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PHYTOCHEMICAL AND ANTIOXIDANT ASSAY OF *ECLIPTA ALBA* (L.) LEAF EXTRACT

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ABSTRACT: Currently, pharmacological activities of *Eclipta alba* (L.) plant extracts and individual phytoconstituents have revealed anticancer, hepatoprotective, snake venom neutralizing, anti-inflammatory and antimicrobial properties. The role of antioxidants is increasing day by day due to their multiple roles to reduce the harmful effects of oxidative stress. Phytoconstituents like wedelolactone and ursolic and oleanolic acids as well as luteolin and apigenin can form the basis of new drugs against cancer, arthritis, gastrointestinal disorders, skin diseases, and liver disorders. Plants have all these activities due to biologically active compounds, and for this, we have analyzed phytochemical screenings and antioxidant activity through the DPPH, ABTS●+, and reducing power assay and we got maximally phenols then flavonoids and flavonols and a good natural antioxidant agent. The best-known compound in *E. alba i.e.*, Wedelolactone. It was analyzed by TLC, and it was present as 0.52 R_f value and present in pet ether extract and acetone extract and minimum in ethanol extract. The antioxidant activity was assessed through DPPH, ABTS●+ free radical scavenging activity and reducing power assay, this was explained in terms of effective concentration EC_{50} / IC_{50} and Anti-oxidant Radical Power (ARP) values. The maximum free radical scavenging activity was showed in pet ether compared to other extracts.

INTRODUCTION: The use of synthetic drugs causes many side effects as well as resistance in pathogenic microbes. Thus, there is a need to focus on developing herbal drugs. Among them, a very well-known plant is *E. alba* (L.) Hassk. It belongs to the family Asteraceae and is native of India and its neighboring countries [1].

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Past ethnomedicinal literature revealed that plant and plant parts are highly medicinal value but still these plants only used with limited areas like respiratory tract disorders (including asthma), gastrointestinal disorders, fever, hair loss and graying of hair, liver disorders (including jaundice), skin disorders, spleen enlargement and cuts and wounds [2].

Many types of research were done and found *E. alba* was effective against many diseases, but still, there is little dark remain which is to be enlightened to the world. A various phytochemical bioactive component was found in this plant [3]. Plant extract of *E. alba* was reported as a protective

effect on the liver and showed various positive effects against hepatic disorder [4, 5]. Ethanolic extract was found more effective and stimulate liver cell regeneration [6]. The plant is also recorded to inhibit the activity of the Hepatitis C virus (HCV). The extract showed that 3 compounds namely, wedelolactone, luteolin and apigenin were responsible for the inhibitory effect on the virus [7]. Petroleum ether and ethanol extract of *E. alba* have been tested in albino rats for promoting hair growth activity [8]. Cardiac inhibitory activity was showed maximum effectiveness by ethanolic extract of *E. alba* on the frog's heart. Ethanolic extract showed negative ionotropic, negative chronotropic effects and also in the reduction of cardiac output. Ethanolic extract of callus showed antagonistic effects against adrenaline [9].

The plant extract was found to decrease pancreatic islet superoxide dismutase (SOD) activity [10]. The extract also helps in the inhibition of α-glucosidase, and aldose reductase was postulated to be the reason behind the other observed effects [11, 12]. Leaves of *E. alba* were used to get rid of ectoparasites and check the recurrence of the disease eczema [13].

The aqueous extract showed an antioxidant and protective effect against cell-damaging by UV irradiation and absorbed the UV-A and UV-B. It also demonstrated to shows protection of human keratinocytes and mouse fibroblasts 3T3 cells against cytotoxicity induces by UV-B [14]. The aqueous and hydro-alcoholic extracts of *E. alba* have been evaluated for sedative, muscle relaxant, anxiolytic, nootropic, and anti-stress activities [15]. Aqueous extract of *E. alba* has been tested for its ability to reduce aggression through foot shock-induced aggression and water com-petition tests [16]. The antimalarial activity of leaf extract of *E. alba* has been tested against *Plasmodium* species causing malaria [17, 18]. The immune-stimulatory effects of feeding aqueous extract of leaves of *E. alba* have been studied in tilapia fish (*Oreochromis mossambicus*) against gram-negative bacteria *Aeromonas hydrophila* regarded as both fish and human pathogens. The plant extract inhibited the pathogen growth, as well as the lysozyme activity of fish, which were significant increases [19]. The Antiepilepsy activity was also reported with methanolic extract and found more effective [20]. Extract of *E. alba* was reported to be inhibiting the effects of the venom of snakebite and found that the effective compound was coumestans, wedelolactone, and dimethyl wedelolactone [21].

Various solvent (petroleum ether, benzene, chloro-form, acetone, methanol, and aqueous) extracts were reported to be effective against oral cancers. The anticancer activity was reported from aqueous and ethanolic extract of *E. alba*. The extracts were inhibiting the cell proliferation in a dose-dependent manner in HepG2, A498 and C6 glioma cell lines [22]. The expression of matrix metalloproteinase (MMP) 2 and 9 was down-regulated significantly. Additionally, the downregulation of nuclear factor κB (NFκB) was also observed. The DNA damage was observed following 72 h of extract treatment, leading to apoptosis [23, 24].

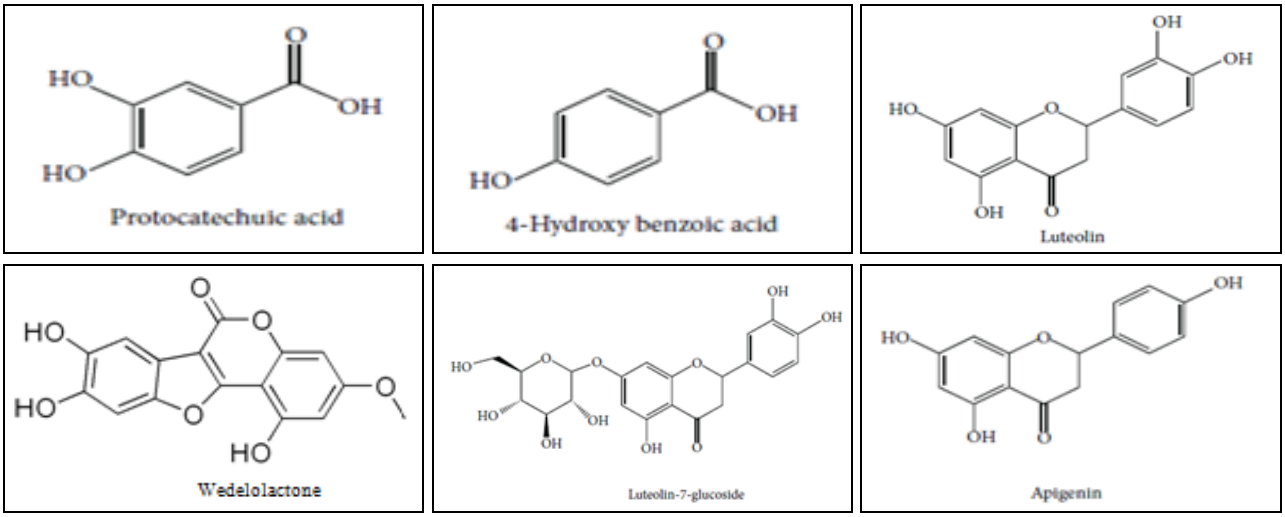

FIG. 1: SOME IMPORTANT PHYTOCONSTITUENTS PRESENT IN *ECLIPTA ALBA*

MATERIALS AND METHODS:

Collection of Materials: The healthy about 1-2 months old plants were collected from the Roxburgh Botanical Garden, Department of Botany, University of Allahabad. The collected plants were thoroughly washed with distilled water and kept in the shade for drying. The collected plants have been gone through identification by the Botanical Survey of India (BSI) and accession no. – BSA104179.

Preparation of Plant Extract: Shade dried leaves of *E. alba* crushed into powdered form. The powdered material was extracted in 3-different solvents of increasing polarity. 10 mg of dried leaf powder was extracted by cold percolation method separately in 100 ml of Petroleum ether, Acetone, and Ethanol respectively for 24 h. After this, all the samples were filtered by the vacuum filtration method in a round bottom flask separately. The collected filtrates were concentrated under reduced pressure at 45 °C on a rotary evaporator (Buchi Rotavapor) and stored in a desiccator. Further, completely dried extracts were used for the estimation of % yield based on crude plant material taken.

$$\% \text{ Yield} = Ep - Eb / \text{Initial weight of plant} \times 100$$

Ep = Weight of Eppendorf tube with metabolites after extraction. Eb= Weight of blank Eppendorf tube [26].

Analysis of Phytochemicals:

Total Phenolic Content: Total phenolic contents in the extracts were determined spectro-photometrically by the Folin Ciocalteau method [29]. Dried extracts were reconstituted in distilled water (1 mg/ml). Folin-Ciocalteau reagent (0.5 ml) was added to the extract solution (0.5 ml), and the total volume was adjusted to 8.5 ml with distilled water. The tubes were kept at room temperature for 10 min, and thereafter 1.5 ml of sodium carbonate (20%) was added. The tubes were incubated in a water bath at 40 ºC for 20 min; the intensity of the blue colour developed was measured by recording the absorbance at 755 nm using a UV-visible spectrophotometer (Varian, CARY-300 Bio). The reagent blank was also prepared using distilled water. For quantification of the total phenolic in the extract, a standard calibration curve was prepared using gallic acid. The total phenolic content of the extract samples was expressed as gallic acid equivalent (GAE) milligrams per gram of the extract.

Total Flavonoid Content: Total flavonoid content was determined using the aluminium chloride colorimetric method with some modifications [30, 31]. A calibration curve for quercetin in the range 20-80 µg/mL was prepared. Plant extract (0.5 mL) and standard (0.5 mL) were placed in separate test tubes and test tubes and 10% aluminum chloride (0.1 mL), 1 M potassium acetate (0.1 mL), 80% methanol (1.5 mL) and distilled water (2.8 mL) added and mixed. A blank was prepared in the same manner but 0.5 mL of distilled water was used instead of the sample or standard. All tubes were incubated at room temperature for 30 min and the absorbance was read at 415 nm. The concentration of flavonoid was expressed as mg quercetin equivalent (QE) per gram extract. Each plant extract was made in triplicate.

Total Flavonol Content: Total flavonol content was determined following the aluminum chloride colorimetric method with some modifications [32, 33]. A calibration curve for quercetin in the range 20-80 µg/mL was prepared. Extract (1 mL) and standard (1 mL) were placed in separate test tubes and 2% aluminum chloride (1mL), 5% sodium acetate (3 mL) added and mixed. The mixture was then centrifuged at 3000 rpm for 20 min to obtain a clear solution. The absorbance was read at 440 nm and the results expressed as mg quercetin equivalent (QE) per gram of extract. Each plant extract was prepared in triplicate.

Thin Layer Chromatography for Wedelolactone

Test Solution: Extract dissolved in methanol.

Standard Solution: Dissolved 1 mg Wedelo-lactone (Wedelolactone standard from Natural remedies Pvt. Ltd., Bangalore, India) in 10 ml of methanol.

Solvent System: Toluene: Acetone: Formic acid (11:6:1).

Procedure: Apply 10 µl each of the test and standard solutions separately on a percolated silica gel 60 F 254 TLC plate (Merck) of uniform thickness of 0.2 mm.

Develop the plate in the solvent system in a twin trough chamber to a distance of 8 cm.

Visualization: Observe the air-dried plate under UV light at 366 nm. Recorded the R_f value and colour of the resolved bands.

Free Radical Scavenging Ability by the use of a Stable ABTS●+ Radical Cation: ABTS●+ cation radical decolorization assay was determined by ABTS●+ radical cation decolorization assay [34]. ABTS●+ was dissolved in water to get 7 mM concentration, and radical cation (ABTS●+) was produced by reacting ABTS●+ solution with 2.45 mM potassium persulphate at room temperature in the dark (12-16 h) before use.

For the assay, ABTS●+solution were diluted with distilled water to an absorbance value of 0.700 ± 0.02 at 734 nm. After the addition of 3ml of diluted ABTS●+ solution to 100 µl of extracts solutions, absorbance was recorded after 6 min. All the tests were performed in triplicate.

The percentage inhibition of the samples was calculated as:

$$\text{Inhibition \%} = (1 - A / A0) \times 100$$

Where, A0 is the absorbance at 734 nm of the negative control, A is the absorbance at 734 nm of the mixture with a sample.

Reducing Power Assay: Reducing power of standard antioxidants and extracts was determined. Different concentrations of extracts were mixed with distilled water (2.5 ml), phosphate buffer (2.5 ml, 0.2 M, pH 6.6), and potassium ferricyanide (2.5 ml, 1%). The resulting mixture was incubated at 50 ºC for 20 min in a water bath.

After cooling, trichloroacetic acid (2.5 ml, 10 %) was added to the mixture. The upper layer of solution (2.5 ml) was taken and mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%). The absorbance was recorded using a UV-visible spectrophotometer at 700 nm.

The increasing absorbance value was interpreted as increased reducing activity [35]. The reaction mixture turns bluish-green depending upon the capacity of reducing power. All the tests were performed in triplicate.

Reducing the power of the sample calculated by using the formula:

$$(1-AS /AC) \times 100$$

Here, AC = absorbance of the standard at maximum concentration tested and AS = absorbance of the sample.

DPPH Scavenging Assay: Different concentrations (equivalent to 200,400, 600, 800, and 1000 ppm) of the extracts were taken in test tubes. The total volume was adjusted to 8.5 ml by the addition of methanol. 5.0 ml of 0.1 mM methanolic solution of DPPH was added to these tubes and mixed well with a vortex mixer. The tubes were retained in the dark for 20 min. The blank was prepared by adding all the solvents without extract, and methanol was used for dissolution and correction. Absorbance was recorded at 517 nm by using a UV visible spectrophotometer. Radical scavenging activity (RSA) was expressed as the inhibition percentage and was calculated using the following formula [36].

%Radical scavenging activity = (Absorbance of blank-absorbance of the sample) / (Absorbance of blank) × 100

Calculation of Efficient Concentration EC_{50}: Efficient concentration (EC_{50}) of DPPH was calculated by a linear equation of the dose inhibition curve obtained by plotting the extract concentrations versus corresponding percent radical scavenging activity using Graph pad prism 5.0.1. Results were obtained as mean ± standard deviation (SD) of three independent experiments for each antioxidant, EC_{50} values were expressed as 95% confidence interval.

Calculation of the Antioxidant Radical Power: The antioxidant radical power (ARP) was also calculated as follows

$$ARP = 1 / EC5037$$

RESULTS AND DISCUSSION:

Phytochemical Analysis: The outcome or result demonstrated that the whole quantity of these alkaloids was considerably different among the solvents, but the ratio pattern of the alkaloid content was established to be helpful in classifying the samples. The total phenol content is maximum in p. ether (92 ± 3) followed by acetone and ethanol 88 ± 3 and 68 ± 2 respectively. Total flavonoid

content is maximum in p. ether extract (60 ± 1) followed by acetone and ethanol 57 ± 1 and 47 ± 2, respectively. Total flavonol condition differs from the above two it is maximum in acetone (28 ± 2) followed by ethanol and pet. ether 17 ± 4 and 16 ± 2.

TABLE 1: YIELD, TOTAL PHENOLICS VALUES OF THE EXTRACTS OF *E. ALBA*

Extracts	Yield (%)	Total Phenolics (mg/g GAE)	Total Flavonoid Content (mg/g QE)	Total Flavonol Content (mg/g QE)
Acetone	2.928	88 ± 3	57 ± 1	28 ± 2
Ethanol	3.264	68 ± 2	47 ± 2	17 ± 4
P. ether	2.152	92 ± 3	60 ± 1	16 ± 2

TLC Analysis: The medicinal importance of *E. alba* is known at a certain level. Some aspects of the study still do not have so, much attention like phytochemical screening and broth microdilution study, by studying several experimental studies; it shows that plants contain bioactive compounds like phenolic, flavonoid, and flavonol [38].

These results are directly in accordance with phytochemical accumulation, suggesting that phytochemicals can effectively be developed into therapeutic inhibitors to overcome diabetes-related complications [39]. Different plant extracts can affect cell envelope structure as the major antibacterial compositions of extracts could penetrate through the cell wall and destroy the cytoplasmic membrane resulting in cell lysis. Total phenolic content is considered an important indicator of the antioxidant potential and in the different biological activity of plant extracts.

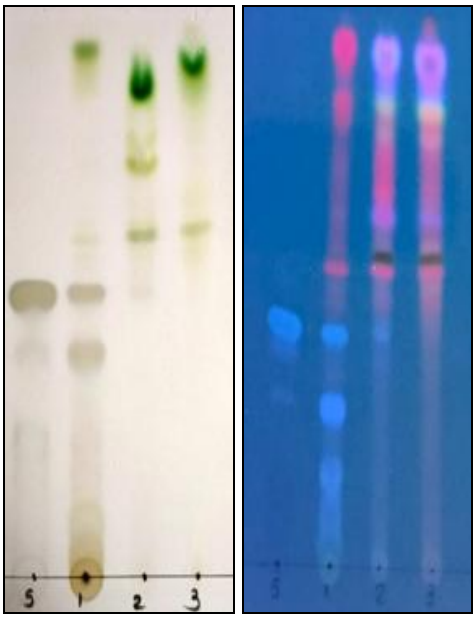

FIG. 2: TLC PROFILE FOR *ECLIPTA ALBA* IN UV LIGHT S-WEDELOLACTONE 1- P. ETHER 2- ACETONE 3- ETHANOL EXTRACTS. 2- TLC PROFILE IN VISIBLE LIGHT

TABLE 2: A B AND (RF0.52) CORRESPONDING TO WEDELOLACTONE IS VISIBLE IN BOTH THE STANDARD AND IN EXTRACTS

R_f Value	Colour of the Band
0.17	Light blue
0.29	Light blue
0.41	Blue
0.52	Blue (Wedelolactone)
0.55	Red
0.60	Red
0.63	Light blue
0.73	Blue
0.79	Red
0.86	Greenish blue

Antioxidant Activity: The EC_{50} / IC_{50} and percentage yield values of acetone extract and ethanol extract, and pet ether extract are listed in **Table 1**. The ethanol extract possesses a more % yield while the lowest in pet ether and moderate in acetone extract.

TABLE 3: IC_{50} / EC_{50} VALUES OF THE EXTRACTS OF *E. ALBA*

Extracts	DPPH EC_{50} (mg/ml)	ARP	$ABTS^{\bullet+}$ IC_{50} (µg/ml)	Reducing Power IC_{50} (µg/ml)
Acetone	2.92 ± 0.02	0.343	79.99	43.83
Ethanol	1.22 ± 0.04	0.819	77.50	37.81
P. ether	0.872 ± 0.02	1.15	36.43	2.93

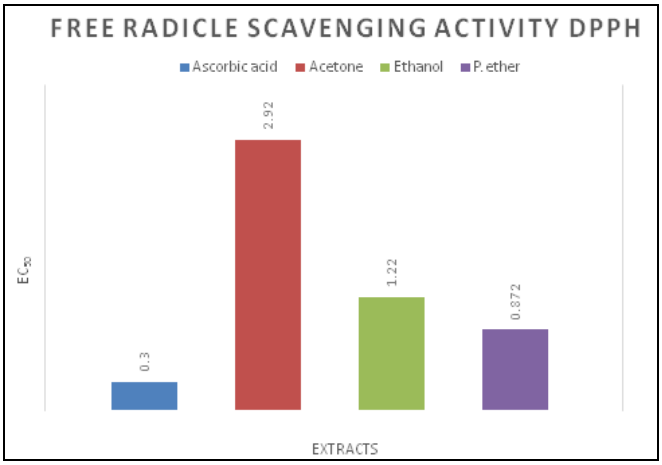

FIG. 3: COMPARISON OF EC_{50} VALUES OF ALL THE THREE EXTRACTS

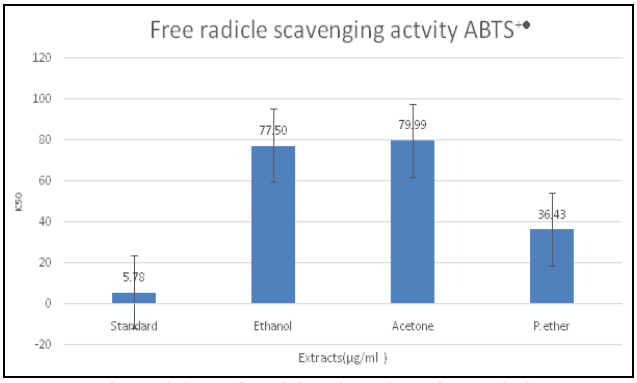

FIG. 4: COMPARISON OF IC_{50}VALUES OF EXTRACTS IN ABTS●+ MEDIATED PERCENT INHIBITION

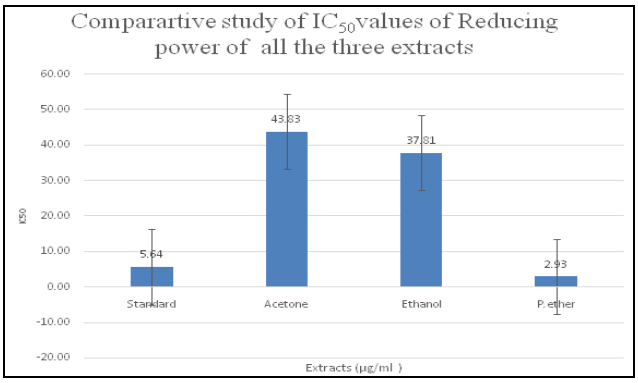

FIG. 5: COMPARISON OF IC_{50} VALUES OF EXTRACTS IN REDUCING POWER ASSAY

The antioxidant activity was assessed through DPPH and $ABTS^{\bullet+}$free radical scavenging activity and reducing power assay, this was explained in terms of effective concentration EC_{50} / IC_{50} and Antioxidant Radical Power (ARP) values **Table 3**.

DPPH (1, 1-Diphenyl-2-picrylhydrazyl radical) and ABTS [2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) assays have been widely used to determine the free radical scavenging activity of various plant extracts and pure compounds. DPPH and $ABTS^{\bullet+}$ both are stable free radicals that easily dissolve in methanol or ethanol, and their colons showed absorption at 517 nm and 734 nm, respectively. When antioxidants scavenge free radicals by hydrogen donation its colours of both the solution become lighter. Electron transfer (ET-based) methods involve two components in the reaction mixture, antioxidants, and oxidants (probe). The probe itself is an oxidant and abstracts an electron from the antioxidant, resulting in colour changes of the probe. The degree of the colour change is proportional to the antioxidant concentration. The IC_{50}value and their concentration were plotted in linearity to get the linear equation of all the three extracts in comparison to the standard.

The reaction is reached to the endpoint when colour change stops. The change of absorbance (ΔA) is plotted against the antioxidant concentration to give a linear curve. The EC_{50} value of ascorbic acid is 0.30, and the corresponding ARP value is 3.34 **Table 3** and **Fig. 3.** The IC_{50} value of pet ether extract showed minimum, *i.e.*, 0.872 and corresponding ARP value 1.15, followed by ethanol extract and acetone extract with 1.22, 2.92 IC_{50} values and 0.819 and 0.343.

In $ABTS^{\bullet+}$ assay, pet ether extract again showed a minimum IC_{50} value of 36.43, followed by ethanol extract and acetone extract with IC_{50} values 77.50 and 79.99 µg/ml, respectively. A similar result was noticed by Then Mozhi *et al.* [40] The reducing power of an extract may serve as a significant indicator of its potential antioxidant activity **Table 3** and **Fig 4**.

It can be seen that the reducing power percentage values of all leaf extracts and the positive control (Trolox) were concentration-related and increased with the increase in sample concentration in the range of the tested concentrations. In reducing power assay again, pet ether extract showed minimum IC_{50} value *i.e.*, 2.93, followed by ethanol extract and acetone extract with IC_{50} value 37.81 and 43.83 µg/ml **Table 3** and **Fig. 5**. The pet ether extract, due to the presence of higher phenolic and flavonoid content, might have higher antioxidant potential compared to other extracts [41].

It is well known that the antioxidant effect of plant products is mainly due to the radicals scavenging activity of phenolic compounds such as tannins, flavonoids, polyphenols, and phenolic terpenes [42].

Correlation of Total Flavonoid and Phenolic Content with Antioxidant Activity: Correlation of total phenolic content with their IC_{50} of DPPH scavenging activities showed that total phenolic content of *Eclipta alba*, gave a significant correlation with their IC_{50} of DPPH scavenging activity **Fig. 6**.

Pet ether extract had more total phenolic content so as the activity. An increase in phenolic content lowers the IC_{50} value of extracts [42].

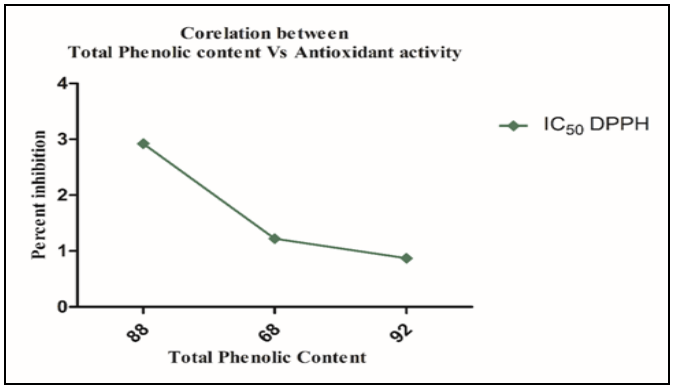

FIG. 6: POSITIVE CORRELATION BETWEEN TOTAL PHENOLIC CONTENT AND PERCENT INHIBITION (IC_{50})

CONCLUSION: The plant, *E. alba,* is regarded by traditional medicinal practitioners as a valuable medicinal plant, particularly for the treatment of liver disorders, gastrointestinal disorders, respiratory tract disorders, hair loss, skin disorders, and fever. In this study, important phytochemicals have been isolated and identified from the plant.

Three extracts of *Eclipta alba* dose-dependently increased the radical inhibition (or reducing power) values, suggesting that *E. alba* possesses antioxidant activity. Therefore, *E. alba* various pharmacological activities and curative effects may be closely correlated with its antioxidant activities. However, the antioxidant activities of three *E. alba* extracts were different within the tested concentration ranges.

In general, Pet ether had relatively high antioxidant activity followed by ethanolic and acetone extract showed moderate activity and high content of phenols flavonols and flavonoid.

This conclusion is expected, as similar observations have been reported in a large number of previous researches. Some previous findings of researchers suggested that this antioxidant activity is due to phenols, indicating the significant contribution of phenolics to these antioxidant assays.

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CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest.

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