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Lalita Singh
Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India

Reena Antil
Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India

Dheeraj Kumar
Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India

Pushpa Dahiya
Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India

Correspondence
Pushpa Dahiya
Department of Botany, Maharshi Dayanand University, Rohtak, Haryana, India

Phytochemical analysis and In-vitro assays for antimicrobial and antioxidant activity of Bhringraj herb *Eclipta prostrata* (L.)

Lalita Singh, Reena Antil, Dheeraj Kumar and Pushpa Dahiya

Abstract
Eclipta prostrata (L.) L. (*Asteraceae*) is a widely distributed annual herb. This plant has been utilized as folk or traditional medicine in various traditional medicine systems in world as well as in India. Powdered stem and leaves of *E. prostrata* were extracted with different solvents i.e. petroleum ether, chloroform, acetone, methanol etc. in soxhlet extraction apparatus. These extracts were further analysed for their antimicrobial and antioxidant appraisal in relation to their phytochemical constituents. Methanolic extract showed more promising antimicrobial as well as antioxidant activity as compared to other extracts. Maximum number of phytochemicals as well as total phenolic and flavonoid contents was also higher in case of methanolic extract followed by the acetonic and chloroform extract. As methanolic extract showed significant antioxidant and antimicrobial efficacy, this extract can be utilized as an effective and safe antioxidant source, on a commercial basis for the development of new phytodrugs.

Keywords: *Eclipta prostrata*, biological, phytochemicals, traditional

1. Introduction
Drugs which are derived from plants have played a significant role in the health benefits of many cultures including ancient as well as modern type. Scientific investigation of plants has often shown that active principles relies in plants are responsible for their therapeutic importance. *Eclipta prostrata* (L.) L. is an annual herbaceous plant, commonly known as the panacea for the hairs belonging to the family *Asteraceae*. Morphologically it is erect or prostrate, branches are present, hairs are present, annual, rooting at the nodes; the leaves are opposite, sessile and lanceolate [1-3]. Mainly it is known in the three major forms of traditional medicinal systems in the Indian subcontinent i.e Ayurveda, Unani, and Siddha, as bhringoraaja, bhangraa, and karissalaankanni, respectively. The Ayurvedic medicine system of India considers this herb as hepatoprotective [4]. *Eclipta* is used in traditional medicine for cirrohosis and infectious diseases. It is believed to prevent aging and rejuvenate hair, teeth, bone, memory, sight, and hearing. *Eclipta* has been reviewed for its pharmacological and insecticidal activities and phytochemical constituents, antioxidant, antihepatotoxic, antihyperlipedemic, anticancer immunomodulatory, analgesic, anti-inflammatory and antidiabetic activities [5-6].
Based on the survey of literature and its pharmocological importance of Bhringraj, the present study was designed to assess the different extracts of *E. prostrata* for its antimicrobial and antioxidant potentials with regards to its phytochemicals and total phenolic and flavonoid contents.

2. Materials and Methods
2.1 Selection, collection and authentication of plant material
The vegetative aerial parts of *E. prostrata* were collected from canal side in Bawani Khera block District Bhiwani, Haryana (India). Collected specimens were identified by Prof. B.D Vashistha, Department of Botany Kurukshetra University, Kurukshetra. Selection of plant was based on pharmacological information and ethanomedicinal uses of *Eclipta* for treatment of various ailments. The material was then shade dried in laboratory (25±2°C) till the constant weight. The dried sample were powdered by electrical grinder and filled in the air tight container and stored at 4°C for further use.

A. Whole plant **B.** Leaves and stem

C. Drying **D.** Extraction

Fig 1: *Eclipta prostrata*

2.2 Preparation of plant extract
The extract of plant bhringraj's aerial parts was prepared in different solvent on the basis of polarity index petroleum ether, chloroform, acetone and methanol by Soxhlet apparatus. In Soxhlet extractor 50gram of grinned powder was taken with 250ml of solvent. After 25-30 cycles of plant material with their solvent extract was prepared by evaporating with rotary evaporator.

2.3 Phytochemical screening
The plant extracts extracted in different solvents were screened out for the detection of various phytochemicals. Qualitative tests were performed as per the standard methods [11-13].

2.4 Total phenolic content or TPC
TPC was assessed by Folin-Ciocalteu method [10]. To 1mg/ml solution of each plant extract, 5ml of Folin-Ciocalteu and 2ml of Na_2CO_3 was added. The solution was vortexes and incubated in dark for 15min. Absorbance was measured at 620nm. Gallic acid was used as standard. The TPC was calculated from calibration curve ($y = 0.0164x+0.0557$, $R^2 = 0.9964$) and result was expressed in terms of mg of gallic acid equivalent (GAE)/ gram dry weight of sample. All tests were performed in triplicates.

2.5 Total flavonoid content or TFC
Total flavonoid content was estimated by Aluminium Chloride Colorimetric method [10]. 3ml of methanol, 0.2ml of 10% aluminium chloride, 0.2ml of 1M potassium acetate and 5.6ml of distilled water was added to each plant extract of 1mg/ml concentration. The mixture was vortexes and left at room temperature for 30 minutes. The absorbance was taken at 420nm. Quercetin was used as standard. All tests were performed in triplicates. Flavonoid contents were determined from the calibration curve ($y=0.0047x+0.0391$, $R^2 = 0.996$) and results were expressed as mg of quercetin equivalent (QE)/ gram dry weight of sample.

3. Antimicrobial studies
Antimicrobial potential of all the plant extracts was evaluated by disc diffusion method and microbroth dilution assay against six different bacterial strains and four different fungal strains.

3.1 Preparation of plant samples
All the extracts were re-constituted in DMSO to obtain the concentrations of 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml and 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml for antibacterial and antifungal studies, respectively.

3.2 Inoculums preparation
The antimicrobial activity of plant extracts against six different bacterial strains (*Staphylococcus aureus* (MTCC-96*)*, *Bacillus subtillis* (MTCC-2057*)*, *E. coli* (MTCC-41). *Streptococcus pyogens* (MTCC-890), *Pseudomonas aeruginosa* (MTCC-2453) and *Chromobacterium violaceum* (MTCC-2656) was assessed. Four different fungal strains used were *Aspergillus niger* (MTCC- 3002*) Rhizopus oryzae* (MTCC-262) *Fusarium oxysporum* (MTCC- 7392) and *Penicillium expansum* (MTCC- 2818). Active cultures for experiments were prepared by dissolving the nutrient broth (6.5 gm, Himedia) in distilled water (500ml) and sterilized by autoclaving at 121º C for 15 minutes. A loopful sterilized powder was added to 10 ml of sterile nutrient broth and was incubated at 37ºC for 24 hours. After this, cultures were adjusted to the 0.5 McFarland (1.5×10^8 CFU/ml) and used for the experiments [11].

3.3 Disc diffusion method
6mm sterilized discs were kept over the nutrient agar media (Hi-media) petriplates and CZ and PDA petriplates, already spreaded with 100µl bacterial inoculums and 20µl fungal strains. 10µl plant extract of different concentration was loaded on the discs. Ampicillin, chloramphenicol and fluconazole (0.1mg/ml) were taken as positive controls and DMSO was taken as negative control. Petriplates were then incubated at 37ºC in BOD incubator, after incubation each petriplate was examined and diameter of the ZOI was measured. The assay was repeated thrice. Method was subjected to antibiotic sensitivity tests by the Kirby-Bauer's disc diffusion method (1996) [12].

3.4 Minimum inhibitory concentration (MIC)
The MIC's of *E. prostrata* were determined by micro broth dilution assay using two fold serial dilutions of plant extracts [13]. 100 µl of nutrient broth and 100 µl plant extracts were added to each well of a 96 welled microtitre plate (12x8). 10µl of standardized inoculums (1.5×10^8 CFU/ml) and 10µl of Rezazurin dye (0.2% W/V) were added in each well and petriplates were incubated at 37°C for 24hrs for the bacterial MIC and rezazurin was not added in case of fungal MIC and petriplates were incubated for 72 hrs. The lowest concentration that inhibited the test organism was recorded as MIC.

4. Non enzymatic antioxidant potential
Non enzymatic antioxidant potential of plant extracts was determined by DPPH and ABTS assays, respectively.

4.1 DPPH Assay
The free radical scavenging activity of plant extracts was measured using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay [14]. A 1mg/ml stock solution of plant extract in their respective solvents was diluted from 10-100 µg/ml. One ml of 0.3mM DPPH solution was added to 1ml of the plant extracts of different concentrations and also to the standard (Ascorbic acid). Samples were incubated in dark for 30minutes. The absorbance was measured at 517nm by UV-Vis spectrophotometer (SHIMADZU 1800, Tokyo, Japan). Three biological mean were recorded. Antioxidant activity was calculated by using the formula given below:

% Inhibition = (Abs control - Abs sample)/ Abs control× 100

4.2 ABTS Assay
ABTS **(**2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid**)** assay was also used to carry out the antioxidant potential of plant extracts [15]. ABTS radical (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM ammonium per sulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS solution was diluted with ethanol to get an absorbance of 0.700 at 745 nm. A 1mg/ml stock solution of each plant extract and standard in their respective solvents and diluted from 10- 100 µg/ml. One ml solution of ABTS was added to the 1ml of plant extracts of different concentration. The absorbance was measured at 745nm. Antioxidant activity was calculated as below:

% Inhibition = (Abs control - Abs sample)/ Abs control× 100

5 Enzymatic assays
5.1 Extraction of plants for enzymatic Assays
0.5 g fresh plant material was collected in liquid nitrogen and brought in the lab immediately. Plant extract was prepared by homogenization of plant material in 5ml of extraction buffer which contains 100mM potassium phosphate buffer of pH-7.0 and 0.1mM sodium ethylene di-amine tetra acetic acid (EDTA). The homogenate was filtered through muslin cloth, and it was further it was centrifuged at 14000 rpm for 20 minute at 4ºC. This supernatant was used for all the enzymatic assays.

5.2 SOD Assay
A 3ml reaction mixture contained 50mM potassium phosphate buffer, methionine(13mM), NBT (75mM), riboflavin (2µM), EDTA(0.1 mM) and 0.1ml plant extract. This reaction mixture was transferred in the test tubes. These test tubes were placed in the light intensity of 5000 lux for 25 min. The absorbance was measured at 560 nm. Reaction mixture placed in dark did not produce any colour, served as the control. One unit of SOD activity can be defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT per unit time at 560nm [16].

5.3 Catalase assay
A 3ml reaction mixture contained 0.1ml plant extract, H2O2 (10mM), and potassium phosphate buffer (50mM) of pH-7.0. Absorbance was taken continuously for 5 min at 240 nm in every 30 seconds**.** Enzyme activity was expressed as µmoles of H2O2 decomposed mg-1 fresh weight min-1. One unit of catalase is the decomposition of 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25 °C [17]. (EC of H2O2 is 39.4 mM-1cm-1).

5.4 GST Assay
A 3 ml reaction mixture contained potassium phosphate buffer (97mM) of pH-6.5, 1mM EDTA (in DW), CDNB (30mM) (prepared in 95% ethanol) and GSH 75 mM (prepared in Buffer). Absorbance was taken at 340 nm till 5 min for every 30 sec**.** One unit of GST activity is the amount of enzyme which produces 1.0 µmol of GS-DNB conjugate per min [18] **(**EC of GS-DNB conjugate at 340 nm is *9.6 mM*-1cm-1).

6. Statistical analysis
Antioxidant activity (IC 50 values) and total phenolic and flavonoid content were correlated using Pearson's correlation coefficient. Statistical analysis was performed using the SPSS version 16.0.

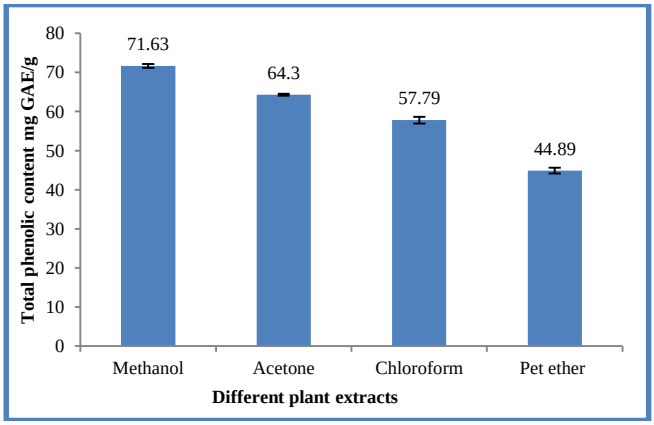
7. Results and Discussion
7.1 Phytochemical Analysis
Phenols and flavonoids were present in all the plant extracts of *E. prostrata*. Methanolic extract showed the maximum number of phytochemicals followed by both acetonic and chloroform extract and least number of phytochemicals was detected in case of petroleum ether extract.

Table 1: Phytochemical analysis of plant extracts of *E. prostrata* prepared in different solvents

S. No	Phytochemicals	Plant extracts			
		Methanol	Acetone	Chloroform	Petroleum ether
1	Alkaloids	+	+	+	-
2	Flavanoids	+	+	+	+
3	Glycosides	-	-	-	-
4	Phenols	+	+	+	+
5	Saponins	+	-	-	-
6	Steroids	-	-	+	+
7	Terpenoids	+	-	-	-

** (+) indicate the presence of phytochemicals, (-) indicate the absence of phytochemicals

7.2 TPC and TPC
Maximum TPC and TFC was estimated by methanolic extract (71.63±0.76; 67±0.76) followed by acetonic extract shown in figure 2 and 3. The least value of TPC (44.89± 0.21) and TFC (39.46 ± 0.11) was observed in case of petroleum ether extract.



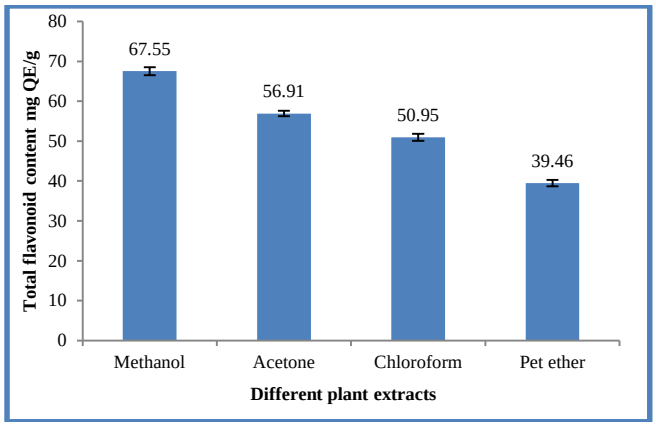
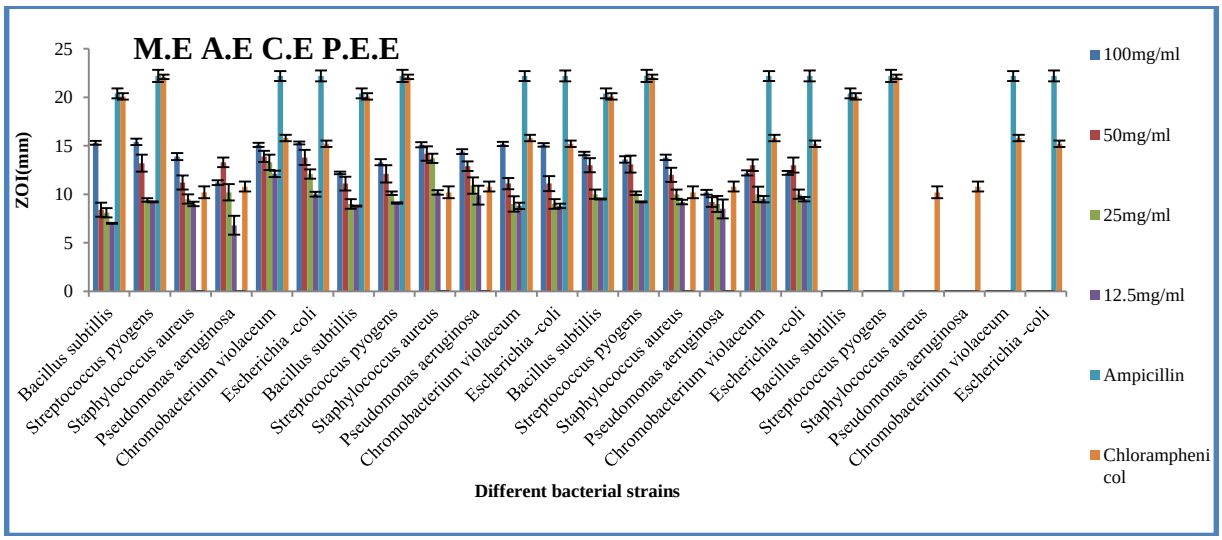


Fig 2: TPC of *E. prostrata* Figure 3-TFC of *E. prostrata*

7.3 Antibacterial activity (Disc Diffusion Assay)

ZOI (in mm) obtained with plant extracts extracted in different solvents, with positive control against different bacterial strains, are represented graphically in Figure- 4. The highest ZOI was obtained with methanolic extract against *B. subtilis* (15.1 ± 0.21mm) followed by *S. pyogens (*15.4 ± 1.02mm). Presence of diverse phytochemicals may be present in the plant extract extracted in methanol in higher amounts. Quantitative analysis also indicate highest phenolic (71.63 ± 0.76 mg GAE/g and flavonoid content (67.55 ± 0.71 mg QE/g) in the methanolic extract, which comply to the statement that methanol, is undoubtedly considered the best solvent for extraction of some phytoconstituents due to the good solubility and polarity of the solvent [19]. Acetonic and chloroform extract also showed good activity against all bacterial strains. petroleum ether extract did not show any ZOI against the bacterial strains used in the present investigation. Highest ZOI obtained with the positive controls i.e. Ampicillin and Chloramphenicol was 22.2±0mm and 22.1±0mm against *B. subtilis* and *S. pyogens*, respectively. *P.aeruginosa* and *S. aureus* were found to be resistant with one of the positive control i.e. Ampicillin.



** M.E methanol extract; A.E- acetonic extract; C.E-chloroform extract; P.E.E- petroleum ether extract

Fig 4: ZOI obtained with different extract of *E. prostrata* and positive controls against different bacterial strains

7.4 MIC values of different extract of *E. prostrata* (mg/ml)

In case of methanolic extract MIC values (table-2) were in the range of 0.78-6.25 mg/ml where as MIC values obtained in case of acetonic extract were in range of 1.56-12.5mg/ml. Petroleum ether didn't show any activity similarly like the disc diffusion against the bacterial strains. Positive control ampicillin showed MIC range from 0.025-0.1mg/ml and *P.aeruginosa* was found resistant against it, where as the MIC values obtained with chloramphenicol were in the range of 0.025-0.1mg/ml.

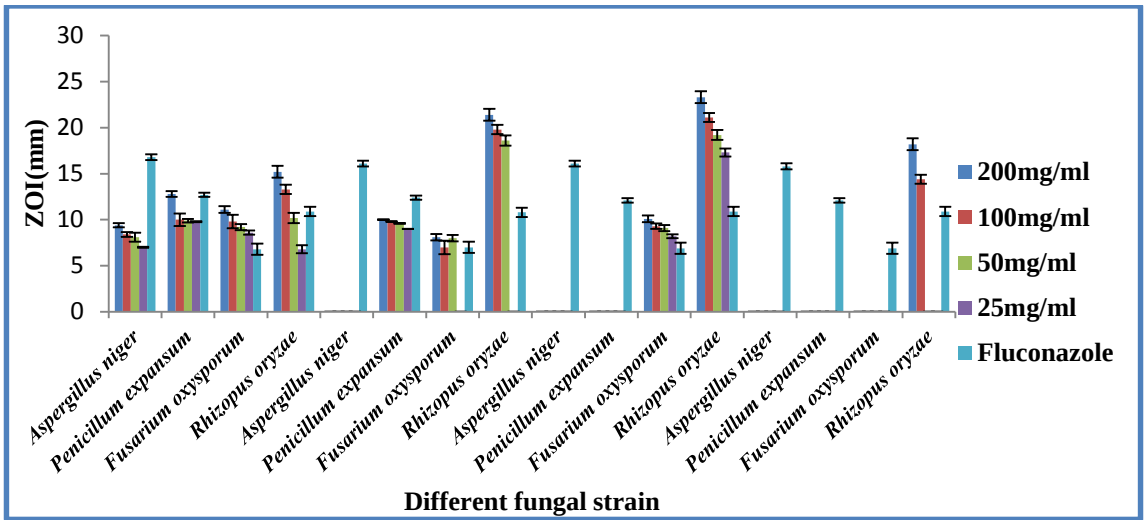
Table 2: MIC values of plant extracts of *E. prostrata* and positive controls i.e. Ampicillin and Chloramphenicol against different bacterial strains.

Microorganisms	M.E	A.E	C.E	P.E.E	A C
B. subtilis	0.78	3.125	6.25	NA	0.05 0.025
S. pyogens	0.78	3.125	1.56	NA	0.05 0.05
S. aureus	3.125	1.56	1.56	NA	N.A 0.025
C. violaceum	6.25	12.5	12.5	NA	0.1 0.1
E. coli	3.125	3.125	1.56	NA	0.1 0.05
P. aeruginosa	3.125	6.25	1.56	NA	N.A 0.05

**MIC (Minimum Inhibitory Concentration) values are expressed as mean (n=3), Unit is mg/ml; M.E methanol extract; A.E- acetonic extract; C.E-chloroform extract; P.E.E- petroleum ether extract; A-Ampicillin; C-Chloramphenicol; NA- no activity; Ampicillin and Chloramphenicol were used as dilutions from 0.1mg/ml up to all 12 wells

7.5 Antifungal activity of *E. prostrata* (Disc Diffusion Assay)

Methanolic extract inhibited all the fungal strain (at 200 mg/ml concentration) (shown in figure -5). Both acetonic and chloroform extract also showed good activity whereas petroleum ether extract exhibited no activity against any fungal strains.



** M.E methanol extract; A.E- acetonic extract; C.E-chloroform extract; P.E.E- petroleum ether extract

Fig 5: ZOI obtained with different extract of *E. prostrata* and positive control against different fungal strains.

7.6 MIC values of plant extracts of *E. prostrata* (mg/ml)

MIC values of different extracts of *E. prostrata* obtained are represented in table -3. Methanolic extract exhibited the significant antifungal activity against all the fungal strains displaying values in range of 3.125-12.5mg/ml. Petroleum ether extract showed activity (25mg/ml) only against *R. oryzae* out of four fungal strains.

Table 3: MIC values of plant extracts and of positive control (fluconazole) against different fungal strains

Microorganism	M.E	A.E	C.E	P.E.E	Fluconazole
A. niger	3.125	NA	NA	NA	0000.05
F. oxysporum	12.5	NA	NA	NA	0.000125
P. expansum	6.25	12.5	12.5	NA	0.00025
R. oryzae	3.125	6.25	6.25	25	0.000125

**MIC (Minimum Inhibitory Concentration) values are expressed as mean (n=3), Unit is mg/ml; M.E methanol extract; A.E- acetonic extract; C.E-chloroform extract; P.E.E- petroleum ether extract; A-Ampicillin; C-Chloramphenicol; NA- no activity; Ampicillin and Chloramphenicol were used as dilutions from 0.1mg/ml up to all 12 wells.

7.7 Non enzymatic assays

7.8 DPPH Assay

The highest antioxidant activity was obtained with the methanolic extract showing the least IC- 50 value i.e.58.63 ± 0.09 followed by acetonic extract (80.05± 0.38) and poorest activity was shown by the chloroform extract having highest IC-50 value (105.4± 0.22).

Strong antioxidant activity of methanolic extract might be due to the presence of high amount of phenolic and flavonoid compounds. The scavenging activity of the phenols is mainly, because of the presence of hydroxyl groups in it. Methanolic extract of *E. prostrata* is found more potent as compared to the other extracts and found similar with some previous reports reported by [20-22]. On a similar pattern our results also showed that highest TPC and TFC are present in methanolic extract. On a similar pattern of antimicrobial results, antioxidant potential was also least in case of petroleum ether.

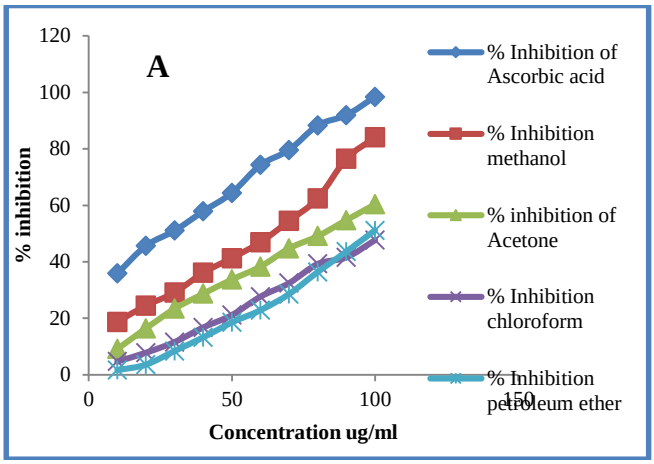


Fig 6(A): Percentage inhibition of DPPH radical by different plant extracts of *E. prostrata*

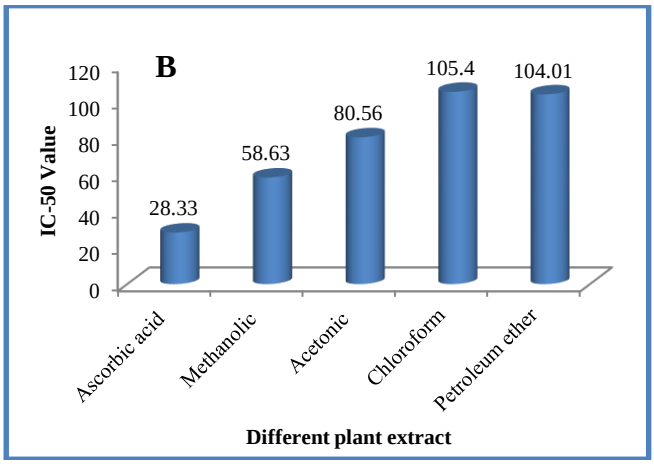


Fig 6(B): IC- 50 values of different plant extracts of *E. prostrata*

7.9 ABTS assay

On a similar pattern of DPPH assay results, antioxidant activities of the extracts towards ABTS radical was also

maximum (Fig-7) with the methanolic extract showing lowest IC- 50 value (73.22± 0.22) followed by acetonic extract (95.97± 0.48) and lowest in case of petroleum ether extract (147.12± 0.23). Our results agree with a previous report of reported by Cao *et al*, 2009 [23].

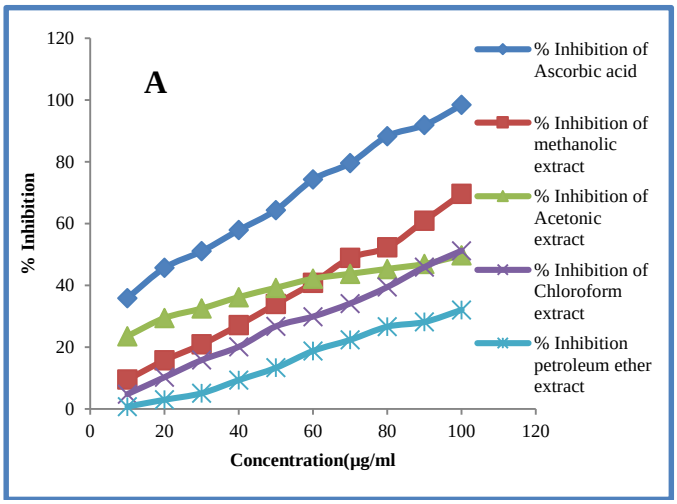


Fig 7(A): Percentage inhibition of DPPH radical by different plant extracts of *E. prostrata*

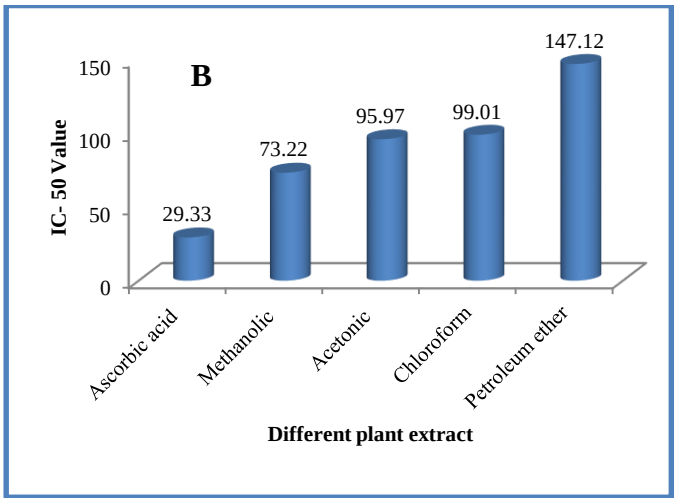


Fig 7(B): IC- 50 values of different plant extracts of *E. prostrata*

7.10 Correlation between antioxidant activities IC_{50} Values, TPC and TFC (Table- 4)

Correlation (Pearson correlation coefficient = r) between the IC_{50} values and TPC and TFC of the extracts are shown in the table -4. There is a positive correlation between the TPC and TFC and negative correlation is there between IC- 50 values and TPC/TFC which means a low IC-50 value is having high antioxidant potential and vice versa.

Table 4: Correlation between IC_{50} Values, TPC and TFC

Pearson correlation coefficient (r)				
TPC and TFC	**TPC and IC_{50} of ABTS**	**TFC IC_{50} of ABTS**	**TPC and IC_{50} of DPPH**	**TFC and IC_{50} of DPPH)**
0.992**	-0.977*	-0.963*	-0.868	-0.906

** Correlation is significant at the 0.01 level (2 tailed)
* Correlation is significant at the 0.05 level (2 tailed)

7.11 Enzymatic assays

The enzyme activity was found to be 0.392±0.12 µmole of H_2O_2 per minute FW for Catalase and 1.434±0.33µmol of GS-DNB conjugate/min FW for Glutathione-S- Transferase while in case of SOD value was found to be 48.94±0.21 SOD (Unit) FW for Superoxide dismutase respectively.

Table 5: Antioxidant enzyme activities of *E. prostrata*

Antioxidant enzymes Activity
Catalase 0 .392±0.12
Glutathione-S- Transferase 1.434±0.33
Superoxide dismutase 48.94±0.21

** Values are expressed as mean (n=3) ±SD.

Units of enzyme activities were expressed as: CAT- One unit of catalase will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25 °C under the assay condition. SOD - One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT per unit time as monitored at 560 nm. GST- One unit of GST activity is the amount of enzyme which produces 1.0 µmol of GS-DNB conjugate/min under the conditions of the assay.

8. Conclusion

Our results suggested that the present study have significant value especially of methanolic extract with respect to its antioxidant potential and antimicrobial activities. The presence of these activities may be because of the presence of high amount of phenolic and flavonoid compounds present in methanolic extract followed by acetonic extract. Present study suggested that the methanolic extract can be use as an effective and safe antioxidant source, as an ethnomedicine and on a commercial basis for the development of drugs. Further, some bioactive elements from the plant should be isolated and assessed for their medicinal properties.

Abbreviations used

E. prostrata- Eclipta prostrata
TPC-Total phenolic content
TFC-Total flavonoid content
MIC-Minimum inhibitory concentration
ZOI-Zone of inhibition
PDA- Potato dextrose agar
CZ- Czapek dox
P.E-Plant extracts
DMSO-Dimethyl sulfoxide
EC-Extinction coefficient
FW- Fresh weight

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