ANTIOXIDANT POTENTIAL OF METHANOLIC AND HYDROLYZED EXTRACTS OF ECLIPTA ALBA

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Summary

Antioxidant therapy is an exciting new concept in the prevention and treatment of countless human diseases such as inflammation, cardiovascular disease, cancer and aging related disorders which originate from free radical derived oxidative stress. In the present investigation, methanolic and hydrolyzed extracts of *Eclipta Alba* Linn. were investigated for its antioxidant potential using *in vitro* and *ex vivo* models. Extraction of the powdered drug of *Eclipta alba* was carried out to obtain methanolic and hydrolyzed extracts. The *in vitro* antioxidant activity of plant extract were assessed on the basis of the radical scavenging effect of the stable DPPH free radical and Nitric oxide radical inhibition activity while *ex vivo* antioxidant activity was assessed by lipid peroxidation inhibitory activity on mice liver homogenate by TBARS method. *In vitro* and *ex vivo* models, demonstrates both methanolic and hydrolyzed extracts of *Eclipta alba* to be powerful scavengers of free radicals and nitric oxide radicals, as well as efficient inhibitors of lipid peroxidation. The methanolic extract of *Eclipta alba* revealed significant antioxidant activity and was much more effective than its isolated constituents (Hydrolyzed extract) as revealed by the IC_{50} values and statistical analysis.. In conclusion, the data provided from in *vitro* and *ex vivo* studies indicate that the *Eclipta alba* plant is a potential source of natural antioxidants, thus could be taken as a platform for further formulating a suitable dosage form which will pave way for economic treatment in various diseases.

Keywords: *Eclipta alba*, Antioxidant activity, DPPH, Griess reagent, TBARS, Lipid peroxidation

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Introduction

Free radicals plays an important contributory role in the pathogenesis of numerous diseases like hypertension, diabetes, arthritis and numerous neurodegenerative disorders including Alzheimer's disease. Improved antioxidant status helps to minimize the risk for developing many chronic age related, free radical induced diseases. [1, 2, 3] Hence, it is logical to speculate that oxidative damage and disease progression could be arrested by augmenting the physiological antioxidant mechanisms. To date, modern science has failed to produce a remedy devoid of side effects even through laboratory synthesis, which has led to a shift in focus from conventional medicine to alternative forms of therapy based on drugs derived from plants. [4] Many of the herbs are promising candidates in the treatment of cancer, atherosclerosis, diabetes, inflammation as well as neurodegenerative disorders. They provide as an excellent natural source of antioxidants. Following this idea, herbs seem to be one of the answers to free radical rebellion! [5]

Eclipta alba (Linn.) Hassk. of the Asteraceae family, is a well known herb in the Indian system of medicine. The major phytoconstituents in *Eclipta alba* are Coumestan derivatives which comprises of Wedelolactone and Demethylwedelolactone. Minor phytochemicals present are Triterpenoids, Flavonoids, Tannins and Phenolic compounds. Various studies have been carried out to indicate that flavonoids and other polyphenolic compounds have a major role to play for antioxidant activity. Alcoholic extracts of the plant shows protective effect on experimental liver damage in rats and mice. The plant extracts exhibit antimyotoxic and antihaemorrhagic effects against crotalid venoms. Wedelolactone is a potent and selective 5-lipoxygenase inhibitor. Wedelolactone and demethylwedelolactone exhibit antihepatotoxic activities in carbon tetrachloride, galactosamine and phalloidin induced liver damage in rats. It is widely used as a bitter, acrid, anthelmentic, febrifuge, anti-inflammatory, aphrodisiac and diuretic. It is good for blackening and strengthening of the hair, for stopping hemorrhages and fluxes and for strengthening of gums. The seeds are good for increasing sexual vigor. Root is used as emetic, purgative. It is applied externally as antiseptic to ulcers and wounds. Also used as tonic, spasmogenic, ovicidal and in healing of fractures. [6, 7] *Eclipta alba* is widely used since ages as hepatoprotective.The purpose of the present study was to evaluate the *in vitro* and *ex vivo* antioxidant activity of *Eclipta alba* as a possible mechanism for hepatoprotective effect.

Materials and methods

Chemicals

DPPH (1,1-diphenyl-2-picryl hydrazyl) was procured from Fluka Chemicals, Ascorbic acid was obtained as a gift sample from BASF Industries and TBA (Thiobarbituric acid reagent) was procured from Hi media. Sodium nitroprusside, Potassium di hydrogen sulphate, Sodium hydroxide, Sulphanilamide, Ortho-phosphoric acid, Napthyl-ethylene diamine, Sodium chloride, Potassium chloride, Tris buffer, Ferrous sulfate and Methanol were procured from Rankem Industries and were of AR (Analytical Reagent) grade, Curcumin was obtained as a gift sample from sigma chemicals.

Plant material
The authenticated powdered material of *Eclipta alba L.* was procured from Zandu Pharmaceutical Works Limited, Mumbai, India

Preparation of methanolic extract
Extraction of the powdered drug of *Eclipta alba* was carried out by two different methods to obtain methanolic and hydrolyzed extracts. The methanolic extract was prepared by subjecting the powdered drug to extraction with methanol for eighteen hours using Soxhlet apparatus. The drug to solvent ratio was 1:8. The extract was then evaporated and concentrated to dryness by using rotary vacuum evaporator and was then reconstituted with methanol for further use and analysis.[8] The percent yield of the methanolic extract was found to be 15.156% w/w

Preparation of hydrolyzed extract
The hydrolyzed extract was prepared by refluxing the powdered drug with 6M hydrochloric acid over one to two hours. The hydrolyzed extract was then filtered. Filtrate was extracted with portions of chloroform to obtain aglycones from the hydrolyzed fraction. The chloroform extract was then evaporated to facilitate complete removal of the solvent and reconstituted with methanol for further use and analysis.The percent yield of the hydrolyzed extract was found to be 0.1071% w/w. The methanolic extract was the whole extract of *Eclipta alba* whereas the hydrolyzed extract contained isolated active principles of *Eclipta alba* i.e. phenolic compounds which are thought to be responsible for the antioxidant activity of the herbals.[9]

Preliminary phytochemical screening of extracts
The extracts were standardized with respect to parameters like color, consistency, pH and percent yield. Preliminary phytochemical investigation was performed on the methanolic extract of *Eclipta alba* using qualitative tests to identify the phytoconstituents in the extract. [10]

Free radical scavenging activity
This assay is based on the measurement of the radical scavenging ability of the test substances towards a stable free radical, DPPH (1, 1-diphenyl-2-picryl hydrazyl). Antiradical activity was measured by a decrease in absorbance at 517 nanometer (nm) of a methanolic solution of coloured DPPH brought about by the sample. A stock solution of DPPH (0.36 mg/ml in methanol) was prepared such that 200µl of this solution in 2.7 ml methanol gave an initial absorbance of 0.65. Decrease in the absorbance in presence of sample extract at different concentrations was noted after 15 minutes. Baseline value of DPPH solution is noted as initial absorbance. After addition of the methanolic/ hydrolyzed extract or ascorbic acid, 15 minutes are allowed for the extract or ascorbic acid to interact with the free radicals which are generated due to DPPH methanolic solution. The final absorbance is noted after 15 minutes. During this period, scavenging of free radicals by the extract has taken place. The decrease in final absorbance as compared to the initial absorbance at 517 nm indicated antioxidant activity of the plant. During this procedure deep violet color of the DPPH free radical solution changes to yellowish color due to scavenging action of antioxidants. Ascorbic acid was used as the reference standard.[11]

Nitric oxide (NO) radical inhibition activity

This assay is based on the generation of nitric oxide from sodium nitroprusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitrite ions. The results of this interaction are determined by the use of the Griess reagent (1% sulphanilamide, 2.35% ortho-phosphoric acid and 0.1% napthyl-ethylene diamine in water). Scavengers of nitric oxide compete with oxygen and reduce the production of nitric oxide. The test system comprised of sodium nitroprusside (10 mM), phosphate buffer and different concentrations of extracts ranged from 1-10 µg/m.This test system was incubated at 25° C for 150 minutes. The above test system without the extracts, (Test extract: Methanolic extract or Hydrolyzed extract of *Eclipta alba*) but with an equivalent amount of solvent served as a control. After the incubation period, 1.5 ml of reaction mixture was withdrawn and 1.5 ml of Griess reagent was added to it. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with napthyl-ethylene diamine, was read at 540 nm. Ascorbic acid was used as the reference standard.[12]

Lipid peroxidation inhibitory activity by TBARS (Thio Barbituric Acid Reacting Substances):

Preparation of mice liver homogenate

These studies were carried out on Wistar strain albino mice of either sex, weighing 25-30 grams and of normal size. The mice were sacrificed using anaesthetic ether. The liver was quickly removed and chilled in ice cold saline. After washing with 0.9% ice cold saline the liver was homogenized in 0.15 mM KCl to get 10% w/v liver homogenate.

Fresh liver homogenate (0.2 ml) was mixed with 0.15 mM KCl (0.1 ml) and Tris buffer (0.4 ml). The extracts were then added in various concentrations ranged from 1-15 µg/ml.. *In vitro* lipid peroxidation was initiated by addition of ferrous sulfate (10µM) and Ascorbic acid (100 µM), 0.1 ml each. After incubation for one hour at 37° C, the reaction was terminated by the addition of Thiobarbituric acid reagent (2 ml) and boiled for 15 minutes for development of a colored complex. The colour was measured spectrophotometrically at 532 nm. Percent reduction of thiobarbituric acid reacting substances was calculated with respect to a control to which no sample had been added. The inhibition of lipid peroxidation was determined by calculating the percent reduction in formation of TBARS (Thio Barbituric Acid Reacting Substances). The reference standard used in this procedure was Curcumin.[13] Curcumin is reported to have anti-lipid peroxidation effects, hence used as a reference standard for this model)

The Inhibitory Concentration 50% (IC_{50}) of the extracts and standard antioxidants were calculated from concentration/effect regression line using the regression equation.

Statistical analysis:

The results are represented as Mean $\pm$ SEM (Standard Error Mean). The mean is calculated as the average of the experiments that were conducted six times each for every model (n=6). Statistical analysis was performed using one-way ANOVA followed by Tukey's test for multiple comparisons. $P < 0.05$ was regarded as significant.

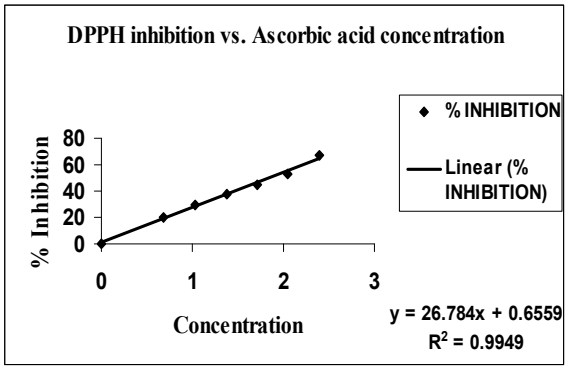
Results:

Preliminary phytochemical screening of extracts

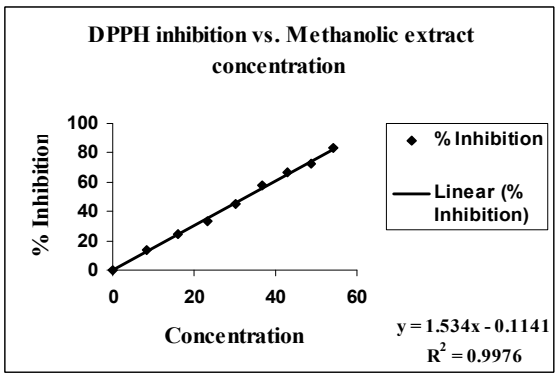
Preliminary phytochemical investigation of the methanolic extract revealed the presence of carbohydrates, flavonoids, tannins and phenolic compounds as the phytoconstituents present in the plant.

Free radical scavenging activity:

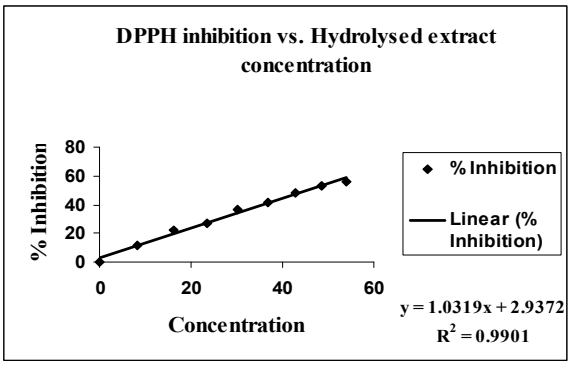
The antioxidant activity using DPPH free radical scavenging method showed an average Inhibitory Concentration (IC_{50}) of the methanolic extract to be 32.847µg/ml and that of the hydrolyzed extract to be 45.88µg/ml. The standard ascorbic acid revealed an average IC_{50} of 1.840µg/ml (Figure 1). At highest concentration of 54.050µg/ml the methanolic extract was found to be statistically better than hydrolyzed extract at 95 % level of significance. Hence the interaction of *Eclipta alba* extract with DPPH established the capability of the constituents to scavenge the free radicals. Thus it indicates that some of the therapeutic actions of *Eclipta alba* may be due to its free radical scavenging activity ($P < 0.05$).



1 A



1 B



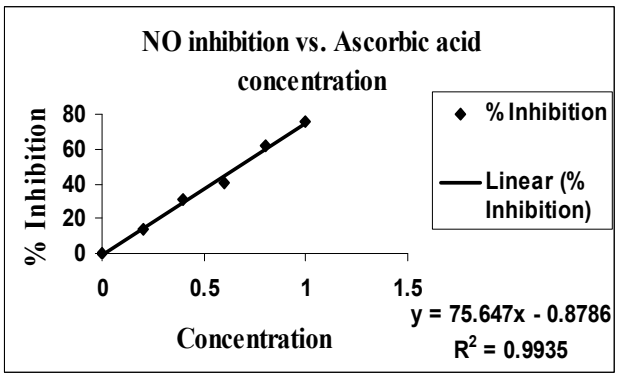
1 C

Figure 1: DPPH free radical scavenging activity of Ascorbic acid standard solution (1 A), Methanolic extract (1 B) and Hydrolyzed extract (1 C) of *Eclipta alba*

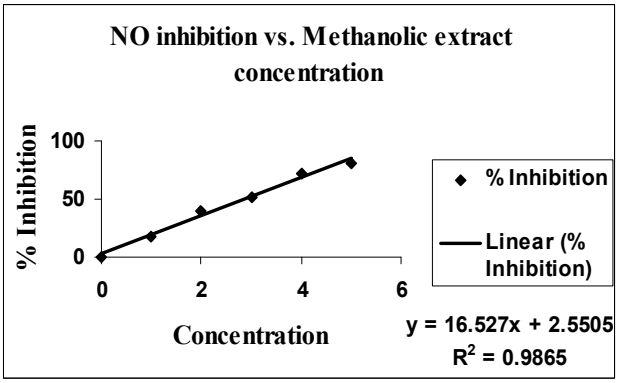
Figure 1 A, B and C depicts the plot of concentration of the extract or standard (Ascorbic acid) vs. % Inhibition. Excellent co-relation coefficient between Concentration and % Inhibition is obtained as indicated by the R^2 values in the graphs. The Inhibitory Concentration 50% (IC_{50}) of the extracts and standard antioxidant were calculated from concentration/effect regression line using the regression equation.

***Nitric oxide (NO) radical inhibition activity*:**

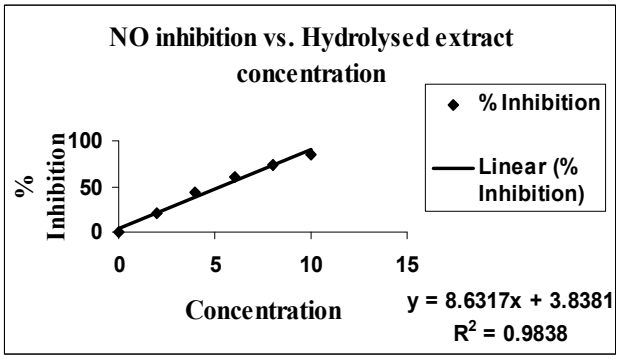
In the Nitric oxide radical inhibition activity procedure, the average IC_{50} of the methanolic extract was found to be 2.871µg/ml and that of the hydrolyzed extract was found to be 5.347µg/ml. The standard of ascorbic acid revealed an average IC_{50} of 0.6725µg/ml. **(Figure 2)**
Hence it can be said that *Eclipta alba* not only quenches free radicals but also efficiently inhibits reactive nitrogen species. At highest concentration of 10.00µg/ml the methanolic extract was found to be statistically better than hydrolyzed extract at 95 % level of significance ($P < 0.05$).



2 A



2 B



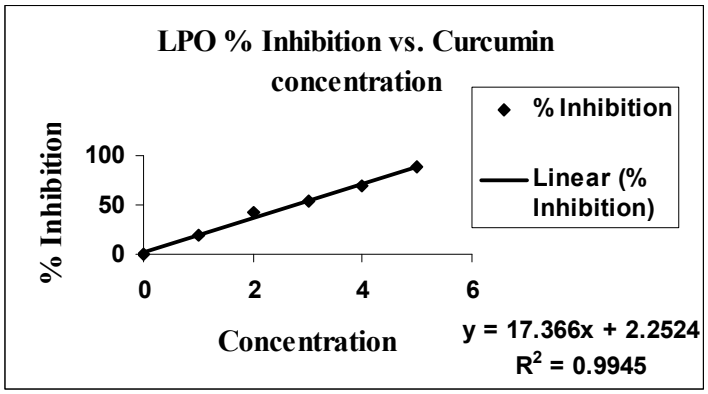
2 C

Figure 2: NO radical inhibition activity of Ascorbic acid standard solution (2 A), Methanolic extract (2 B) and Hydrolyzed extract (2 C) of *Eclipta alba*

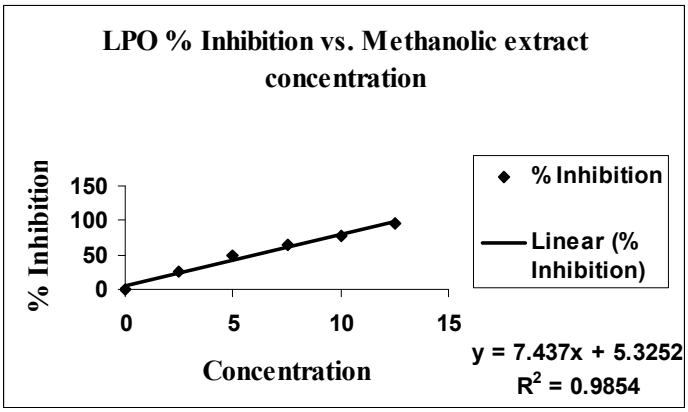
Figure 2 A, B and C depicts the plot of concentration of the extract or standard (Ascorbic acid) vs. % Inhibition. Excellent co-relation coefficient between Concentration and % Inhibition is obtained as indicated by the R^2 values in the graphs. The Inhibitory Concentration 50% (IC_{50}) of the extracts and standard antioxidant were calculated from concentration/effect regression line using the regression equation.

Lipid peroxidation inhibitory activity by TBARS (Thio Barbituric Acid Reacting Substances):

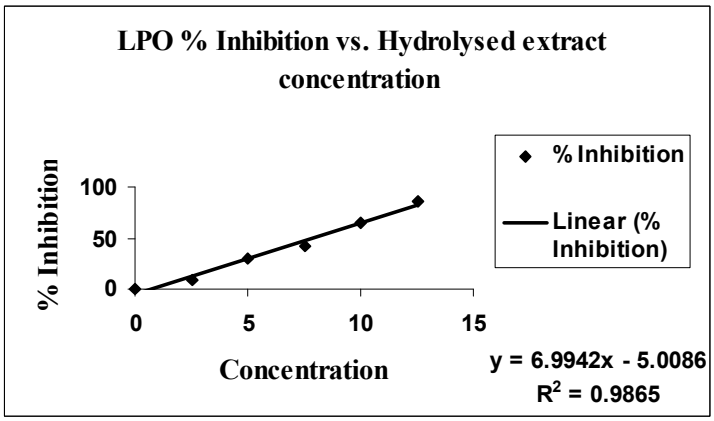
In the lipid peroxidation studies, the average IC_{50} of the methanolic extract of was found to be 5.994µg/ml and that of the hydrolyzed extract was found to be 7.866µg/ml. The standard of Curcumin revealed an average IC_{50} of 2.749µg/ml**(Figure 3).***Eclipta alba* was found to significantly inhibit ferrous sulfate induced lipid peroxidation in mice liver homogenate. Since this plant has been used in the traditional medicine as a hepatoprotective agent, it may be that its proposed efficacy could be partially attributed to its anti-lipid peroxidation activity. At highest concentration of 12.5µg/ml the methanolic extract was found to be statistically better than hydrolyzed extract at 95 % level of significance ($P < 0.05$).



3 A



3 B



3 C

Figure 3: Anti-lipid peroxidation activity of Curcumin standard solution (3 A), Methanolic extract (3 B) and Hydrolyzed extract (3 C) of *Eclipta alba*

Figure 3 A, B and C depicts the plot of concentration of the extract or standard (Curcumin) vs. % Inhibition. Excellent co-relation coefficient between Concentration and % Inhibition is obtained as indicated by the R^2 values in the graphs. The Inhibitory Concentration 50% (IC_{50}) of the extracts and standard antioxidant were calculated from concentration/effect regression line using the regression equation.

Discussion

The antioxidant activity of the methanolic and hydrolyzed extracts of *Eclipta alba* was carried out using *in vitro* and *ex vivo* models. The studies done in present research reveal that the methanolic and hydrolyzed extracts of Eclipta alba Linn. (Hassk.) are effective antioxidants and their activities are comparable with the standards which are potent antioxidants.

It was found that the methanolic extract of *Eclipta alba* possessed significant antioxidant activity when compared to the hydrolyzed extract in *ex vivo* and *in vitro* models. The IC_{50} values of the methanolic extract was much less compared to the IC_{50} values of the hydrolyzed extract. In the DPPH model, at highest concentration of 54.050µg/ml the methanolic extract was found to be statistically significant than the hydrolyzed extract at 95 % level of significance. In NO model, at highest concentration of 10.00µg/ml the methanolic extract was found to be statistically significant than the hydrolyzed extract at 95 % level of significance. In lipid peroxidation model, at highest concentration of 12.5µg/ml the methanolic extract was found to be statistically significant than the hydrolyzed extract at 95 % level of significance.

The methanolic extract comprised the whole extract of *Eclipta alba* herb whereas the hydrolyzed extract contained isolated active principles of phenolic compounds, which are thought to be responsible for antioxidant activity. Thus, the whole methanolic extract was much more effective than its isolated constituents (Hydrolyzed extract) as revealed by the IC_{50} values and statistical analysis. It is suggested that once an active principle has been isolated from the natural product without its synergic constituents to support and/or balance its action, it may lose its character as present in its natural form. The natural or holistic approach attempts to solve problems by taking these phytoconstituents in their entirety, with all their interlinkages and their complexity. The methanolic extract of *Eclipta alba* revealed good antioxidant activity which could be attributed to synergism and the role of many phytoconstituents together in the methanolic extract for attaining the desired therapeutic effects. The concept of isolating an active group of compounds did not achieve the desired results as compared with the whole extract. Thus the concept of synergy is central to the holistic approach.[14]Thus the data provided from in *vitro* and *ex vivo* models, demonstrates both methanolic and hydrolyzed extracts of *Eclipta alba* to be powerful scavengers of free radicals and nitric oxide radicals, as well as efficient inhibitors of lipid peroxidation. These results also indicate that the *Eclipta alba* plant is a potential source of natural antioxidants, since the antioxidant activity of extracts is comparable with well-known potent antioxidants.

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