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RESEARCH ARTICLE

Antioxidant and Anti-proliferative activity of methanolic leaf extract of *Eupatorium glandulosum*. L

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Abstract

From olden days it was believed that natural plants and its products have given the cure of every disease. Furthermore, plant extracts have been revealed to possess health-promoting properties. In the present study leaf extract of *Eupatorium glandulosum* was used to evaluate antioxidant (DPPH, metal chelating ions, hydroxyl radical, hydrogen peroxide, reducing power and nitric oxide radicals) and anti-proliferative properties. Powdered plant leaf was extracted with methanol solvent and tested for the antioxidant activity and analyzed for the result. The plant extract of *E. glandulosum* possessed the highest antioxidant activities in higher concentration (100 mg/ml). Anti-proliferative activity against mitogen induced lymphocytes using MTT assay showed the most potent activity. Plant extracts and plant-derived antioxidant compounds potentiate body's antioxidant defense and are of choice because of their lower toxicity and side effects.

Introduction

The antioxidants are radical scavengers which protect the human body against free radicals. A free radical is a species containing one or more unpaired electrons. Free radicals are electron deficient species, but they are usually uncharged. These free radicals are generated in living systems as a part of the normal metabolic process. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Cancer preventive phytochemicals have been shown to suppress or block carcinogenesis by a variety of mechanisms including acting as antioxidants or antiproliferative agents. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources and these free radicals may oxidize nucleic acids, proteins, lipids which can initiate degenerative disease. The present study aims at the determination of antioxidant and antiproliferative properties in *Eupatorium glandulosum.*

MATERIALS AND METHODS:

Plant material collection

Eupatorium glandulosum L. (Asteraceae) leaves were collected from Ooty (Hill station), India during the month of February 2012.

Extraction

Freshly collected leaves of *E. glandulosum* were shade dried and then powdered using a mechanical grinder. 10 grams of pulverized leaf material were soaked in 100 ml of methanol (LR grade, Merck, India) and kept on a rotary shaker for 24 h. The extract was filtered through a Whatman No. 1 Filter Paper and the process was repeated until all soluble compounds had been extracted. The collected portions of the filtrate were subjected to screening for further studies.

Assaying methods

DPPH free radical scavenging assay

The ability of the plant extracts to scavenge the stable free radical DPPH (2, 2-diphenyl-2-picryl hydrazyl), was assayed by the method of Larrauri et al., (1998). DPPH, a stable free radical acted upon by an antioxidant is converted into diphenyl-picryl hydrazine which involves a colour change from deep violet to light yellow. This can be quantified spectrophotometrically at 517 nm to indicate the extent of DPPH scavenging activity by the plant extracts.

0.1 ml of the extract in various concentrations (1mg, 10 mg, 50 mg, and 100 mg/ml) was added to 2.9 ml of methanolic DPPH solution. The control contained only the reaction reagents. The absorbance was measured at 517 after 30 minutes incubation. The percentage inhibition values were calculated from the absorbance of the control (A_c) and the sample (A_s) by the equation,

$$\textbf{Inhibition (\%)} = (1 - A_s) / A_c \times 100$$

Ascorbic acid, BHT was used as positive control. The values are presented as the mean of duplicate analyses.

$$\underset{\text{(Purple colour)}}{\text{DPPH} + \text{AH}} \longrightarrow \underset{\text{(Yellow colour)}}{\text{DPPH-H} + \text{A}^-}$$

Ability of chelating ferrous ions

The ferrous ion (Fe^{2+}) chelating ability of the leaf extract was measured by the formation of ferrous iron-ferrozine complex method described by Dinis et al., (1994). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted. As a result the formation of red color complex is decreased. The formation of Ferrozine-Ferrous ion complex is not complete in the presence of plant extract, indicating their ability to chelate the ion.

The extracts were dissolved with methanol to prepare various concentrations (1mg, 10 mg, 50 mg, and 100 mg/ml). The reaction mixture containing sample (100µl), 1mM Ferric chloride ($Fecl_2$ -100µl), 1mM ferrozine (200µl) and methanol of 1.3 ml was mixed and then incubated for 10 min at 25 ± 2°C. The change in the absorbance was recorded at 562 nm. Ascorbic acid, BHT, was used as a positive control. The ability of the extract to chelate ferrous ion was calculated using the equation

$$\textbf{Inhibition (\%)} = (1 - A_s) / A_c \times 100$$

Hydrogen peroxide scavenging activity

The scavenging activity of hydrogen peroxide (H_2O_2) by the plant extracts was determined by the method of Lcin (2005). The UV light absorption of hydrogen peroxide can be easily measured at 230 nm. On scavenging of hydrogen peroxide by the plant extracts, the absorption decreases at this wavelength, which property can be utilized to quantify their H_2O_2 scavenging ability.

The extract was dissolved in methanol to prepare various concentrations (1mg, 10 mg, 50 mg, and 100 mg/ml). To the assay, reaction mixture containing sample (100µl), 1.5 ml of phosphate buffer (0.05M, pH 7.4) and 0.6 ml of H_2O_2 (40mM) are added. The change in the absorbance was measured at 230 nm. BHT and Ascorbic acid was used as positive control. The percentage were calculated from the absorbance of the control (A_c) and the sample (A_s) by the equation,

$$\textbf{Inhibition (\%)} = (1 - A_s) / A_c \times 100$$

Hydroxyl radical scavenging activity

The DNA damage induced *in vitro* by hydroxyl radicals generated by hydrogen peroxide in the presence of plant extracts was quantified by the production of TBARS (thiobarbituric acid reactive substances) spectrophotometrically as per the procedure given by Singh et al., (2002).

The hydroxyl radical scavenging activity can be measured by studying the competition between deoxyribose and the plant extracts for hydroxyl radicals generated with Fe^{3+}/ ascorbate / EDTA / H_2O_2 system. The hydroxyl radicals attack deoxyribose, which eventually result in TBARS formation, which can be quantified spectrophotometrically.

The reaction mixture contained sample (100µl), Iron EDTA (1 ml), 0.018 % of EDTA (500µl), 0.85 % of DMSO prepared in 0.1 ml of phosphate buffer (1000µl) and 0.22 % of Ascorbic acid (500µl). The mixture was incubated in water bath for 15 min in 80-90°C. Then it was taken to room temperature and 17.5% of ice cold TCA (1000µl) was added along with 3ml of Nash reagent. The mixture was again left for 15 min at 25± 2° C. The absorbance was measured at 412nm. The absorbance of control was determined by replacing the sample with methanol. BHT, EDTA, and ascorbic acid were used as a positive control. The scavenging activity on hydroxyl radical was calculated as follows,

$$\textbf{Inhibition (\%)} = (1 - A_s) / A_c \times 100$$

Reducing power activity

The reducing power of the extract was determined according to the method of Oyaizu (1986). In this assay, the yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of the extract. The presence of reductants (antioxidants) in the plant extract causes the reduction of Fe^{3+}/ Ferricyanide complex to ferrous form. Therefore, Fe^{2+} can be monitored by measuring formation of Perl's Prussian blue at 700 nm (Chung et al., 2002).

Extract solution (0.1ml) was mixed with sodium buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1 %). This was incubated at 50°C for 20 min. After incubation 2.5 ml of 10 % Trichloroacetic acid was added, and kept for centrifugation for 10 min. After centrifugation 5 ml of aliquot of the upper layer was taken and to this 5 ml distilled water and 1000 µl of 0.1 % ferric chloride were added. Absorbance was measured at 700 nm. Increasing absorbance of the reaction mixture indicates increasing reducing power.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging assay was performed according to the method described by Sreejayan et al., (1997) with slight modification.

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. The absorbance of the chromophore formed was measured at 546 nm.

The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate buffered saline, and extract of various concentrations (1, 10, 50 and 100 mg/ml) were incubated at 25°C for 150 min. After incubation added 0.5 ml of Griess reagent. The absorbance was measured at 546 nm. BHT and Ascorbic acid were used as a positive control.

$$\textbf{Inhibition (\%)} = (1 - A_s) / A_c \times 100$$

Antiproliferative assays

Establishment of lymphocyte culture

Isolation of peripheral blood lymphocytes

Venous peripheral blood was collected aseptically from healthy donors using EDTA as anticoagulant. The blood was diluted with equal volume (3 ml) of RPMI 1640 medium. Peripheral blood lymphocytes were separated by density gradient centrifugation at 400 g using lymphocyte separation medium. The white layer formed intermittently was taken and washed by using RPMI 1640. After centrifugation at 160-200 g for 10 minutes, the pellet was collected. Isolated peripheral blood lymphocytes were suspended in RPMI medium with 10 % sterile fetal bovine serum (FBS), 2mM glutamine – streptomycin solution (Suganthy et al., 2010).

Cell viability

The cell suspension was mixed with equal volume of 0.4 % tryphan blue and loaded in to a hemocytometer for cell count (Freshney, 1994). The reactivity of tryphan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Hence all the cells which exclude the dye are viable.

Lymphocyte culture

The lymphocytes were grown to log phase by overnight culture in RPMI 1640 containing 10% heat inactivated bovine serum and 1% antibacterial antimycotic solution (Sigma). After checking the viability 1.0 X 10^6 cells / ml was seeded in six well plates and made up to 3.0 ml with complete media.

Mitogen induction of Isolated Lymphocytes

The lymphocytes were checked for the viability and 1.0 X 10^6 cells /ml were seeded in six well plates with Concanavalin A (10 µg /ml) to induce cell proliferation.

MTT Assay

Cytotoxic activity was studied by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 -diphenyltetrazolium bromide) assay. Solvent chosen for extraction is methanol. Evaporated plant sample was weighed according to the concentration 1 mg/ml, 10 mg/ml, 50 mg/ml, 100 mg/ml and dissolved in DMSO (Di Methyl Sulphoxide). Quercetin (1 mg/ml) was used as a positive control and DMSO as negative control. The cells in the range of 1.5 x 10^3 cells in 100µl were added to 96 well plates followed by different concentration (1, 10, 50 and 100 mg/ml) of extracts to a volume of 20 µl. The culture was incubated for 44 hours, followed by addition of 10µl MTT (1mg/ml) and incubated for four hours. The cells were spin at 3000 rpm for 20 min followed by adding 100µl of DMSO to all the wells. After 15 minutes incubation absorbance was read at 540 nm. The control well had no extract and blank had no cells and extracts (Manosroi et al., 2006).

RESULT AND DISCUSSION

DPPH free radical scavenging assay

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts (Nanjo et al., 1996). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns yellow which indicates that the electron of DPPH radical has paired with hydrogen from a free radical scavenging antioxidant.

Among various concentrations, DPPH free radical scavenging activity for *E. glandulosum* (Table-1 & Fig. 1) was high with 100 mg/ ml of sample (66.8 ± 0.70 %). Similar results have been observed in Ulam raja (*Cosmos caudatus*) leaves (Wong et al., 2005). DPPH radical scavenging activity of leaves of *Sauropus androgynous* and *Altrananthera pungens* species reveals that they have got profound antioxidant activity (Ghosh et al., 2011). Fruit and leaf methanol extracts showed the highest scavenging effects of 90.16 ± 0.01 % and 82.24 ± 0.02 %, respectively at concentration of 5 mg / ml, with IC_{50} value of 0.9 mg / ml and 1.5 mg / ml, respectively in the sample *Ardisia crispa* (Jindal et al., 2012).

Ability of chelating ferrous ions

Ferrous ion, commonly existed in food systems, is well known as an effective pro-oxidant (Hsu et al., 2003). Ferrozine can quantitatively form complexes with Fe^{2+}.

In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of complex is decreased. The formation of Ferrozine - Fe^{2+} complex is not fulfilled in the presence of plant extract, indicating their ability to chelate the iron.

The chelating agents which form ○¯ bond with a metal are effective secondary antioxidants, because they decrease the redox potential there by steadying the oxidized form of the metal ion (Duh et al., 1999). Non phenolic metal chelators include phosphoric acid, ascorbic acid, carnosin, some amino acids, peptides and proteins such as transferrin are also responsible for metal chelation (Lee et al., 2004).

Among various concentrations of the leaf extracts of *E. glandulosum* (Table-1 & Fig. 1) an extreme chelating activity was observed at100 mg/ml (70.5 ± 0.98). Maximum chelation of metal ions was detected from the leaf methanolic extract of *C. occidentalis* (77.12 ± 0.10 in 1.0 mg / ml) followed by seed and stem extracts (Arya et al., 2011).

Hydrogen peroxide scavenging activity

Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals which is very toxic to the cell (Halliwell, 1991). Thus, scavenging of H_2O_2 is a measure of the antioxidant activity of the fractions.

Hydrogen peroxide scavenging activities of the leaf extract can possibly be due to the presence of phenolics, which donate electrons to H_2O_2, thereby neutralizing it into water. Although H_2O_2 itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radical in the cell. Hence it is worth removing H_2O_2 throughout food systems.

The hydrogen peroxide activity of *E. glandulosum* (Table-1 & Fig. 2) leaf showed 66.8 ± 1.1% in 100 mg/ml. The extract was able to scavenge hydrogen peroxide in a concentration dependent manner. Higher concentration exhibited higher scavenging activity. Similar studies in *Ziziphus mucronata* leaf extracts revealed high antioxidant activity in higher concentration of extract (Kwape and Chaturvedi, 2012).

Hydroxyl radical scavenging assay

Hydroxyl radical is the most deleterious and reactive among ROS and it bears the shortest half – life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid (Halliwell et al., 1987). The hydroxyl radical is an extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells (Halliwell, 1997; Waling, 1975). Hydroxyl radicals are short-lived, but the most damaging radicals within the body. This type of free radical can be formed from O_2- and H_2O_2 via the Harber - Weiss reaction. The interaction of copper or iron and H_2O_2 also produce OH^- which was first observed by Fenton. Generally molecules that inhibit deoxyribose degradation are those that can chelate the iron ions and thereby prevent them from complexing with deoxyribose and render them inactive in a Fenton reaction (Smith et al., 1992).

100 mg / ml of the leaf extract of *E. glandulosum* (Table-1 & Fig. 2) showed high hydroxyl radical activity (92 ± 0.97 %). The sample extract possess better and high scavenging activity even at low concentration. Similar results were observed in case of *Avicennia officinalis* species, where the ethanol leaf extract examined exhibited better antioxidant activity (82.75 ± 2.63 in 2 mg/ml) compared to standard ascorbic acid (88.54 ± 3.54 in 2 mg/ml) (Thirunavukkarasu et al., 2011). Related results were also obtained for seed, stem and leaf extracts of *C. occidentalis* (Arya et al., 2011).

Reducing Power activity

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The reductive activities were measured by the potassium ferricyanide reduction method, and quantified by absorbance measurement at 700 nm. The transformation of Fe^{3+} into Fe^{2+} in the presence of various fractions was measured to determine the reducing power ability. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom (Meir et al., 1995).

The antioxidant principles present in the fractions of *E. glandulosum* (Table-1 & Fig. 3) caused the reduction of Fe^{3+} / ferricyanide complex to the ferrous form, and thus proved the reducing power ability. Increased absorbance of the reaction mixture indicated increased reducing power. Similar studies in pomegranate (*Punica granatum*) carried out with seed, peel, leaf and flower extracts exhibited higher activity with increased concentrations. When compare to peel and flower, leaf showed high reducing power, with significant results (Elfalleh et al., 2012).

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological processes (Lata et al., 2000). Additional concentration of NO is associated with several diseases (Ross R 1993; Ialenti et al., 1993). Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions which act as free radicals (Sainani et al., 1997). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada et al., 1991).

The various concentration of *Eupatorium glandulosum* leaf effectively reduced the generation of nitric oxide from sodium nitroprusside (Table 1 & Fig. 4). Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent. The extract showed higher activity in higher concentration when compare to the synthetic antioxidant like BHT and Ascorbic acid the activity is less. Similar studies in *Coccinia grandis* leaf extract showed strong nitric oxide scavenging activity (Umamaheswari et al., 2008).

Antiproliferative assays

The antitumor area has the greatest impact on plant derived drugs, where drugs like vinblastine, vincristine, taxol and camptothecin have improved the chemotherapy of some cancers (Newman et al., 2003). Plants are exploited for new and novel chemotherapeutics. The continuing search for new anticancer compounds in plant medicines and traditional foods is a realistic and promising strategy for its prevention (Wei et al., 2009). Numerous groups with antitumor properties are plant derived natural products including alkaloids, phenyl propanoids and terpenoids (Park et al., 2008). The present study was conducted to evaluate the antiproliferative activity of fractionated leaf extracts from *E. glandulosum.*

Cell Viability

The quantity of lymphocytes obtained by density gradient centrifugation was equivalent to 1.15×10^6 cells per ml. The viability of the isolated lymphocytes was analyzed by tryphan blue dye exclusion method and the cell viability was highly significant ranging to 98.2%. The percentage of viable cells was good and therefore used as such for further analysis.

Comparison of cell quantity in normal and mitogen induced human peripheral blood lymphocytes

The cells were cultured overnight in two different plates one with Concanavalin A (mitogen induced) and another one without Concanavalin A (normal). It shows that there is 70.86% increase in the cell number in mitogen induced culture (Table 2).

Cytotoxicity

The percentage control of growth by the extract (cytotoxicity) was found to be significant in leaf extract of *Eupatorium glandulosum* (Table 3 & Fig 5). High cytotoxic effect was observed at 100 mg/ml concentration of plant extract. Quercetin is used as a positive control.

Fig. 1. DPPH and Metal Chelating Radical Scavenging Assay

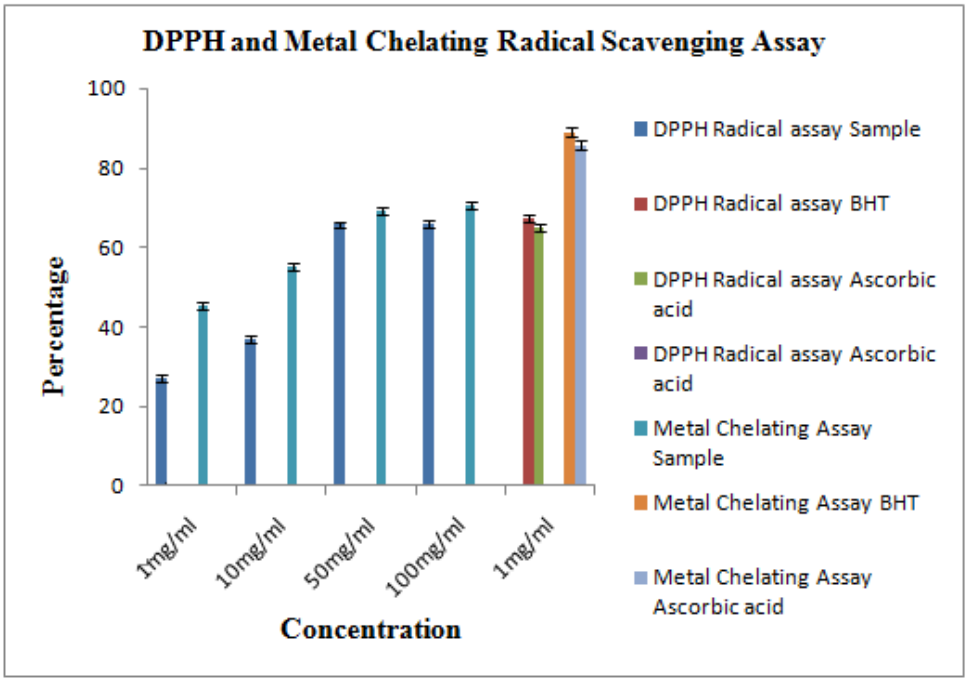


Fig. 2. Hydrogen peroxide and Hydroxyl Radical Scavenging Assay

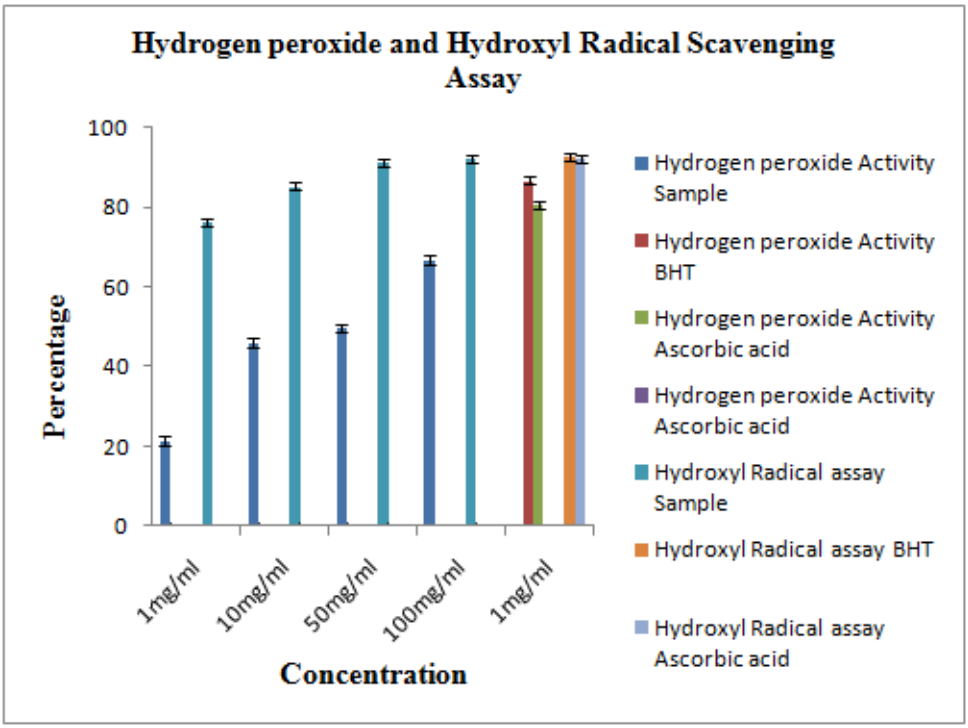


Fig. 3. Reducing Power activity

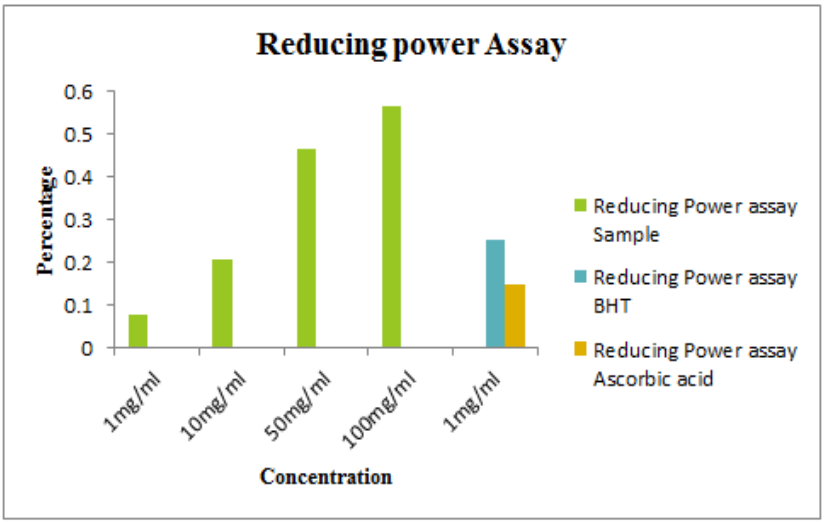


Fig. 4. Nitric oxide radical scavenging activity

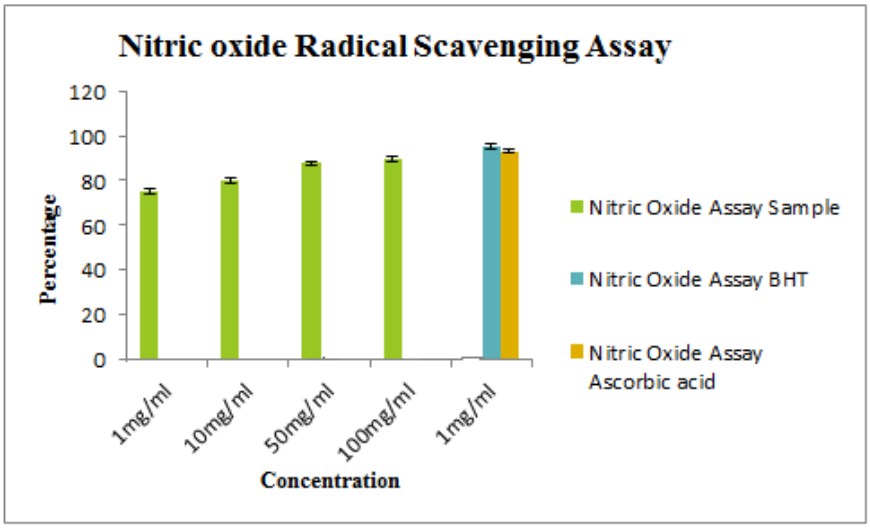


Table: 1 Antioxidant analysis of Methanol leaf extract of *Eupatorium glandulosum*

Antioxidant assays	Standards		Concentration				IC_{50} value for samples
	BHT 1mg/ml	Ascorbic acid 1mg/ml	1mg/ml	10mg/ml	50mg/ml	100mg/ml	
DPPH assay	67.37%	65.11%	27.2%	37.0%	65.59%	66.8%	60.83
Metal chelating assay	88.9%	85.90%	45.3%	55.1%	69.5%	70.8%	56.49
Hydrogen peroxide activity	86.7%	80.8%	21.2%	46.0%	49.6%	66.8%	65.35
Hydroxyl radical activity	92.0%	93.0%	76.0%	85.0%	91.0%	92.0%	42.92
Reducing power activity	0.2525	0.147	0.072	0.202	0.436	0.561	-
Nitric oxide activity	95.3%	93.0%	75.6%	79.9%	87.9%	89.0%	44.46

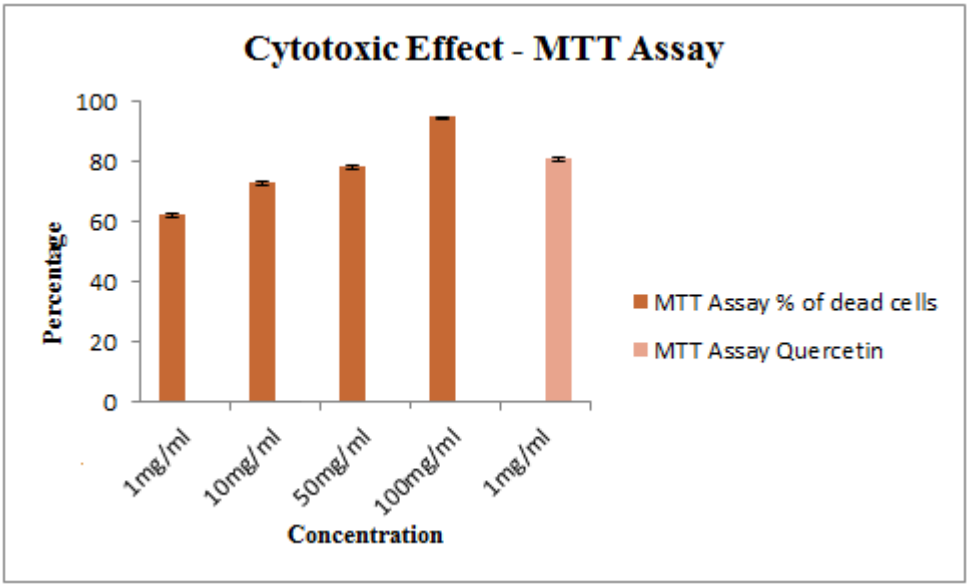
Table: 2 Comparison of the viability of normal lymphocytes and mitogen induced lymphocytes

S.No	Lymphocyte Culture	Cells/ ml
1	Normal lymphocytes	5.4×10^5
2	Mitogen induced lymphocytes	7.62×10^5

Table: 3 CYTOTOXICITY ASSAY

S. No	Concentration	% of Live Cells	% of Dead Cells	Dissolved in
1	1 mg/ml	37.83	62.16	DMSO
2	10 mg/ml	27.02	72.97	DMSO
3	50 mg/ml	21.6	78.37	DMSO
4	100 mg/ml	5.4	94.59	DMSO
5	Quercetin (+ve control) 1mg/ml	18.9	81.08	Water

Fig. 5. Cytotoxic effect of leaf extract on mitogen induced human peripheral blood lymphocytes (*E. glandulosum* Leaf extract dissolved in DMSO-Dimethyl sulfoxide)



SUMMARY

Antioxidant ability is broadly used as a parameter to characterize food or medicine plant and their bio-active components. Modern patrons ask for natural item for consumption, free of synthetic additives. According to nutritionists some synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have side effects with carcinogenic effects in living organisms. Hence there is a growing interest into the analysis of potentially active natural phytochemicals. Plant extracts and plant-derived antioxidant compounds potentiate body's antioxidant defense and are of choice because of their lower toxicity and side effects.

Due to different antioxidant potentials of different compounds, the antioxidant activity of extract strongly dependents on the extraction solvent and among them, methanol and ethanol are preferred more. Our results conclude that methanolic leaf extract of *E. glandulosum* has antioxidant capacity to various free radicals.

The leaf extract of *E. glandulosum* showed a strong antiproliferative activity against Mitogen induced lymphocyte. This seems that the leaf extract can be used as natural antiproliferative agent.

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