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ECLIPTA PROSTRATA L.: ANTIBACTERIAL AGENT AGAINST MULTIPLE DRUG RESISTANT (MDR) PATHOGENS ISOLATED FROM CLINICAL SPECIMEN

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ABSTRACT

The present study was carried out for evaluation of antibacterial activity of *Eclipta prostrata* L. against multiple drug resistant (MDR) bacteria isolated from clinical specimen. The antibacterial activity of *Eclipta prostrata* L. were evaluated on MDR strains such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Salmonella spp., Enterococcus faecalis, Citrobacter freundii, Acinetobacter baumannii, Streptococcus pneumoniae, Enterococcus faecium* and *Enterobacter cloacae.* Antibacterial activity of five different solvent extracts (Methanol, acetone, ethanol, petroleum ether and n-Hexane) were prepared by using Soxhlet extractor. *In-vitro* antibacterial activity was performed by agar well diffusion method. The phytochemical anyalsis showed that the presence of Saponins, Glycosides, Alkaloids, Flavonoids, Phenolic substance Di & Tri-terpenes and Steroids in both methanolic and ethanolic extracts. The ethanolic and methanolic extract showed good inhibitory activity against all tested bacteria. The methanolic extract showed highest zone of inhibition against *S. typhi* (20 mm), *P. mirabilis* (19 mm), *S. aureus* (18 mm), *K. pneumoniae* (18 mm), *E. coli* (17 mm) and *P. aeruginosa* (16 mm). The lowest MIC value of methanolic extract was found against *E. coli, S. typhi, A. baumannii* and *E. faecium* (6.25mg/ml) and highest MIC value was found 12.5 mg/ml against remaining bacteria. The ethanolic extract showed zone of inhibition against *S. pneumoniae* (18 mm) followed by *P. mirabilis* (18 mm), *S. aureus* (16 mm), *S. typhi* (16 mm), *E. coli* (14 mm), *K. pneumoniae* (14 mm) and *C. freundii* (14 mm). The lowest MIC value was observed against 12.5mg/ml and highest MIC value was 25mg/ml against tested

bacteria. Acetone extract also showed good inhibitory activity against tested bacteria. The maximum zone of inhibition was found against *S. pneumoniae* (15 mm) after that *S. aureus* (14 mm), *P. aeruginosa* (14 mm),*S. typhi* (14 mm) and *C. freundii* (14 mm).

KEYWORDS: MDR, Antibacterial Activity, *Eclipta prostrata* L., Soxhlet extractor.

INTRODUCTION

Antibiotics are used extensively for treating diseases caused by bacteria, yet these biological weapons do not always accomplish their mission. Antibiotics resistance is the ability of a microorganism to withstand the effects of an antibiotic. This resistance may develop through gene action or plasmid exchange between bacteria of the same species.[1] If a bacterium carries several resistant genes, it is called multiresistant or, as it is often described, a 'superbug".[1] In the early 1970s, physicians were forced to abandon their belief that, given the vast array of effective antimicrobial agents, virtually all bacterial infections were treatable.[2] Their optimism was shaken by the emergence of resistance to multiple antibiotics among such pathogens as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*.[2] Resistance to antibiotics is a serious worldwide problem which is increasing and has implications for morbidity, mortality, and health-care both in hospitals and in the community [Franco *et al.*, 2009]. The irony of this trend toward progressively more resistant bacteria is that it coincides with a period of dramatically increased understanding of the molecular mechanisms of antimicrobial resistance.[2] Unfortunately, while this insight has resulted in the identification of novel drug targets in herbal medicine, it has not yet resulted in effective new chemotherapeutic agents. [2] Several studies have demonstrated that, patterns of antibiotic usage can have dramatic effects on the prevalence of resistant organisms.[1] Irrational use of antibiotics in humans and animal species, insufficient patient education when antibiotics are prescribed, lack of guidelines for treatment and control of infections, inadequate dissemination of scientific information for physicians on the rational use of antibiotics, and lack of official government policy on the rational use of antibiotics in public and private hospitals, have all contributed to antibiotic resistance.[3] Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants. Several screening studies have been carried out in different parts of the world.

Eclipta prostrata (L.) Hassk commonly known as False Daisy and Bhringraj, is a plant belonging to the family Asteraceae. It is also named "Kehraj" in Assamese, Maka (Marathi), Bhangra (Hindi) and Karisalankanni in Tamil. It is a perennial herb usually found spreading easily in moist tropical countries. The branches are hairy, reddish brown and can grow up to 40 cm high. The roots are found growing at the thickened nodal points. The leaves are opposite, lance like wit a toothed edge and hairy. When the leaves are cut with iron knife, it turns the sap black. The flowers are white, small and arranged in small clusters. The flowering stalk arises from the axis of the leaf. The dry fruits are formed by fusion of two carpels, which do not break open and each has just one seed. Root well developed, cylindrical and grayish.[4] *Eclipta prostrata* L. occurs throughout the whole of India. It is widely distributed throughout India, China, Thailand and Brazil.

Phytochemicals

Eclipta prostrata L. contains wide range of active principles, which includes coumestans, alkaloids, flavonoids, glycosides, polyacetylenes, triperpenoids. The leaves contain stigmasterol, a-terthienylmethanol, wedelolactone, demethylwedelolacone and demethylwedelolactone-7-glucoside. The roots give hertriacontanol and heptcasanol. The roots contain polyacetylene substitutued thiophenes. The arial part was reported to contain a phytosterol, P-amyrin in the n-hexane extract and luteolin-7-glucoside, P-glucoside of phytosterol, a glucoside of a triterpenic acid and wedelolactone in polar solvent extract. The polypeptides isolated from the plant yield cystine, glutamic acid, phenylalanine, tyrosine and methionine on hydrolysis. Nicotine and nicotinic acid were reported to occur in this plant.[5]

Traditional Uses

Eclipta prostrata L. has been traditionally used for blackening, promoting hair growth and strengthening the hair. It is believed by some people that if it is taken internally as well as applied externally the hair will eventually hair black. As a dye it has also been used in tattoos. In Ayurveda medicine, the leaf extract is considered a powerful liver tonic and rejuventative. It has traditional external uses, like althelete foot, eczyma and dermatitis, on the scalp to address hair loss and the leaves have been used in the treatment of scorpion stings. It is used as anti-venom against snake bite in China and Brazil. It is reported to improve hair growth and colour. *Eclipta prostrata* is one of the important medicinal herbs with a role in the traditional medicine systems of the East. It is reported to possess antiseptic, analgesic, antipyretic, antispasmodic, antimicrobial and antiviral properties. *Eclipta prostrata* is

reported to be effective for the retrieval of memory.[6] It is hepatoprotective, anti-inflammatory.[7] and antimalarial.[8] This plant is considered rejuvenative and good for hair, and a blackening dye for hair is obtained from this plant. The leaves of *Eclipta prostrata* are used against snake bites and scorpion stings. This plant is an important constituent of the polyherbal cardioprotective drug called abana.[9] *Eclipta prostrata* is also reported to have antianaphylactic,[10] antihyperglycemic,[11] and antioxidant.[12] properties. Wedelolactone present in *Eclipta alba* has been reported to be useful for treating hepatitis and cirrhosis and to be antibacterial and antihemorrhagic.[13,14].

MATERIALS & METHODS

1) Plant Materials

Medicinal plants and their parts were collected from different areas of Nagpur city. This plant then authentified from P.G. Department of Botany, R.T.M. Nagpur University, Nagpur. Leaves were collected washed with sterile distilled water and air dried at room temperature. Dried leaves were coarsely powdered using a mortar and pestle and were further reduced to powder using an electric blender. The powder was transferred into closed containers for further use.

2) Herbal preparations

The dried plant materials (20 gm) were extracted with 200 ml of each solvent separately by using Soxhlet extractor for 2 to 5 hr at a temperature not exceeding the boiling point of the Solvent. The solvents used for the study were methanol, ethanol, petroleum ether, acetone and n-hexane. The extracts were filtered and then concentrated to dryness. The extract were transferred to glass vials and kept at 4ºC before use. The extracts were dissolved in 20% aqueous dimethyl sulfoxide (DMSO) to produce a stock solution of 100 mg/ml. The stock solutions were stored in a refrigerator until needed.[15]

3) Phytochemical analysis

The phytochemical screening of all the extracts was carried out to determine the presence of the following compounds; alkaloid, flavonoids, polyuronides, reducing sugars, cyanogenic glycoside, saponins, terpenes, anthracenosides, phytosterols and phenols as described below.[16]

3.1: Saponins (the Froth test)

2 ml of the extract was added to distilled water and shaken vigorously. A froth (foam) that persisted for more than 10 minutes indicated the presence of saponins.

3.2: Glycosides

To the solution of extract in glacial acetic acid few drops of ferric chloride and conc. H_2SO_4 are added and observed for reddish brown coloration at the junction of 2layers and bluish green color in upper layer.

3.3: Polyuronides / Polyamides

Ten milliliters of acetone was added to 2ml of the extract in a test tube. The appearance of a Precipitate indicated the presence of polyuronides.

3.4: Reducing sugars

Two milliliters of the extract was diluted in 2ml of distilled water and Fehling's solutions (A+B) added to the mixture. A brick red precipitate after standing in the heat or water bath indicated the presence of reducing sugars.

3.5: Alkaloids

Twenty milliliters of the alcohol extract was evaporated to dryness on a water bath. Five to ten milliliters of 10% hydrochloric acid (HCl) and $CHCl_3$ were added to the extract. Concentrated ammonia was added to the aqueous layer to obtain a pH of between 8 and 9. The solution was then extracted in a separating tube with chloroform or ether. The a polar solvent was evaporated to dryness in an evaporated dish in a water bath and the residue was dissolved with 5ml of HCl (2N) and the solution was divided into three separate test tubes. Two to three drops of Mayer's reagent was added to one and the same amount of Bertrand's reagent to the other, while the third test tube served as a reference. The appearance of an opalescent or yellow-white precipitate with the reagents indicated the presence of alkaloids.

3.6: Anthracenocides

Four milliliters of the extract was concentrated to 2ml with 2ml of 25% of ammonia solution added and shaken. A cherry red colour of the alkaline layer indicated the presence of emodols (aglycones of anthracenosides) in an oxidized form–Borntrager's reaction.

3.7: Flavonosides

Five milliliters of the extract was evaporated to dryness. The residue was dissolved in 2ml of 50% methanol by heating and 4 grams of metal magnesium and 6 drops of concentrated HCl added. A red solution indicated the presence of flavonoids, while an orange solution indicated the presence of flavones.

3.8: Phenolic substances

Two to three drops of 10% Ferric chloride solution was added to 5ml of extract in a test tube and observed. Dark Green color was develops indicated positive results.

3.9: Sterols and Triterpenes

Ten milliliters of the extract was evaporated to dryness. The residue was dissolved in 0.5ml of acetic aldehyde and 0.5ml of $CHCl_3$ added and transferred into a dry test tube. About two milliliters of concentrated sulphuric acid (H_2SO_4) was added to the bottom of the tube using a pipette. A brownish red or violet ring at the contact zone of the two liquids indicated the presence of sterols and triterpenes. The greenish and brownish red (wine) nature of the supernatant indicated the presence of sterols and triterpenes respectively.

3.10: Test for Tannins

To 0.5 ml of extract solution 1 ml water and 1-2 drops of ferric chloride solution was added. Blue color was observed for garlic tannins and greenish black for catecholic tannin.

3.11: Test for amino acids

1 ml of plant extract add 2 ml of Ninhydrins. For positive results indicates forming purple color.

3.12: Test for proteins

1 ml of dilute extract add 1 ml of 5% $CuSO_4$ add 1% of 1ml of NaOH. Deep blue color indicates positive results.

3.13: Test for Saponin

To 50 mg powder and add 20 ml distilled water shake for 15 minutes. Forming 2 cm foam was produced in measuring cylinder indicated positive results.

4. Bacterial Isolates

Multiple drug resistant bacteria were isolated from different clinical specimen such as urine, blood, wound swabs/pus, cerebrospinal fluid and sputum. The MDR strains were identified on the basis of their morphology, cultural, biochemical characteristics as well as antibiotic susceptibility test. These all MDR bacteria were resistant to more than 10 antibiotics. The MDR strains used for the antibacterial activity were *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Salmonella spp., Enterococcus faecalis, Citrobacter freundii, Acinetobacter baumannii, Streptococcus pneumoniae, Enterococcus faecium and Enterobacter cloacae.*

5: Determination of the potency of the herbal preparation

The agar diffusion method was used to investigate the antibacterial activity of the crude extracts. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterilized swab was aseptically dipped into the suspension. The dried surface of a Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface with bacteria. A sterilized cock borer of an internal diameter of about 6 mm was used to punch holes in the medium and plant extracts were dispensed into the respective labeled holes. 20 % v/v DMSO was used as negative controls. Triplicates of each plate were made and the procedure was repeated for the other microorganisms. The plates were kept in the refrigerator for about 4 hours for complete diffusion of the extract and incubated at 37ºC for 24 hours. After the incubation period, the diameter of each zone of inhibition was measured in millimeters (mm) with zone measuring scale. [15]

6: Determination of minimum inhibitory concentration (MIC) of the crude extracts

MIC for each test organism was determined by following the modified agar well diffusion method. A twofold serial dilution of each extract was prepared by first reconstituting the dried extract (100 mg/ml) in 20% DMSO followed by dilution in sterile distilled water (1:1) to achieve a decreasing concentration range of 50mg/ml to 0.195 mg/ml. A 100 µl volume of each dilution was introduced into wells (in triplicate) in the agar plates already seeded with 100µl of standardized inoculum (10^6cfu/ml) of the test microbial strain. All test plates were incubated aerobically at 37^0C for 24 hrs and observed for the inhibition zones. MIC, taken as the lowest concentration of the test extract that completely inhibited the growth of the microbe, showed by a clear zone of inhibition (>8mm), was recorded for each test organism.[17]

RESULTS AND DISCUSSION

In Ayurveda medicine, the leaf extract is considered a powerful liver tonic and rejuvenative. It has traditional external uses, like athlete foot, eczema and dermatitis, on the scalp to address hair loss and the leaves have been used in the treatment of scorpion stings. It is used as anti-venom against snake bite.[18] *Eclipta prostrate* L. contains wide range of active principles, which includes coumestans, alkaloids, flavonoids, glycosides, polyacetylenes, triterpenoids.[19] This study showed that the presence of Saponins, Glycosides, Alkaloids, Flavonoids, Phenolic substance Di & Tri-terpenes and Steroids in both methanolic and ethanolic extracts [Table No. 1]. It has been observed that the extract exhibited strong activity with the increase in polarity (with reference to organic solvent), indicating that polyphenols or flavanone or flavanoids may play important roles in the activities. The present findings are in agreement with the report of Tepe *et al.* (2005).[20]

The antibacterial activity was perfomed against all MDR strains. The ethanolic and methanolic extract showed good inhibitory activity against all tested bacteria the methanolic extract showed highest zone of inhibition against *S. typhi* (20 mm), *P. mirabilis* (19 mm), *S. aureus* (18 mm), *K. pneumoniae* (18 mm), *E. coli* (17 mm) and *P. aeruginosa* (16 mm). The methanolic extract also showed 14mm zone of inhibition against *E. faecalis*, *A. baumannii*, *S. pneumoniae* and *E. faecium* [Table No. 2]. The lowest MIC value of methanolic extract was found against *E. coli, S. typhi, A. baumannii* and *E. faecium* (6.25mg/ml) and highest MIC value was found 12.5 mg/ml against remaining bacteria [Table No. 3]. The ethanolic extract showed zone of inhibition against *S. pneumoniae* (18 mm) followed by *P. mirabilis* (18 mm), *S. aureus* (16 mm), *S. typhi* (16 mm), *E. coli* (14 mm), *K. pneumoniae* (14 mm) and *C. freundii* (14 mm). The lowest MIC value was observed against 12.5mg/ml and highest MIC value was 25mg/ml against tested bacteria [Table No. 3] Acetone extract also showed good inhibitory activity against tested bacteria. The maximum zone of inhibition was found against *S. pneumoniae* (15 mm) after that *S. aureus* (14 mm), *P. aeruginosa* (14 mm),*S. typhi* (14 mm) and *C. freundii* (14 mm). The other solvent such as petroleum ether and n-hexane showed least antibacterial activity against tested bacteria [Table No. 2] Some study showed that the extracts revealed the presence of various compounds like Hydrazine carboxamide, Naphthoquinones, Glycine, Carbamic acid etc., Hydrazine carboxamide is reported to possess antimicrobial activity and showed inhibition against some bacterial strains like *S. aureus, K. pneumoniae, E. coli, P. aeruginosa*,[21] which is also exhibited in this study where the extracts were found to be highly antimicrobial in nature which may be attributed to the

compounds present in various extracts. The study conducted by Karthikumar, S *et. al.* (2007) showed the ethanolic extract of leaves of *E. prostrata* recorded significant antibacterial activities against all tested bacterial strains, while ethyl acetate extract recorded medium activity and no significant results were recorded in both hexane and petroleum ether extracts. [22] Though ethanol extract showed broad spectrum of activity against all tested organisms (*E. coli, K. pneumoniae, S. dysenteriae, S. typhi, P. aeruginosa, S. aureus*). *S. typhi* is highly sensitive to ethanol extract similar results were found when comparing with the present study. The results disagree with those reported by Karthikumar *et al.*, (2007) and Devi *et al.*, (2009) which found that least inhibitory activity against *Salmonella typhi* whereas the present study results agree with those reported by Lenza *et al.*, (2009).[22,23,24] The data concerning the antibacterial activities of petroleum ether methanol and water extracts from *Eclipta alba* against Gram-positive *Staphylococcus aureus*.

Table No. 1 : Phytochemical analysis of *Eclipta prostrata* L.

Sr. No.	**Phytochemical analysis**	***Eclipta prostrata* L.**	
		Solvents	
		Methanol	**Ethanol**
1	Saponins	+	+
2	Glycosides	+	-
3	Polyamides	-	-
4	Reducing Sugars	-	-
5	Alkaloids	+	+
6	Steroids	+	-
7	Flavonoids	+	+
8	Phenolic substance	+	-
9	Di & Tri-terpenes	+	+
10	Tannins	+	+
11	Amino acids	-	-
12	Proteins	+	+

Table No. 2: Antibacterial activity of *Eclipta prostrata* L. against multiple drug resistant (MDR) bacteria.

Plant Part used	Solvent	Zone of inhibition in mm											
		S. aureus	***E.coli***	***P. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***S. typhi***	***E. faecalis***	***C. freundii***	***A. baumannii***	***S. peumoniae***	***E. faecium***	***E. cloacae***
Leaves	Ethanol	16	14	12	14	18	16	12	14	12	18	12	11
	Methanol	18	17	16	18	19	20	14	12	14	14	14	10
	Petroleum Ether	12	10	8	NZ	8	10	11	12	11	10	10	8
	Acetone	14	12	14	10	10	14	12	14	12	15	10	10
	n-Hexane	10	8	9	NZ	8	8	10	8	10	12	8	8

NZ – No Zone

Table No. 3: MIC of *Eclipta prostrata* L. against Multiple drug resistant (MDR) bacteria.

Plant Part used	Solvent	Minimum Inhibitory Concentration in mg/ml											
		S. aureus	***E.coli***	***P. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***S. typhi***	***E. faecalis***	***C. freundii***	***A. baumannii***	***S. peumoniae***	***E. faecium***	***E. cloacae***
Leaves	Methanol	12.5	6.25	12.5	12.5	12.5	6.25	12.5	12.5	6.25	3.125	6.25	12.5
	Ethanol	25	12.5	12.5	12.5	25	12.5	12.5	25	25	12.5	12.5	25

CONCLUSION

This study was concluded that the extract of *Eclipta prostrate* L can be alternative source for potential antimicrobial agents for the treatment of diseases caused multiple drug resistant pathogens. This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity.

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