant extract.

Assay of superoxide dismutase

The assay of superoxide dismutase was carried by the method of Beauchamp and Fedovich[11]. To 0.5ml of plant extract, 1.0ml of 0.125 M sodium carbonate, 0.4 ml of 25µM NBT and 0.2 ml of 0.1mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1.0mM hydroxylamine hydrochloride and absorbance was measured at 560nm using spectrophotometer (Hitachi, Germany). Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed as units per mg protein.

Assay of catalase

The catalase activity was assayed by the titrimetric method described by Radhakrishnan and Sarma[12]. To 2.5ml of 0.1M phosphate buffer, pH 7.5, 2.5ml of 0.9% H_2O_2 (v/v) in the same buffer and 0.5 ml of the plant extract was added and incubated at room temperature for 3 min. The reaction was then arrested by adding 0.5ml of 2N H_2SO_4 and the residual H_2O_2 was titrated with 0.1N potassium permanganate solution. Units of enzyme activity were expressed as ml of 0.1 N potassium permanganate equivalents of H_2O_2 decomposed per mg protein.

Assay of peroxidase

Assay of peroxidase activity was carried out according to the procedure of Malik and Singh[13]. To 3.5ml of 0.1M phosphate buffer, pH 6.5, 0.2ml of plant extract and 0.1ml of freshly prepared 0.1% O-dianisidine solution was added. Then reaction was initiated by adding 0.2ml of 0.2M H_2O_2 and the absorbance read at 430nm for 3min with an interval of 30 sec. A graph was plotted with the increase in absorbance against time. From the linear phase, the change in absorbance per min was calculated and activity was expressed as units per mg of protein.

Assay of glutathione peroxidase (GPx)

Glutathione peroxidase was assayed by the method or Rortruck *et al.,* [14]. 0.2 ml each of 0.8mM EDTA, 10mM sodium azide, 1.0 mM GHS, 2.5nm H2O2, 0.32M phosphate buffer, pH 7.0 and plant extract were mixed in the final volume of 1.2ml and incubated at 37°C for 10min. The reaction was arrested by the addition of 0.5ml of 10% TCA and the tubes were centrifuged. To 0.5ml of supernatant, 3.0 ml of 0.33m phosphate solution and 1.0 ml of

0.6mM DTNB reagent were added and the absorbance was read at 420nm. Graded amount of standard were also treated similarly. Glutathione peroxidase activity is expressed as µg of glutathione utilized per mg protein.

Assay of ascorbate oxidase

Assay of Ascorbate oxidase activity was carried out according to the procedure of Vines and Oberbacher[15]. To 3.0 ml of the ascorbate solution (0.003%), 0.1 ml of the plant extract as added and the change in absorbance at 265nm was measured at an interval of 30 seconds for a period of 5min. One unit of enzyme activity was expressed as 0.01 OD change per mg of protein.

Estimation of ascorbic acid

Ascorbic acid content was determined by the procedure described by Sadasivam and Balsubraminan[16]. To 5.0ml of ascorbate solution (10µg per ml), 10ml of 4% oxalic acid was added and titrated against 0.025% dichlorophenol indophenol. The amount of the dye consumed is equivalent to the amount of ascorbic acid present in the plant extract. Similar titration was carried out with 5.0ml of plant extract.

Reduced glutathione

Reduced glutathione was determined by the Boyne and Ellman method[17]. 1.0 ml of the plant extract was treated with 4.0ml of precipitating solution containing 1.67g of glacial metaphosphoric acid, 0.2g of EDTA and 30g of NaCl in 100ml water. After centrifugation 2.0 ml of the protein free supernatant was mixed with 0.2ml of 0.4M disodium hydrogen phosphate and 1.0ml of DTNB reagent. Absorbance was read at 412 nm within 2min. GHS concentration was expressed as n mol per mg protein.

Analysis of total phenolics

The total phenolics were determined using the Folin Cio-calteau reagent as reported by Javanmardi *et al.,*[18]. To 50 µl of the plant extract of diluted Folin Cio-calteau reagent and 2.0ml of 7.5% (w/v) sodium carbonate was added incubated at 45°C for 15min. The absorbance values of all samples were measured in a spectrophotometer at 765 nm. The results were expressed as mg of gallic acid equivalents per gm weight.

Antioxidant ability assays

Ferric reducing of antioxidant power assay (FRAP)

The total antioxidant power of the sample was assayed by the method of Benzie and strain[19]. 3.0ml of FRAP working reagent was taken in a test tube then 100 µl of plant extract was added, this is vortex mixed, and the absorbance was read at 593nm against a reagent blank after 1min. The results are expressed as ascorbic acid equivalents (µ moles/ml) of FRAP units.

Iron (III) to Iron (II) reducing activity (or) reducing power assay

The ability of the extracts to reduce ion (III) was assessed by the method of Oyaizu[20]. 1.0ml of plant extract was mixed with 2.5 ml of 0.2M phosphate buffer, pH 6.6 and 2.5 ml of 1% aqueous K_3Fe (CN_6) solution. After 30 min of incubation at 50°C, 2.5ml of 10% trichloroacetic acid was added, and the mixture was centrifuged for 10min. Finally, 2.5ml of the upper layer was mixed with 2.5ml of water and 0.5 ml of 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700nm. The results were expressed as ascorbic acid equivalents (AscAE) in milligrams of ascorbic acid per gm of extract. Butylated hydroxy toluene (BHT) and ascorbic acid were used as positive controls.

Diphenyl picryl hydrazyl radical scavenging assay (DPPH Assay)

The DPPH assay was carried out as described by Cuendet *et al.,*[21]. 5.0 ml of DPPH solution (0.004%) in methanol was added to 50 µg of plant extract. After 30min of incubation at 37°C, the absorbance was read against control at 517nm. BHT and Rutin were used as positive controls. Percentage of inhibition=(Absorbance of control-Absorbance of test/Absorbance of control) × 100.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the extract for hydroxyl radical's generated Fenton reaction, a method carried out by Gulhan *et al.,*[22]. 0.1 ml of plant extract was added to the reaction mixture containing 0.1ml of 3.0 mM deoxyribose, 0.5ml of 0.1mM $FeCl_3$, 0.5ml of 0.1mM EDTA, 0.5ml of 0.1mM ascorbic acid, 0.5ml of 1mM H_2O_2 and 0.8 of 20mM phosphate buffer, p^H7.4, in a

final volume of 3.0ml. The reaction mixture was incubated at 37°C for 1h. The thiobarbituric acid reactive substances (TBARS) formed were measured by treating with 1.0ml of TBA (1.0%) and 1.0ml of TCA (2.8%) at 100°C for 20min. After the mixtures were cooled, absorbance was measured at 532nm against a control. Percentage of inhibition was calculated as I=(Absorbance of control-Absorbance of test/Absorbance of control) × 100.

Inhibition of lipid peroxide formation

The lipid peroxidation was induced by $FeSO_4$-ascorbate in sheep liver homogenate by Bishayee and Balasubramaniyam[23] and the formed TBRAS was estimated by the method of Ohkawa *et al.,*[24]. The reaction mixture consisting of 0.1ml each of 25%(w/v) sheep liver homogenate in 40mM Tris-HCl buffer, p^H7.0, 30mM KCl, 0.16 mM ferrous iron ($FeSO_4$), plant extract or positive control and 0.06mM ascorbic acid. The reaction mixture was then incubated at 37°C for 1h. After incubation, 0.4ml of the above reaction mixture was taken and treated with 0.2ml of sodium dodecyl sulfate, 1.5ml of 1.0% TBA, and 1.5ml 20% acetic acid, adjust the p^H to 3.5. The total volume was made up to 4.0 ml by adding distilled water and the reaction mixture was kept in water bath at 95°C for 1h. To the pre cooled reaction mixture, 1ml of distilled water and 5ml of n-butanol and pyridine mixture (15:1 v/v) was added and was shaken vigorously. After centrifugation at 4000rpm for 10min, the organic layer was taken and its absorbance at 532 nm was measured. % of inhibition (I)= (absorbance of control-absorbance of test/absorbance of control) × 100.

Estimation of total protein

Total protein was estimated by the method of Lowry *et al.,*[25]. 0.1ml of plant extract and different concentrations of standards were taken. The volume was made up to 1.0ml with distilled water. To all the tubes, 5.0ml of alkaline copper reagent was added and left at room temperature for 10 min. Then 0.5 ml of Folin's phenol reagent was added and the blue color developed was read after 20min at 620 against a reagent blank. Protein concentration is expressed as mg/ml, mg/gm of sample (or) µg/ml.

RESULTS AND DISCUSSION

Studies on enzymatic antioxidant levels of some medicinal plants

The results obtained on the enzymatic antioxidant levels of some medicinal plants were shown in the Table 1. All the enzymatic antioxidant activities were increased with increasing concentrations from 25 to 100 mg/ml. The results also indicated that significant superoxide dismutase activity was detected in both the plants of present study. Highest SOD activity was detected in *T. purpurea* with a value 1.99 ± 0.02 where as lowest value is observed in *A. sessilis* with 1.66 ± 0.064 units/mg. The maximum activity of catalase was noticed in *T. purpurea* (0.348 ± 7 0.005 units/mg) and minimum activity was observed in *A. sessilis* (0.14 ± 0.01 units/mg). High peroxidase levels were detected in *T. purpurea* with 0.465 ± 0.021 units/mg and low levels were observed to be low in *A. sessilis* (0.222 ± 0.005 units/mg). Glutathione peroxidase activity was observed to be low in *A. sessilis* with 79 ± 0.95 units/mg while the activity is high in *T. purpurea* (101.8 ± 1.113 units/mg). Maximum activity of ascorbate oxidase was reported in *A. sessilis* with 1.47 ± 0.1 units/mg while minimum activity is observed in *T. purpurea* (0.5 ± 0.03 units/mg). The predominant activity of oxidase in *A. sessilis* is associated with low levels of axoclate activity of catalase, peroxidase and glutathione peroxidase emphasizing that the importance of Ascorbate system in *A. sessilis*. Antioxidants may also act by raising the levels of endogenous defense by up regulating the expression of genes encoding the enzymes such as SOD, catalase, peroxidase, glutathione peroxidase and ascorbate oxidase. Studies shown that these antioxidant defense enzymes protect the aerobic cells against oxygen toxicity and lipid peroxidation. Superoxide dismutase detoxifies the disproportionately superoxide radicals and hydrogen peroxide is destroyed by catalase and different kinds of peroxidases. A major H_2O_2-detoxifying system in plants is the Ascorbate-Glutathione cycle that includes peroxidase, glutathione peroxidase, ascorbate oxidase and glutathione reductase[26]. These peroxidases scavenge the high reactive lipid peroxide in the aqueous phase of cell membrane.

The similar studies of enzymatic antioxidant were reported in *Murrayya koeingii*[27], *Vitis vinifera* (grapes), *Emblicus officials* (goose berry), *Citrus sinensis* (orange) and *Lycopersicum esculentum*[28]. The total protein content of plant extracts were increased with increasing concentrations from 25 to 100 mg/ml. The total protein was found to be maximum in *T. purpurea* with 17.2 ± 0.57 gm/mg and minimum in *A. sessilis* with 12.0 ± 0.25 gm/mg.

Studies on enzymatic antioxidant levels of some medicinal plants

The levels of non-enzymatic antioxidants were increased with increase in the concentration of the plant extract were shown in the Fig. 1-3. The results show that reduced glutathione was found to be maximum in *T. purpurea* with 66.27 ± 1.01 nanomoles/mg where as minimum levels are found in *A. sessilis* with 40 ± 1.00 nanomoles/mg Fig.1. *T. Purpurea* with high levels of glutathione and glutathione peroxidase may play an important role in the prevention of lipid peroxidation[29]. Vitamin C content is found to be high in the leaf extract of *T. purpurea* (101.65 ± 3.48 mg/gm) compared to *A. sessilis* (59.10 ± 1.00 mg/gm) Fig.2. These results were comparable with the vitamin C content of Goose berry, orange, tomato is 41.62,57.31 and 26.09 respectively[28]. Ascorbic acid may function as reductant for many free radicals[30]. Phenolics are well known antioxidants of medicinal plants *T. purpurea* is best known antioxidant medicinal plant having total phenolics of 30.0 ± 0.7 GAE and *A. sessilis* has 6.0 ± 0.51 GAE units Fig.3. There is a strong relationship between total phenolics and antioxidant activity and plants with high total phenolics may exhibits high antioxidant activity[31,18]. Comparable relationship between phenolics and antioxidant activity was also reported in roschip extracts[31] and *Ocimum bascilicum*[18].

Antioxidant ability assays

Antioxidant activity of neutraceutical and pharmaceutical preparations is quantified by *invivo* and *invitro* studies where the focus is mainly on the role in scavenging reactive oxygen species. In order to know the antioxidant properties, one can determine the antioxidant ability of these plants extracts was also determined and results are given in table 2. All the antioxidant ability assays were increasing with increasing the concentration of the extracts from 25 to 100 mg/ml and maximum antioxidant ability was shown by the plant extract with a concentration of 100 mg/ml. The results indicate that highest total antioxidant power was found in *T. purpurea* with 1480 FRAP units followed by and *A. sessilis* with 880 FRAP units. We elected to use the FRAP analysis for several reasons because the FRAP assay is the only assay that directly measures antioxidant or reductants in sample. Similar total Antioxidant power was reported

Table 1: Various levels of enzymatic antioxidants

Name of the plant	Conc. (mg/ml)	SOD U/mg	Catalase U/mg	Peroxidase U/mg	Glutathione Peroxidase	Ascorbate Oxidase
T. purpurea	25	0.79 ± 0.02	0.148 ± 0.005	0.165 ± 0.021	39.8 ± 1.13	0.15 ± 0.37
	50	1.23 ± 0.04	0.233 ± 0.01	0.260 ± 0.01	69.8 ± 1.47	0.26 0.05
	100	1.99 ± 0.02	0.348 ± 0.005	0.465 ± 0.021	101.8 ± 1.13	0.5 ± 0.037
A. sessilis	25	0.86 ± 0.06	0.044 ± 0.01	0.123 ± 0.005	39.9 ± 0.95	0.47 ± 0.10
	50	1.18 ± 0.02	0.089 ± 0.01	0.175 ± 0.011	44.4 ± 0.87	0.87 ± 0.2
	100	1.66 ± 0.06	0.14 ± 0.01	0.222 ± 0.005	79 ± 0.95	1.47 ± 0.10

(All the above values are an average of three determinations and expressed as mean ± S.D)

Graphs showing non-enzymatic antioxidants

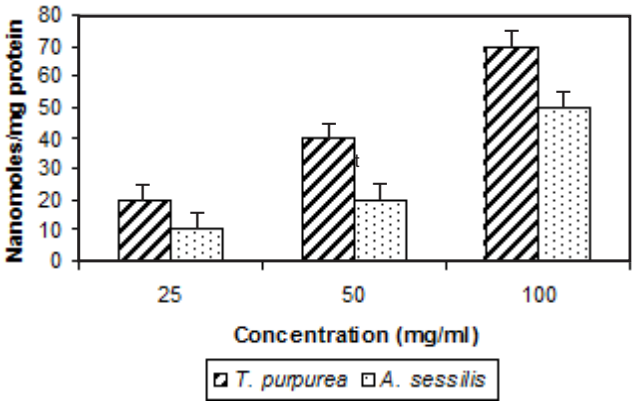

Fig. 1: Reduced glutathione (RG) levels of *A. sessilis* and *T. purpurea*

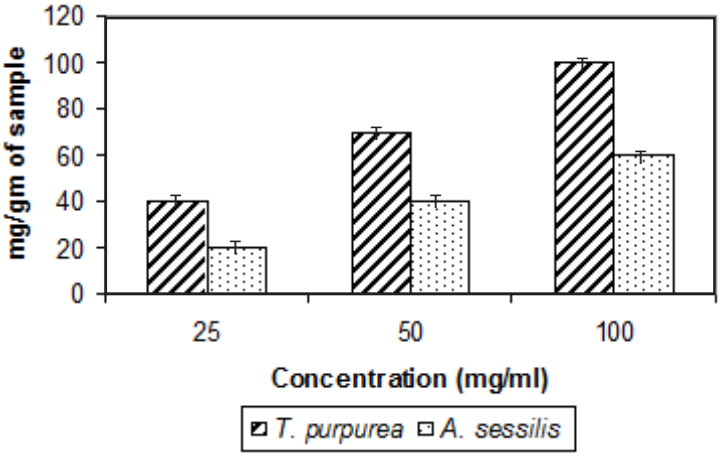

Fig. 2: Vitamin C content of *A. sessilis* and *T. purpurea*

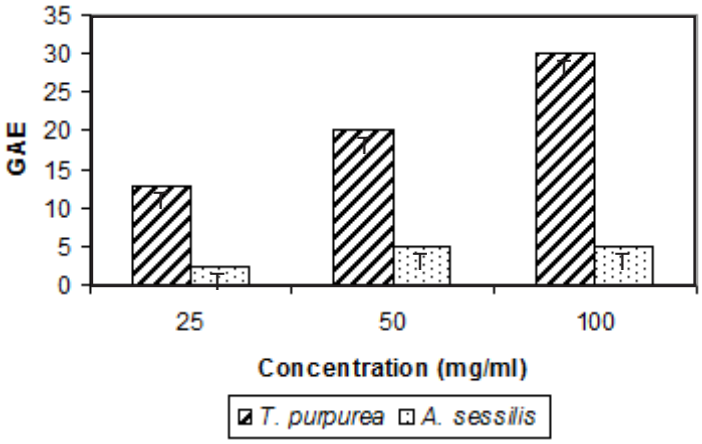

Fig. 3: Total phenolic contents of *A. sessilis* and *T. purpurea*

Table 2: Antioxidant ability assays

Name of the plant	Conc. (mg/ml)	FRAP Assay FRAP Units	Fe(III) to Fe(II) AscAE	DPPH Assay % of inhibition	Hydroxy Radical Scaveng ing %of inhibition	Inhibition of Lipid Peroxidation %of inhibition
T. purpurea	25	484	116	9.3	26.9	23.1
	50	860	224	18.8	45.6	36.3
	100	1480	410	24.5	66.5	63.5
A. sessilis	25	244	110	6.2	16.5	19.6
	50	496	162	10.6	19.8	29.4
	100	880	260	16.2	26.4	39.8
Positive controls	1mg/ml			BHT-76.4 Rutins-40	BHT-76 AscA-76.4	BHT-82.6 AscA-70

in shoot, stem and leaves of some Labiatae members[32]. Reductones are reported to be terminators of free radical chain reactions[33] and the antioxidant activity of an aqueous extract may be related to its reductive activity. Studies shows that highest reducing activity is observed in *T. purpurea* with 410 AscAE and *A. sessilis* with 260 AscAE. There was strong relation between the reducing activity and DPPH radical scavenging assay. This may be due to system solubility and a common underpinning mechanisms i.e., electron of hydrogen donation. The plants extracts, demonstrated electron-donating properties thus may act as free radical chain terminators, transforming reactive free radical species into more stable non radical product[34]. The Di phenyl picryl hydrazyl radical scavenging (DPPH radical scavenging) is a stable radical and used to evaluated the antioxidant capacity of plant extracts. The strongest effect of 1% inhibition of DPPH radical is observed in rutin and BHT, the positive controls with 40.0% and 76.2% of inhibition respectively compared to the plant extracts of *T. purpurea* with 24.5% and *A. sessilis* with a minimum of 16.0. The DPPH free radical scavenging of antioxidant is due to their hydrogen donating ability and showed that plants with high hydrogen donating ability and shown the plants with high hydrogen donating capacity have high DPPH free radical scavenging activity[35]. Hydroxy radicals are one of the quick initiators of the lipid peroxidation process by abstracting hydrogen atom from unsaturated fatty acids as simply auto oxidation of poly unsaturated fatty acids found primarily in membranes and the increase in these peroxide radicals in tissues therefore, reflects membrane damage[36]. Percentage of inhibition of hydroxy radical is highest in positive controls of BHT with 76% and Ascorbic acid with 76.4% when compared to plant extracts of *T. purpurea* with 66.5% and *A. sessilis* with a low 26.4%. The hydroxy radical scavenging activity and inhibition of lipid peroxidation of the extracts is due to the free radical quenching activity of the extracts, which can be attributed to the presence of a number of polyphnolics in the extract. Polyphenolics exhibit a wide spectrum of pharmacological effects as reported earlier[37]. Radical scavenging was reported earlier in *Ternstroemia japonica*[38], *Kappaphycus alvarezii*[39], *Thymus pectinatus*[22]. Percentage of inhibition of lipid peroxidation is highest in positive controls of BHT with 82.6% and Ascorbic acid with 70% when compared to plant extracts of *T. purpurea* with 63.5% and followed by *C. ternate* with 57.8%, *A. sessilis* with 38.9% and *E. prostrata* with a minimum of

18.4%. The inhibition of lipid peroxidation was reported earlier in Flax seed[40], *Gymnema montanum*[41], *Clay sage*[42].

To summarize, the radical scavenging and inhibition of lipid peroxidation by the extracts was due to the quenching free radical of reduction of Fe^{+3} to Fe^{+2}, which can be attributed to the presence of a number of polyphenolics, reduced glutathione, vitamin C and enzymatic antioxidants in the extracts.

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