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Antioxidant activity of *Eclipta alba* extract

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Abstract
Eclipta alba (Asteraceae) is used traditionally in ayurvedic system of medicine in India for the treatment of liver diseases and also used as a liver tonic. *In vitro* antioxidant activity of hydro-alcoholic extract of *Eclipta alba* was evaluated by studying superoxide radical scavenging activity, hydroxyl radical scavenging activity, nitrous oxide radical scavenging activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing ability and Fe^{+2} chelating ability using standard procedure. Identification and quantification of the wedelolactone, one of the active constituents of the *Eclipta alba* plant extract, was carried out by HPLC analysis. Result of the present study indicates that the *Eclipta alba* extract shows high amount of phenolics, flavonoids, tannins and ascorbic acid contents. Hydro-alcoholic extract of *Eclipta alba* effectively scavenged free radicals at all different concentrations and showed potent antioxidant potency and effects were in a dose-dependent manner. *Eclipta alba* extract possess potent antioxidative properties.

Keywords: *Eclipta alba*, Antioxidants, free radical scavenging, 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

Introduction
Medicines derived from plants have played a pivotal role in the health care of many cultures, both ancient and modern. Scientific evaluation of plants has often shown that active principles in these are responsible for therapeutic success. Medicinal plants may serve as a vital source of potentially useful new compounds for the development of effective therapy to combat a variety of ailments. Plant derived natural products such as flavonoids, terpenoids, steroids, etc. have diverse pharmacological properties including antioxidant activity. Antioxidants are compounds that help to inhibit many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, peroxyl and hydroxyl radicals etc., thereby preventing or delaying damage to the cells and tissues. Antioxidant compounds have potential to mitigate the effect of free radicals and play an important role as a health protecting factor. [1]

Eclipta alba Linn. occurs throughout the whole of India. It is widely distributed throughout India, China, Thailand, and Brazil. *Eclipta alba* (L.) has been used in various parts of tropical and sub-tropical regions like south America, Asia, Africa. In India, the plant is known as bhangra, "bhringraj" or bhringraja. The branches are hairy, reddish brown and can grow up to 40 cm height. The roots are found growing at the thickened nodal points. The leaves are opposite, lance like with toothed edge and hairy. The flowers are white, small and arranged in small clusters. The flowering stalk arises from the axis of the leaf. The dry fruit is formed by fusion of two carpels, which do not break open and each has just one seed. Root well developed, cylindrical and greyish. [2] In Ayurveda the plant is considered as a rasayana for longevity and rejuvenation. Plant is bitter, hot, sharp, dry in taste and is used in Ayurveda and "Siddha" for the treatment of kapha and vata imbalances. *Eclipta alba* Linn. has been traditionally used for blackening, promoting hair growth and strengthening the hair. It is useful in inflammations, hernia, eye diseases, bronchitis, asthma, leucoderma, anaemia, heart, skin diseases and syphilis etc. It is popularly used to enhance the memory and has a reputation as an anti-aging agent. The plant has been reported to contain phytosterol, β-amyrin, triterpenes such as ecalbatin, echinocystic acid, flavones such as luteolin and coumarin such as wedelolactone. [2]

The main aim of the present study was to evaluate antioxidative properties of the hydro-alcoholic extract of *Eclipta alba.*

Materials and methods
Chemicals
The chemicals used in the entire study were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India; Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Sigma-Aldrich,

St. Louis, MO, USA and was of analytical grade. HPLC grade methanol, acetonitrile and acetic acid were obtained from Merk specialities Pvt. Ltd., Mumbai, India.

Plant material

The whole plant of *Eclipta alba* was collected during September-October, 2011 from the Botanical garden of the Gujarat University, Ahmedabad. Herbarium specimens were prepared and authenticated by Dr. Hitesh Solanki, Associate Professor, Department of Botany, Gujarat University, and Ahmedabad.

Extract preparation

The extract was prepared according to World Health Organization protocol CG-06 (1983) with slight modifications. [3] The whole plant materials were shade dried and powdered. 5 gm of whole powdered plant material was extracted by overnight soaking method using 100 ml of 50% aqueous-ethanol (v/v). The content was filtered successively through ordinary and then Whatman filter paper No. 1. Extraction procedure was repeated. Both the fractions were pooled, dried and stored in dark bottle at 4 ^{0}C. During the experiment known amount of dried extract was redissolved in double distilled water and used. The percent yield of the extract was calculated.

Qualitative and quantitative analysis

The hydro-alcoholic extracts of *Eclipta alba* were subjected to phytochemical analysis for the detection of the major chemical groups. Qualitative and quantitative estimation of phytochemical constituents were done as described below.

Qualitative analysis

Qualitative analysis for determining the presence of phenolics, flavonoids, tannins, and ascorbic acid in the plant extracts were carried out using standard methods.

Test for phenolics

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride ($FeCl_3$) solution was added. A dark green colour indicated the presence of phenolic contents.

Test for flavonoids

5 ml of dilute ammonia solution was added to plant extract, followed by addition of concentrated H_2SO_4. A yellow colouration indicated presence of flavonoids.

Test for tannins

0.5 gm of extract was dissolved in 20 ml distilled water in a test tube and then filtered. A few drops of 0.1% $FeCl_3$ was added and observed for brownish green or blue black colour.

Test for ascorbic acid

To the extract, one drop of 2, 6 dichlorophenolindophenol (DCPIP) solution was added. Formation of blue to red colour indicates the presence of ascorbic acid.

Quantitative analysis

Total phenolic content

Total phenolic content of the extract was estimated by the method as described by Singleton *et al*. (1999). [4] Briefly extract react with Folin-Ciocalteu reagent in the presence of sodium carbonate to form a blue coloured complex which was read at 760 nm. Various concentrations of gallic acid were used to plot standard curve. Total phenolic content of extract was expressed as mg gallic acid equivalent/gm dry wt. of extract.

Flavonoid content

The flavonoid content of the plant extract was estimated by the method of (Lamaison and Carnat, 1990). [5] Briefly 1.0 ml of plant extract was mixed with 1.0 ml of aluminium chloride reagent and resultant colour was read at 430 nm. The flavonoid content of the extract was expressed as mg quercetin equivalent/gm dry wt. of extract.

Tannin content

Tannin content of the extracts was estimated by the method as described by (Price and Butler, 1977). [6] Plant extract was allowed to react with K3Fe(CN)6-$FeCl_3$ reagent for five min and the intensity of colour developed was measured spectrophotometrically at 720 nm. The tannin content of the extract was expressed as mg rutin equivalent/gm dry wt. of extract.

Ascorbic acid content

Ascorbic acid, also known as vitamin C, is one of the most abundant antioxidant present in plant was quantified by the method of Jagota and Dani (1982). [7] The ascorbic acid content of plant extracts were expressed as µg/gm dry wt. of extract.

Antioxidative potency

Antioxidative potency of the plant extract (*Eclipta alba*) was estimated by various chemical assay systems as described below:

Superoxide radical scavenging assay

Superoxide radical scavenging activity was assessed by the method of Liu *et al*. (1997). [8] In the PMS/NADH-NBT system, superoxide anion derived from dissolved O_2 by PMS/NADH coupling reaction reduces NBT. Addition of various concentrations of hydro-alcoholic extract resulted in decreased colour intensity which was read at 560 nm against blank to determine the quantity of the formazon generated. IC_{50} values of the extract (concentration required to scavenge 50% of the radicals) was calculated.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extract was estimated by the method of Halliwell *et al*. (1987), where radicals were generated from Fe^{+3}/ ascorbate/ EDTA/ H_2O_2 system by Fenton's reaction. [9] Briefly different concentrations of plant extracts were made to react with 2-deoxy-2-ribose, H_2O_2, $FeCl_3$ and EDTA. The reaction was initiated by the addition of ascorbic acid. After incubation for 90 min the reaction was terminated by addition of thiobarbituric acid (TBA) and resulting colour was read at 590 nm. Percent inhibition by various concentrations of plant extract and IC_{50} of the extract was calculated.

Nitrous oxide radical scavenging assay

Nitrous oxide radical scavenging activity was measured using the method of Sreejayan and Rao (1997). [10] Various concentrations of plant extract was incubated with 10 mM sodium nitroprusside for 150 min. After incubation, Griess reagent was added to the tubes and absorbance of chromophore formed was read at 590 nm. IC_{50} values and percent inhibition by various concentrations of plant extract

was calculated comparing the absorbance values of control and test compounds against blank.

DPPH radical scavenging assay

Ability of plant extract to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured by the method of Gyamfi *et al*. (1999). [11] DPPH is a purple colour radical compound which changes to stable compound having yellow colour by reacting with antioxidant compounds. Addition of 0.1 mM DPPH solution in various concentrations of extracts in presence of tris-HCl buffer (50 mM, pH-7.4) resulted in decreased absorbance which was measured at 517 nm. Percent inhibition was calculated by measuring the absorbance of plant extract treated samples against blank. IC_{50} values for the extract was calculated.

Reducing ability

Reducing ability of *Eclipta alba* plant extract was evaluated using method of Yildirim (2000). [12] Briefly various concentrations of the extract was mixed with potassium phosphate buffer and potassium hexacyanoferrate $[K_3Fe(CN)_6]$ and incubated for 30 min. Reaction was terminated by addition of trichloroacetic acid (TCA) followed by addition of $FeCl_3$. Antioxidants present in the tested plant samples reduces Fe^{+3}/ferricyanide complex to the ferrous form (Fe^{+2}) resulting in formation of perl's Prussian blue colour which was read at 700 nm.

Fe^{+2} chelating ability

The Fe^{+2} chelating activity of the plant extract was estimated using the method of Dinis *et al*. 1994. [13] Plant extract was allowed to react with ferrozine (5 mM) in presence of $FeCl_3$ (2 mM). Blue coloured Fe^{+2} – ferrozine complex formed was read at 562 nm. Chelating ability of the extract was compared with EDTA (0.01 mM). Percent inhibition and IC_{50} value for the extract was calculated by comparing test samples with the control.

Hi-performance liquid chromatography (HPLC)

Identification and quantification of Wedelolactone from the hydro-alcoholic extract of *Eclipta alba*

Standard stock preparation: Standard stock of wedelolactone was prepared by dissolving 10 mg of dry extracts in 10 ml milique water.

Plant extract preparation: Whole plant extract of *Eclipta alba* was prepared by dissolving 10 mg of dry extract in 10 ml milique water.

Chromatographic conditions

A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10ADVP pump, SIL-HTc autosampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. Inertsil ODS-C18 (150 mm length × 4.6 mm inner diameter, 5µ particle diameter) analytical column from Phenomenex Inc. (Torrance, CA, USA) was used. Column oven temperature was 30°C, UV detector was used, separation mode was isocratic, mobile phase was acetonitrile-methanol (70:30 v/v) and flow rate was 1 ml/min. Total chromatographic run time was 30 min and injection volume was 20 µl. Plant extract and wedelolactone standard were separately run on chromatographic column. Based on the retention period of wedelolactone standard and its corresponding peak in the crude extract chromatogram identification and quantification of the same was achieved. Spiking the standard wedelolactone solution with the plant extract *Eclipta alba* was done to confirm presence of wedelolactone in the extract.

Statistical analysis

The results were expressed as the means ± standard error of the mean (SEM). The IC_{50} values were calculated by probit analysis. The data were statistically analysed using GraphPad Instat software, version 5.03. The values were statistically analysed by one way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test at a significance level $p<0.05$. Also, linear regression analysis (R^2 value) was performed.

Results

Phytochemical analyses of the plant extract

Qualitative analysis

Table 1 depicts the results of phytochemical analysis of *Eclipta alba* extract. The qualitative analysis of *Eclipta alba* extract indicated the presence of phenolics, flavonoids, tannins and ascorbic acid contents, which were later determined quantitatively using standard methods.

Table 1: Qualitative and quantitative analysis of the hydro-alcoholic extract of *Eclipta alba*

Phytochemical parameters	*Eclipta alba*
Extract yield (%)	15.03 ± 1.38
Qualitative Analysis	
Phenolic content	+
Flavonoid content	+
Tannin content	+
Ascorbic acid content	+
Quantitative Analysis	
Phenolic content	98.39 ± 2.39
Flavonoid content	86.53 ± 1.90
Tannin content	40.00 ± 5.20
Ascorbic acid content	2.24 ± 0.24

Results are expressed as mean ± SEM; n = 6.

Units: Phenolics – mg gallic acid equivalent/gm dry wt. of extract; Flavonoids – mg quercetin equivalent/gm dry wt. of extract; Tannins – mg rutin equivalent/gm dry wt. of extract; Ascorbic acid - µg/gm dry wt. of extract.

Quantitative analysis

Total phenolic content

The concentration of TPC determined in hydro-alcoholic extract of *Eclipta alba* was 98.39 mg gallic acid equivalent/gm dry weight calculated using equation that was obtained from standard gallic acid graph (Table 1).

Flavonoid content

Quantification of flavonoid content showed that hydro-alcoholic extract of *Eclipta alba* contained 86.53 mg quercetin equivalent/gm dry weight of extract (Table 1).

Tannin content

Standard curve for tannin estimation was plotted using various concentrations of rutin. Tannin content of hydro-alcoholic extract of *Eclipta alba* was found to be 40 mg rutin equivalent/gm dry weight of tannin (Table 1).

Ascorbic acid content

Ascorbic acid content of hydro-alcoholic extract of *Eclipta alba* was found to be 2.24 µg/gm dry weight of extract (Table 1).

Antioxidative potency

This study was designed to assess the potential of *Eclipta alba* extract to scavenge free radicals using chemical models.

Superoxide radical scavenging assay

Superoxide radicals generated from PMS/NADH-NBT system were strongly scavenged by various concentrations of *Eclipta alba* extract. The decrease in colour intensity was observed with increasing concentration of extract indicating consumption of the radicals in the reactions mixture (Fig. 1). Maximum effect was achieved at 300 µg/ml concentration of the extracts. Maximum scavenging effect found with *Eclipta alba* extract was 84.19%. The effect was concentration-dependent for the extracts (R^2 = 0.9874). Concentration required to scavenge 50% (IC_{50}) of the radicals was 150 µg/mL for *Eclipta alba* extract.

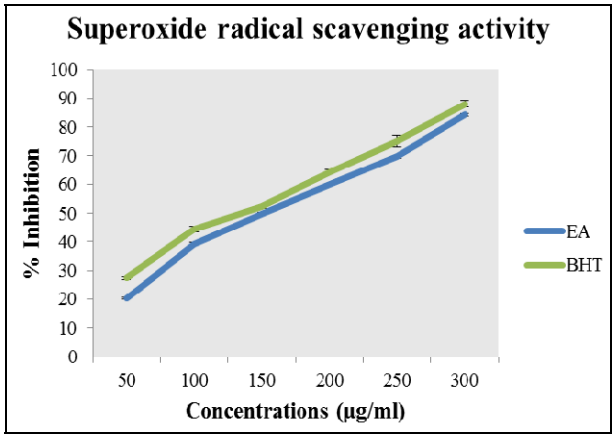

Fig 1: Superoxide radical scavenging activity of *Eclipta alba* extract

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity of extract is directly proportional to its antioxidative potency. The percent inhibition of hydroxyl radical increased significantly (p<0.05) with increasing concentrations of hydro-alcoholic extract of *Eclipta alba* and (Fig. 2). Maximum protection for *Eclipta alba* extract was 71.84%. The protective effect was concentration-dependent (R^2 = 0.9956) and was highest at 50 µg/ml concentration (fig. 2). IC_{50} values of *Eclipta alba* extract was 30 µg/ml.

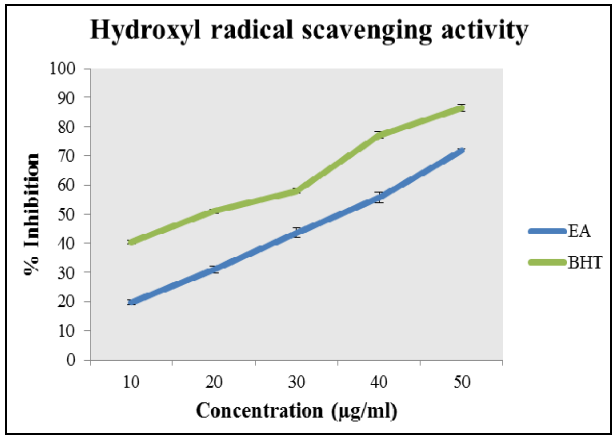

Fig 2: Hydroxyl radical scavenging activity of *Eclipta alba* extract

Nitrous oxide radical scavenging assay

Nitrous oxide radicals generated from sodium nitroprusside at physiological pH were significantly (p<0.05) inhibited by *Eclipta alba* extract. Percent inhibition was concentration-dependent (R^2 = 0.9035) and maximum at 250 µg/ml concentration of the extract. Highest scavenging effect found with *Eclipta alba* extract was 85.55%, (fig. 3). IC_{50} values for nitrous oxide scavenging activity was 50 µg/ml for *Eclipta alba* extract.

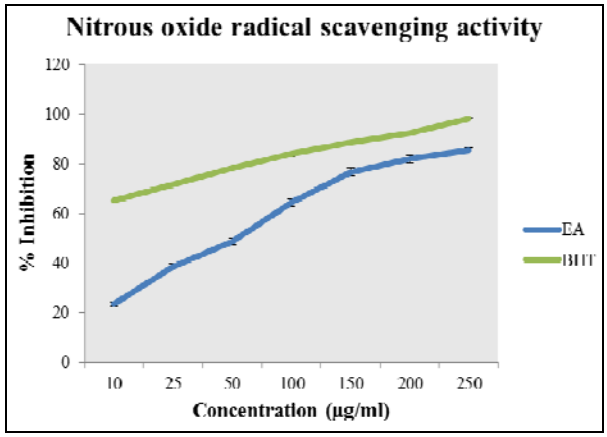

Fig 3: Nitrous oxide radical scavenging activity of *Eclipta alba* extract

DPPH radical scavenging assay

DPPH radical scavenging activity of various concentrations of *Eclipta alba* was found statistically significant (p<0.05). Decrease in absorbance due to antioxidative effect of soluble solids of *Eclipta alba* was highest at 250 µg/ml concentration (fig. 4). *Eclipta alba* was found to be potent (82.47%). Scavenging effect of the *Eclipta alba* extract was concentration-dependent (R^2 = 0.8216,). IC_{50} value for *Eclipta alba* extract was 50 µg/ml.

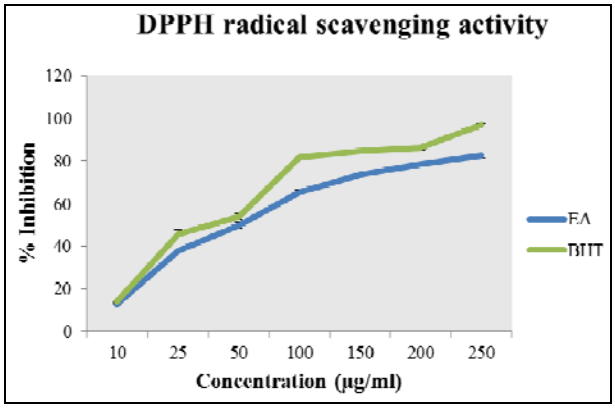

Fig 4: DPPH radical scavenging activity of *Eclipta alba* extract

Reducing ability

The presence of reductant (antioxidant) in the tested extract of *Eclipta alba* resulted in the reduction in Fe^{+3}/ferricyanide complex to ferrous form (Fe^{+2}). Increasing concentrations of the extracts resulted in simultaneous increase of reducing power (fig. 5). Highest reducing ability was found with *Eclipta alba* extract (75.59%) at 250 µg/mL concentration (fig. 5). Increase in reducing ability of the extracts was concentration-dependent (R^2 = 0.9863). IC_{50} values for reducing ability was 100 µg/mL for *Eclipta alba* extract.

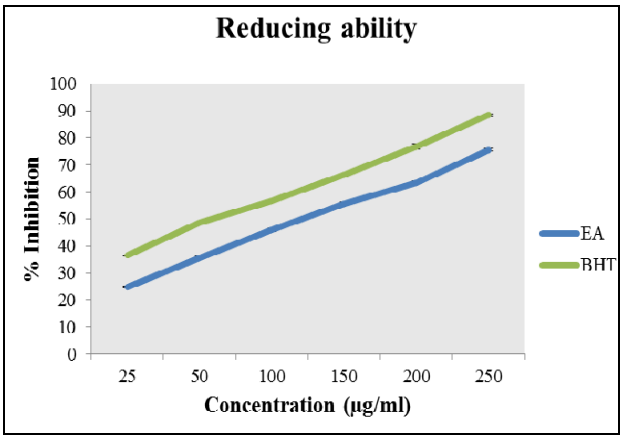

Fig 5: Reducing ability of *Eclipta alba* extract

Fe*$^{+2}$ *chelating activity

Ferrozine - Fe^{+2} complex produces violet colour in the reaction mixture in presence of metal ions which was significantly reduced by addition of *Eclipta alba* extract. Formation of coloured chromophore is interrupted in the presence of chelating agents of *Eclipta alba* extract and resulted in decreased optical density (fig. 6). Maximum inhibition achieved with *Eclipta alba* extract was 78.77%. *Eclipta alba* extract chelated metal ions in a concentration – dependent manner (R^2= 0.9656). IC_{50} values for *Eclipta alba* extract was 100 µg/ml.

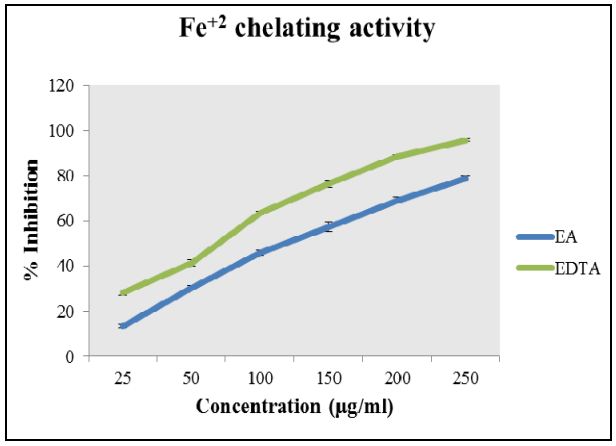

Fig 6: Iron chelating activity of *Eclipta alba* extract

Identification and quantification of active component of plant extract

Standardization of the plant extract was done by quantifying the major active components of the plant extract. The major active component wedelolactone is present in plant the plant *Eclipta alba* was separated from other constituents by reverse phase HPLC analysis. Under the optimized chromatographic conditions, the reaction time for standard wedelolactone was 2.223 as shown in Fig.7. Chromatogram of hydro-alcoholic extract of *Eclipta alba* showed five peaks of 1.627, 2.233, 2.515, 2.833 and 3.068 min as shown in fig. 8. The peak of 2.233 in the crude extract correspond to wedelolactone, which was confirmed by spiking the sample with standard stock solution of wedelolactone (1000 µg/mL) as depicted in Fig. 8. Percent concentration of wedelolactone present in *Eclipta alba* extract was 4.47%.

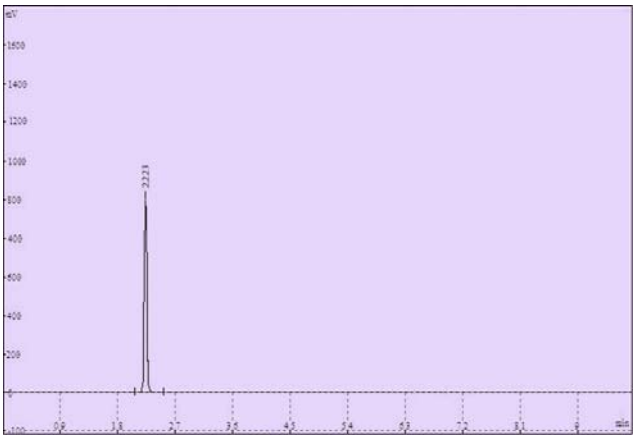

Fig 7: Chromatogram of Wedelolactone

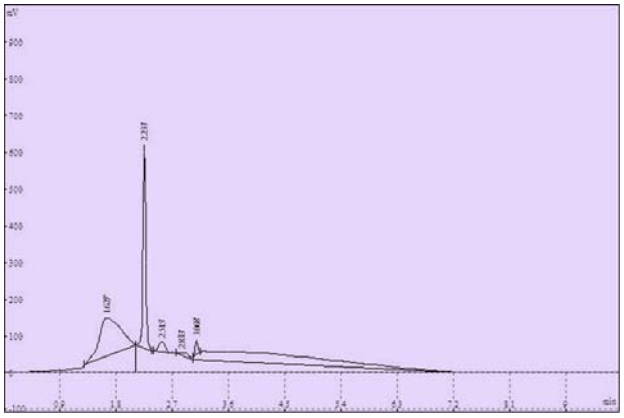

Fig 8: Chromatogram of *Eclipta alba* extract

Discussion

In the present study qualitative assessment of phytochemical constituents of *Eclipta alba* plant extract showed presence of phenolics, flavonoids, tannins and ascorbic acid contents. The quantitative analysis revealed presence of phenolic content in *Eclipta alba* extract. Phenolic as well as flavonoids were in high amount as compared to other phytoconstituents analysed. The presence of alkaloids, phenolic compounds, flavonoids, tannins have been associated with various degrees of antioxidant activities. [14, 15] Phenolics are antioxidants by nature due to their redox properties and metal chelating effects. [16, 17] Polyphenols from plant source are excellent free radical scavenger and widely distributed in the herbs of Lamiaceae species. [18]

In the present study quantitative estimation of crude polyphenols from hydro-alcoholic extracts of *Eclipta alba* revealed presence of significantly high amount of phytochemicals principally responsible for its protective effect (Table 1). Hepatoprotective activity present in these plants may be due to the activities of one or a combination of some of the classes of compounds present. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. [19]

The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular diseases. [20] Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS [21, 22], continuous

exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage. [23] Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated. [24] In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity. [25-28]

In vitro assessment of antioxidative properties of various plant extracts using chemical models provides biochemical basis for the *in vivo* ethnopharmacological uses of the plants. *Eclipta alba* extract was found to be potent scavenger of superoxide (Fig. 1), hydroxyl (Fig. 2), nitrous oxide (Fig. 3) and DPPH (Fig. 4) radicals. Free radical scavenging activity of *Eclipta alba* extract was reported by Prabu *et al*., 2011. [29] These scavenging properties are generally due to high reducing capacity of the polyphenols acting as primary antioxidants. [30] 1, 1-Diphenyl-2-pecryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of Nitrogen Bridge. [31] 1, 1-Diphenyl-2-pecryl-hydrazyl (DPPH) assay has many advantages, such as good stability, credible sensitivity, simplicity and feasibility. [32] Metal ions play central role in reactive oxygen species generation as they can change the state from reduced to oxidised causing removal of electron from various biomolecules. [33] *Eclipta alba* plant extract showed good metal chelating activity as indicated in Fig. 6.

Medicinal values of the plants depend on the bioactive constituents exerting desirable physiological action in humans. Antioxidative and hepatoprotective effect of *Eclipta alba* extract is principally denoted by the phytochemicals acting as reductant and free radical scavenger. An attempt was made in the present study to isolate and characterize the major active component from the indigenous medicinal plant *Eclipta alba*. Wedelolactone was isolated from the whole plant extract *Eclipta alba* (Fig. 8) by RP-HPLC analysis. Isolation of considerable amount of wedelolactone from the methanolic extract of *Eclipta alba* has been reported by Kumar and Dhanani, 2013. [34] Pharmacological screening of various extracts of *Eclipta alba* revealed that wedelolactone is immunomodulatory, antimyotoxic, antiphlogistic, antihemorrhagic and antihepatotoxic. [35-37]

Conclusion

From the present investigation, it can be concluded that *Eclipta alba* plant extract possess potent antioxidative properties.

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