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Research Article

Antimicrobial and toxicity studies on *Breynia disticha* J.R.Forst. & G.Forst. and *Vernonia elaeagnifolia* DC.

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Abstract

The present study was aimed to investigate the phytochemistry, toxicity and antimicrobial activity of the two plants Breynia disticha and Vernonia elaegnifolia. Tests for all the major secondary metabolites were carried out whereas acute toxicity studies on rabbits and cytotoxic studies on Artemia salina were performed. Antibacterial and antifungal activities were tested using disk diffusion method. The phytochemical investigation revealed the presence of flavonoids, tannins and other important compounds in the two plants. Antibacterial and antifungal activity was found to be present in both plants. Both plants exhibited no toxicity in in-vitro and in-vivo assays. The present work thus proves the medicinal use of the two safe and potent plants.

Keywords: Plants; Extracts; Activity; Phytochemistry; Alkaloids; Flavonoids; Glycosides.

Introduction:

Universal role of plants in human life is well established. On one hand they provide us oxygen, an essential element required for life and on other hand they provide us food, medicines, dyes, shelter, fuel and pleasure. The importance of medicinal plants is increasing day by day not only in the developing countries but also in developed and technological advanced countries. Surveys indicate that usage of medicinal plants had increased from 3% to 37% by the American people during 1991 to 1998 (Brevoort, 1998). About 80% people all over the world depend on medicinal plants to prevent, manage and cure various types of illnesses. According to WHO list, about 20,000 medicinal plants are being used for such purpose (Farnsworth, 1988; Laloo *et al*., 2000).

Breynia disticha belongs to the family Phyllanthaceae. It exists in the form of or small trees. The leaves of the plant present an abstract of green, pink, purple and white color. It is extensively found in gardens and has been traditionally used to treat menstrual cramps. *Vernonia elae+9agnifolia* (Parda Bail) belongs to family Asteraceae. It is a creeper commonly grown in gardens of houses for screening purposes. The plant is traditionally renowned for its leech repellant activity (De Boer, 2012; Lorence *et al*., 1995; Matthew, 1995).

Previously for the two plants, we studied the antioxidant activity and antimicrobial action on some strains (Sulayman and Saad, 2015). The present work comprises of five different studies namely, phytochemical, acute toxicity, cytotoxicity, anti bacterial and antifungal studies in order to evaluate the therapeutic efficacy of the plants extensively.

MATERIALS AND METHODS

Plant material

Mature whole plants of *Breynia disticha* and *Vernonia elaeagnifolia* were collected from Arain Nursery, Model Town, Lahore and were identified and authenticated by Dr. Ajaib, Government College University Lahore, Pakistan as *Breynia disticha* and *Vernonia elaeagnifolia*. The voucher

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specimens were deposited at Herbarium of Government College University. The voucher number for *Breynia disticha* and *Vernonia elaeagnifolia* were GC.Herb.Bot.2282 and GC.Herb.Bot.2283 respectively.

Extraction

The aerial parts of *B. disticha* and *V. elaeagnifolia* were dried under shade and ground into coarse powder. The plant material 150g was extracted twice using 750 ml methanol. The combined extract was concentrated in a rotary evaporator (Heidolph Laborota 4000eco) at 35ºC and air dried to obtain a solid mass. The extraction of *B. disticha* and *V. elaeagnifolia* yielded 16.3 g and 15.1 g of crude methanol extract respectively.

Chemicals

Methanol was purchased from Panreac, Spain. Dimethyl Sulfoxide (DMSO), Ethanol and hydrochloric acid were purchased from Sigma Aldrich, USA. Toposar (Etoposide) was obtained from Pfizer pharmaceuticals. All other chemicals were of analytical grade.

Experimental Animals

Healthy rabbits (*Oryctolagus Coniculus*) of either sex weighing 1.5-2.0 Kg were purchased from the local market of Lahore. The animals were kept at room temperature and were allowed for two weeks to acclimatize with experimental environment. Controlled lighting was provided consisting of 12 hour light and dark period. They were fed freely with food (cauliflower, cabbage, banana and green fodder) and tap water *ad libitum*. The study protocol was approved by the ethical committee (AREC016-13) of the Faculty of Pharmacy, The University of Lahore.

Microorganisms tested

The clinical isolates of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsella pneumoniae*, *Proteus mirabilis*, *Bacillus cereus*, *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Saccharomyces cerevisae* and standard cultures of *Pseudomonas aeruginosa* (ATCC-35151), *Staphylococcus aureus* (ATCC-29213), *Escherichia coli* (ATCC-25922), *Bacillus subtilis* (ATCC-6633), *Staphylococcus epidermidis* (ATCC-12228), *Aspergillus niger* (ATCC-16404), *Candida albicans* (ATCC-10239) and *Saccharomyces cerevisae* (ATCC-9763) were used for the antimicrobial assay. All the microbial strains were kindly supplied by Institute of Molecular Biology and Biotechnology, The University of Lahore.

Phytochemical screening

Phytochemical tests for different secondary metabolites such as alkaloids, glycosides, phenolics, saponins etc. were performed according to the methods described in the literature (Harborne, 1973; Raaman, 2006; Trease and Evans, 1983).

Acute toxicity

Sample solutions of *B. disticha* (BME) and *V. elaeagnifolia* (VME) were prepared by dissolving 250mg, 500 mg and 1000mg of methanolic extract in 2 ml of distilled water. In order to facilitate the dissolution, two drops of Tween 80 were added. Three hours prior to experiment, food was withheld and only tap water was supplied. The rabbits were distributed randomly into 7 groups (n=5). Group I served as control group and received vehicle only. Group II, III and IV received 250mg, 500 mg and 1000mg of VME respectively whereas Group V, VI and VII received 250mg, 500 mg and 1000mg of BME respectively through oral route. After the administration of doses the animals were

observed continuously for any toxic symptoms, illness, behavioral changes and mortality for the period of 24 hours. The dose at which no toxic symptoms, illness, behavioral changes and mortality occurred was considered to be safe. (Khan *et al*., 2008; Wadood *et al*., 1989).

Cytotoxic activity

The cytotoxic activity was determined using Brine Shrimp Lethality Assay (Hussain *et al*., 2010). Artificial sea water (38g sea salt in 1000ml distilled water; pH 7.4) was prepared and Artemia cysts were hatched under controlled conditions (28±2 ^{o}C; bright white fluorescent light). Ten shrimps were added to each test tube and 5ml of sea water was added containing 10, 100 and 1000µg/ml BME and VME. The shrimps were incubated for 24 hours under the same conditions in which they were hatched and the total shrimps survived after incubation were counted. The experiment was carried out in triplicate. Etoposide 100µl was used as standard.

Antibacterial and antifungal activity

The antibacterial assay was carried out according to the disk diffusion method described by Bauer *et al*., (1966). Briefly 20mg of extract was dissolved in 2ml DMSO. 20µl (0.2 mg) of this extract was impregnated to sterile filter paper disks (5mm diameter) and placed over prepared Mueller-Hinton Agar (MHA) media swabbed by bacterial suspension in Nutrient broth. Neomycin sulfate and Chloramphenicol disks were used as standard.

Antifungal assay was carried out according to the disk diffusion method described by Saratha and Subramanian, (2010). The procedure of the assay was quite similar to the antibacterial assay described earlier. Sabouraud Dextrose Agar Media (SDA) was used instead of MHA and fungal suspension for swabbing was prepared in normal saline. Nystatin and Amphotericin B disks were used as standard. The results were expressed as mean of zone of inhibition of triplicates.

Statistical analysis

The data was expressed as mean ± standard deviation (SD). For Brine shrimp lethality test LD_{50} was calculated by Probit analysis using SPSS statistical package.

RESULTS AND DISCUSSION:

The results of phytochemical analysis are given in table 1. In case of BME, glycosides, phenolics, tannins, flavonoids and saponins were detected whereas in case of VME, alkaloids, phenolics, tannins and flavonoids were identified. Phytochemical study prove the plants to be rich in secondary metabolites and therefore of high therapeutic activity.

In acute toxicity study VME was found to be safe and no death was observed at the dose of 1000mg/kg in any of the 12 rabbits. Similarly, BME was also found safe at the dose of 500 mg/kg however at the dose of 1000mg/kg all 12 rabbits died within the time period of 24 hours.

Table 1: Results of preliminary phytochemical screening

Secondary Metabolite class	Test	BME	VME
Alkaloid	Mayer's test	-	++
	Wagner's test	-	++
	Hager's test	-	++
	Dragendorff's test	-	+++
Glycoside	Borntrager's test	++	-
	Legal's test	++	-
Phenolics and tannins	Ferric chloride test	+++	+++
	Gelatin test	++	+++
	Lead acetate test	+++	+++
Flavonoids	Alkaline reagent test	++	+++
Gums and	Precipitation test	-	-

mucilages			
Steroids	Salkowski's test	-	-
Catechin	Wood test	++	++
Saponin	Frothing test	++	-

BME *Breynia distichia* methanolic extract; **VME** *Vernonia elaeagnefolia* methanolic extract; "+" indicates presence while "-" indicates absence

The results from cytotoxic assay show that both plants and their mixture possess no cytotoxicity (Table 2). The absence of cytotoxicity on one hand show absence of anticancer potential while on other hand proves its safety for use in animal models (Jahanzeb *et al*., 2014).

Table 2: Cytotoxic activity of crude plant extracts

Sr. No	Test Material	LD_{50} (µg/mL)	Toxicity Profile
1.	Etoposide	7.4625	Highly cytotoxic
2.	BME	6921.7	Non cytotoxic
3.	VME	4936.053	Non cytotoxic
4.	BME + VME mixture	5488.1	Non cytotoxic

BME *Breynia distichia* methanolic extract; **VME** *Vernonia elaeagnefolia* methanolic extract; Values are the mean of triplicate studies (mean ± SEM), n = 30 (number of shrimps); Highly cytotoxic < 20 µg/mL; Cytotoxic 20 µg/mL - 1000 µg/mL; Non cytotoxic > 1000 µg/ml

Antibacterial assay was carried out on both the clinical isolates and the standard strains. BME was found to be highly active against *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) and *E. coli* (clinical isolate) where the zone of inhibition was recorded to be 12mm. VME exhibited a broad spectrum antibacterial action. Only *S. epidermidis* was found to be resistant to the plant extract. The most sensitive strains towards VME included the standard and clinical isolate of *E. coli* having zones of 13mm and 14mm respectively. The zones of inhibition of the standard drugs are given in table 3.

Table 3: Antibacterial activity of plant extracts against Gram positive strains

Sr. no.	Bacterial strain	Zone of inhibition (mm)		
		BME	VME	Standard
1	*S. aureus* (Clinical isolate)	8.0±0.1	7.5±0.1	19.5±0.2[b]
2	*S. aureus* (ATCC 29213)	12.0±0.3	7.0±0.2	20.0±0.35[b]
3	*B. subtilis* (Clinical isolate)	8.0±0.1	10.0±0.1	15.0±0.4[b]
4	*B. subtilis* (ATCC 6633)	8.5±0.4	10.0±0.1	23.5±0.1[a]
5	*S. epidermidis* (Clinical isolate)	-	-	18.5±0.2[a]
6	*S. epidermidis* (ATCC 12228)	-	-	19.0±0.1[a]
7	*E. coli* (Clinical isolate)	12.0±0.5	14.0±0.3	20.0±0.1[b]
8	*E. coli* (ATCC 25922)	12.0±0.1	13.0±0.7	21.0±0.5[b]
9	*P. aeruginosa* (Clinical isolate)	-	8.0±0.4	9.0±0.1[b]
10	*P. aeruginosa* (ATCC 35151)	-	7.0±0.2	10.0±0.3[b]

BME *Breynia distichia* methanolic extract; **VME** *Vernonia elaeagnefolia* methanolic extract; **a**= Neomycin sulfate (10µg); **b**= Chloramphenicol (30µg)

The results of antifungal activity are given in table 4. Both plant extracts were found to be active against *S. cerevisae* only. VME produced larger zone of inhibition (10.5mm) than BME (8.0mm) in case of the standard strain of the fungus whereas in case of clinical isolate the zone was found to be 8.0mm. BME however, did not affect the clinical strain of the fungus.

Table 4: Antifungal activity of plant extracts

Sr. no.	Fungal strain	Zone of inhibition (mm)		
		BME	VME	Standard

1	*A. niger* (Clinical isolate)	-	-	26.0±0.3[b]
2	*A. niger* (ATCC 16404)	-	-	18.0±0.5[b]
3	*C.albicans* (Clinical isolate)	-	-	28.0±0.5[b]
4	*C. albicans* (ATCC 10239)	-	-	25.0±0.1[b]
5	*S. cerevisae* (Clinical isolate)	-	8.0±0.1	20.0±0.2[b]
6	*S. cerevisae* (ATCC 9763)	8.0±0.2	10.5±0.5	17.0±0.1[a]

BME *Breynia distichia* methanolic extract; **VME** *Vernonia elaeagnefolia* methanolic extract; **a**= 10 µg Nystatin; **b**= 10 µg Amphotericin B

CONCLUSION

The importance of natural products especially plants cannot be ignored. Numerous drugs from plant sources have been proven to be superior, both in terms of safety and efficacy, as compared to synthetic drugs. The present study emphasis on the medicinal potential of the two unnoticed plants. The different tests carried out showed significant and promising results. Further studies may be carried out to identify the compounds responsible for different pharmacological activities.

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