International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 4 | OCT-DEC | 2018 | 462-469
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

IN VITRO ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF VARIOUS EXTRACTS OF *EUPHORBIA NIVULEA* HAM

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ABSTRACT

Traditional information possess that the juice of the leaf is used as a purgative, diuretic etc. The paste of the leaf made with neem oil is applied externally in rheumatism. Plant latex is used for treating jaundice, dropsy, enlargement of liver and spleen, and applied to hemorrhoids. Coagulated latex is used for bronchitis. Phytochemical studies indicated that the methanol and ethyl acetate extracts contain a broad spectrum of secondary metabolites like terpenes, flavonoids phenolic compounds, alkaloids, and tannins. Phenol, tannins and flavonoids were predominantly found in ethyl acetate and methanol solvent extracts and petroleum ether and chloroform exhibits phytosterols, fixed oils and terpenoids. In the present study the in-vitro anti-bacterial and anti-fungal activities of petroleum ether, chloroform, ethyl acetate and methanol extracts of Euphorbia nivulea Ham (Euphorbiaceae) was evaluated against various strains of bacteria and fungi. The aerial parts of the plant extracts were tested for the anti-bacterial activity against gram positive (Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis) and Gram negative (Escherichia coli, Salmonella paratyphi, Pseudomonas aeroginosa, Klebsiella pneumoniae, Vibrio cholera) bacteria. The anti-fungal potency was tested against Aspergillus fumigatus, Aspergillus niger, Monococcus purpura and Candida albicans. The preliminary anti-microbial activities were done by agar well diffusion method. Petroleum ether and chloroform extracts displayed very less anti-microbial activity; whereas ethyl acetate and methanol extracts showed very good anti-microbial activity with widest zone of inhibition which was comparable to standard drug. Hence these two extracts were further tested for their MIC by micro broth dilution method. From the study it was found that ethyl acetate and methanol extracts exhibited remarkable anti-microbial activity against the tested micro-organism.

KEY WORDS

Euphorbia nivulea, fluorescence characteristics, pharmacognostic studies, phytochemical screening, antibacterial activity.

INTRODUCTION

People of inaccessible villages and tribal areas are reliant upon the practice of folk medicines (Nadkarni, 1982). Ethnobotanically plant latex has a great potential with respect to its medicinal value. Latex has been reported to occur in 12000 plant species belonging to 900 genera. A common feature that can be found in the latex of *Euphorbiaceae* is the presence of obvious digestive enzyme activity. *Euphorbia* is a large genus consisting of about over 2000 species distributed all over the world. Approximately 195 species of *Euphorbia* have been recorded from India (Basak *et al.,* 2009). This genus includes herbs, shrubs and trees in widely diverse habitats. *Euphorbia nivulea* Buch – Ham, a member of this family Euphorbiaceae is a wild, thorny, xerophytic, succulent plant, commonly used in fencing of the

agricultural field and also in dry barren areas. It has different biological activities for the treatment of several ailments of man. One such plant, *Euphorbia nivulea* Buch. -Ham invites attention of the researchers worldwide for its biological activities. There is not much literature available on the biological activities of *Euphorbia nivulea.* Also, the earlier reviews on *Euphorbia* plants lack satisfactory information regarding its biological activities. The aim of the present review is to provide the updated information on the biological uses of *Euphorbia nivulea*. Emphasis is being laid on the areas of the most recent interest and those which have not been presented in earlier reports.

In the present study the *in-vitro* anti-bacterial and anti-fungal activities of petroleum ether, chloroform, ethyl acetate and methanol extracts of *Euphorbia nivulea* Buch. -Ham was evaluated against various strains of bacteria and fungi. The aerial part of the plant extracts were tested for the anti-bacterial activity against gram positive (*Staphylococcus aureus, Micrococcus luteus*, *Bacillus subtilis*) and Gram negative (*Escherichia coli*, *Salmonella paratyphi*, *Pseudomonas aeroginosa, Klebsiella pneumoniae*, *Vibrio cholera*) bacteria. The anti-fungal potency was tested against *Aspergillus fumigatus*, *Aspergillus niger, Monococcus purpura Candida albicans* and *Tinearubrum.* The preliminary anti-microbial activities were done by agar well diffusion method. Petroleum ether and chloroform extracts displayed very less anti-microbial activity; whereas ethyl acetate and methanol extracts showed very good anti-microbial activity with widest zone of inhibition which was comparable to standard drug. Hence these two extracts were further tested for their MIC by micro broth dilution method. From the study it was found that ethyl acetate and methanol extracts exhibited very good anti-microbial activity against the tested micro-organism.

MATERIALS AND METHODS

Plant collection, Drying, Pulverizing and Preparation of Extract

The *Euphorbia nivulea* Ham shrub was collected from the moist places near Thirunelveli district, Tamilnadu and identified and authenticated by Mr.V.Chelladurai Research Officer of Botany, Central Council for Ayurveda and Siddha, Government of India. The voucher specimen was preserved in our laboratory for future reference. *Euphorbia nivulea* is a tall shrub with cylindrical stem and branches. Stipular spines, glabrous, straight, paired, often blackish. Leaves appear only during rainy season, 8.5 – 20 ×3.5 – 6.5 cm, crowded at the end of branches, obovate oblong or spathulate, glabrous. Cymes – 3- flowered, born from above the leaf scars on the tubercles. Capsules are glabrous, trigonous, seeds globose, dorsally lined and smooth. Flowering and fruiting period is March to July (Khare, 2007; Patil, 2003). After the collection of the plant, the root was removed; the aerial part was washed thoroughly in tap water and dried in shade for about 10 days under controlled temperature (25±2°C), powdered and passed through a 40-mesh sieve and stored in a well closed container for further use. Foreign matter, loss on drying, ash value and extractive values were determined as per the standard procedures (Anonymous, 1996; Anonymous, 1998). Coarsely powdered dried aerial plant (1.1 kg) was successively soxhlated using petroleum ether, chloroform, ethyl acetate and methanol for 72 h at room temperature respectively. The extracts were filtered, and the solvents evaporated to dryness under reduced pressure in an Eyela rotary evaporator at 40 to 45°C. The percentage yield was noted as 1.28 for petroleum ether, 2.68 for chloroform, 5.91 for ethyl acetate and 7.83 for methanol (Table 3).

Analysis of Fluorescence Pharmacognostic Characters

Fluorescence analysis was carried out with powders prepared from shade dried plants as well as in petroleum ether, chloroform, ethyl acetate and methanol extracts as described by Thomas *et al*. (2008). The powders were treated separately with 1N aqueous NaOH, 1N ethonolic NaOH, 1 N H2SO4 and 1N HNO3. The supernatants were examined under ultraviolet light and ordinary day light. Pharmacognostic characters of *Euphorbia nivulea* were analyzed by employing standard method as described in Pharmacopeia of India (Anonymous, 1996).

Phytochemical Screening

Phytochemical screening was carried out to assess the qualitative chemical composition of crude extracts using commonly employed precipitation and coloration to identify the major natural chemical groups such as steroids, reducing sugars, alkaloids, phenolic compounds, saponins, tannins, flavonoids, amino acids, anthrquinone, cardiac and iridoide glycosides (Harborne 1998; Kokate 2001). General reactions in this analysis revealed the presence of these compounds in the crude extracts tested by Brindha *et al.,* 1981). Crude extracts

in various solvents were prepared and stored in a refrigerator were used for the phytochemical tests.

Test Organisms

Gram positive bacteria, *Micrococcus luteus* NCIM 2169, *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063 and Gram negative bacteria; *Escherichia coli* NCIM 2065, *Salmonella paratyphi* NCIM 2501, *Pseudomonas aeroginosa* NCIM 2200, *Klebsiella pneumonia* NCIM 2707, were used as test organisms. The strains were obtained from Nation Collection of Industrial Microorganism, Pune. The fungal strains *Aspergillus fumigates* MTCC1811, *Aspergillus niger* NCIM 1207 *Monococcus purpura* MTCC 1090 and the yeast *Candida albicans* MTCC 3100, were collected from Microbial type culture collection, Chandigarh.

Preparation of Culture Media

Dehydrated media were purchased from Hi-Media Laboratories Ltd, India. All the media were prepared in sterile glass petri plates (4 mm thickness) according to the manufacturer's instructions. Chloramphenicol (10 µg/ml) and Griseofulvin (25 µg/ml) was used as standard drug for comparison of anti-bacterial and anti-fungal activity respectively. DMSO was used as a solvent.

Determination of antimicrobial activities of extract

The anti-microbial activities of petroleum ether, chloroform, ethyl acetate and methanol extracts were determined by agar well diffusion method (Hufford *et al.,* 1975). All bacterial and fungal strains were grown in nutrient broth (NB) and Sabouraud dextrose broth (SDB) for 4-6 hours at specified temperatures. The turbidity of the broth culture was adjusted to 0.5 McFarland units. This gives a suspension containing approximately 1-2 x 106cfu/ml (Mackie and Mac Cartney, 1996). An aliquot of microbial culture was added to agar medium at 45°C and poured into the petri plate. After solidification of the agar, medium was punched with a sterile corkborer (5.0 mm diameter) to cut uniform wells. Different concentrations of the extracts (125, 250 and 500 µg/ml) were prepared using DMSO as solvent and added to the wells. Bacterial cultures were incubated at 37°C for 24 hours and fungal cultures at 25°C for 48 hours. Anti-microbial activity was determined by measuring the zone of inhibition surrounding the well. The zones of inhibition were then measured, recorded and compared with positive standard controls, Chloramphenicol (10 µg/ml) and Griseofulvin (25 µg/ml) for anti-bacterial and anti-fungal activity respectively. The assays were carried out under aseptic conditions. DMSO was used as a negative control.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the extracts was determined by micro broth dilution method (Eloff, 1999; McGaw*et al*., 2002; Rabe *et al*., 2002; Eloff *et al*., 2005). For MIC, four-fold serial dilutions of the extracts were prepared (15.625, 31.25, 62.5 and 125 µg/ml) in microtire wells. Incubation of the microtire plates was carried out at 37oC for 24 hours for bacteria and at 25°C for 48 hours for fungi. After incubation, microtire wells were observed for any visible growth. The bacterial suspensions were used as positive control and extracts in broth were used as negative control. The MIC was interpreted as the lowest concentration of the extract that did not show any visible growth when compared to control tubes.

RESULTS

The results of quality control evaluation of aerial parts of the plant of *Euphorbia nivulea* were presented in Table 1 and were helpful in evaluating the pharmacognostic value of the medicinal plant. The results of fluorescence analysis of the powder and UV range have been shown in Table 2. The Loss on drying, total ash, acid insoluble ash, water soluble ash contents were found to be 3.4%, 31.4 %, 3.82% and 19.3 % for crude plant materials respectively. The percentage yield was noted as 1.22 for petroleum ether, 4.66 for chloroform, 5.77 for ethyl acetate and 7.46 for methanol (Table 3). Higher extractive value was found in methanol extract when compared to other solvents.

Table 1: Quality Control Evaluation of Aerial Parts of the Plant of *Euphorbia nivulea* Buch-Ham

Quality Control Parameters	Amount in Percentage
Foreign matter	3.4%
Loss on drying	5.8%
Total ash	31.8%
Acid insoluble ash	3.82%
Water soluble ash	19.3%
Ethanol soluble extractive value	5.28%
Water soluble extractive value	22.62%

Table 2: Analysis of Fluorescence Characters of Powder *Euphorbia nivulea* Buch-Ham

Sl.No	Treatment category	Under Ordinary Day	Light Under UV Light
1	Powder as such	Greenish brown colour	Greenishyellowfluorescence
2	Powder + 1N NaOH (aqueous)	No colour change	No colour change
3	Powder + 1N NaOH (alcoholic)	Light yellow colour	Yellowish green fluorescence
4	Powder + 1N HCl	Dark brown colour	Green fluorescence
5	Powder + H_2SO_4(1:1)	Black colour	Green fluorescence
6	Powder + HNO_3(1:1)	Yellowish brown	yellowish fluorescence
7	Powder + Ammonia (1:1)	No colour change	Green fluorescence
8	Powder + Iodine (1:1)	No colour change	No fluorescence
9	Powder + 5% $FeCl_2$(1:1)	Dark brown colour	Brown fluorescence
10	Powder + Acetic acid	Brown colour	No fluorescence
11	Petroleum ether extract 60°-80 °C	Green colour	Green fluorescence
12	Chloroform extract	Dark green colour	Green fluorescence
13	Ethyl acetate extract	Greenish colour	Light yellowish fluorescence
14	Methanol extract	Greenish brown	Brown fluorescence

Table 3: Extract Value of *Euphorbia nivulea* Buch-Ham

Name of the Extract	Percentage of Extractive Value
Petroleum ether 60°-80°C	1.28%
Chloroform	2.68%
Ethyl acetate	5.91%
Methanol	7.83%

Phytochemical Screening

The phyto chemical screening of aerial part of the plant different extracts revealed petroleum ether (60-80˚C), chloroform, ethyl acetate and ethanol extracts of aerial plant of *Euphorbia nivulia* were investigated for the presence of phytoconstituents by characteristic chemical test. Petroleum ether (60-80˚C) and chloroform extracts produced pink color on reaction with tin and thionyl chloride indicating the presence of terpenoids. Libermann-Burchard reaction petroleum ether (60-80˚C) and chloroform extracts produced reddish-blue color in the chloroform layer and green fluorescence in acid layer, suggesting the presence of phytosterols. Salkowski reaction produced reddish violet ring at the junction of the two layers revealed the presence of triterpenoids in petroleum ether (60-80˚C) and chloroform extracts of *Euphorbia nivulia.* A blue colouration and greenish black colour resulting from the addition of 5% ferric chloride reagent to the filtrate of methanol and ethyl acetate extract indicated the presence of tannins and phenols respectively. A magenta color obtained in Shinod's test and yellow color precipitate obtained with 10% solution of lead acetate solution confirmed the presence flavonoids in ethyl acetate and methanol extract. The petroleum ether (60-80˚C), chloroform, ethyl acetate and methanol extracts of entire plant of *Euphorbia nivulia* does not produce any positive response in the presence of alkaloids, cardiac glycosides, anthroquinone glycosides, investigations revealed the presence of pharmacologically important bio-molecules such as flavonoids, tannins, terpenoids, amino acids and carbohydrates (Table. 4).

Table 4: Phytochemical Screening of Various Extracts of *Euphorbia nivulea* Buch-Ham

Plant Constituents Test/ Reagent used	Petroleum Ether (60-80˚C) extract	Chloroform Extract	Ethyl Acetate Extract	Methanol Extract
1.Alkaloids				
i) Mayer's test	–	–	–	–
ii) Dragendorff's test	–	–	–	–
iii) Hager's test	–	–	–	–
iv) Wagner's test	–	–	–	–
2. Phytosterols	+	+	–	–

Plant Constituents Test/ Reagent used	Petroleum Ether (60-80˚C) extract	Chloroform Extract	Ethyl Acetate Extract	Methanol Extract
Libermann-Burchard reaction				
Salkowski test	+	+	–	–
3.Test for Terpenoids				
Tin and Thionyl chloride test	+	+	–	–
4.Anthraquinone Glycosides				
i)Borntrager's test	–	–	–	–
ii) Baljet test	–	–	–	–
iii) Legal's test	–	–	–	–
5.Cardiac glycosides				
i)Keller Kiliani Test	–	–	–	–
ii)Antimony trichloride test	–	–	–	–
6.Test for Cyanogenetic Glycosides				
Sodium picrate test	–	–	–	–
8.Test for Flavonoids				
i) Ferric chloride test	–	–	+	+
ii) Shinod's test	–	–	+	+
iii) Zinc-HCl reduction test	–	–	+	+
iv) Lead acetate solution test	–	–	+	+
9. Amino acids				
i) Million's reagent	–	–	+	+
ii) Ninhydrin reagent	–	–	+	+
10.Carbohydrates	–	–	–	–
ii) Fehling's solution	–	–	–	–
iii)Benedict's reagent	–	–	–	–
11.Phenolic Compounds and Tannins				
Aqueous potassium dichromate solution	–	–	+	+
Lead acetate solution	–	–	+	+
12. Saponins				
i) Foam test	–	–	–	–
ii) Haemolytic test.	–	–	–	–
13.Fixed oils and fats				
i) Spot test	–	–	–	–
ii) Saponification test	–	–	–	–

Note: + Traces, ++ Positive, +++ Strongly positive, --- Absent.

Antimicrobial Activities

The results of the preliminary anti-microbial activities (zone of inhibition) are presented in Table 5. DMSO was used as the negative control which not showed any zone of inhibition against tested bacteria and fungi. All test strains of bacteria were found to be sensitive to Chloramphenicol and fungal strains were sensitive to Griseofulvin. Petroleum ether extract does not display any anti-microbial activity against tested micro-organism at all three tested concentration; whereas chloroform extract does not show activity against *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Aspergillus fumigatus*, *Monococcus purpura*, *Candida albicans* at all three tested concentration. Ethyl acetate and methanol extracts displayed anti-bacterial and anti-fungal activity against

all the tested bacteria and fungi at all three tested concentrations (125, 250 and 500 µg/ml). Against all the tested micro-organism methanol extract displayed better activity than ethyl acetate extract. The highest activity was observed at 500 µg/ml concentration of methanol. *Klebsiella pneumonia, Bacillus subtilis* and *Escherichia coli* show higher activity among the all the tested organism. *Candida albicans* show better activity for methanol extracts. Minimum inhibitory concentration (MIC) was tested for the ethyl acetate and methanol extracts of *Euphorbia nivulia* and the results are presented (Table 6).

Table 5: Anti- microbial Activity of Four Extracts of Aerial Part Extracts of *Euphorbia nivulea,* Buch-Ham

Bacteria Gram (-) /(+)	Extract Concen-tration(µg/ml)	Growth inhibition zone diameter(mm) Peroleum Ether	Chloro-form	Ethyl Acetate	Methanol	Antibiotic Control (Chloramph-enicol 10 µg/ml)	Fungal Control Griseofulvin 25 µg/ml
S. aureus (+)	125	--	--	--	8	22	
	250	--	--	--	9		
	500	--	--	--	13		
M.luteus (+)	125	--	--	--	8	20	
	250	--	--	--	8		
	500	--	--	7	13		
B.subtilis (+)	125	--	--	10	13	19	
	250	--	--	12	15		
	500	--	--	15	17		
E.coli (-)	125	--	--	--	--	23	
	250	--	--	8	10		
	500	--	--	9	13		
S.paratyphi (-)	125	--	--	--	7	20	
	250	--	--	8	9		
	500	--	--	9	12		
P.aeroginosa (-)	125	--	--	--	9	22	
	250	--	--	10	12		
	500	--	8	11	14		
K.pneumonia (-)	125	--	--	10	15	24	
	250	--	--	13	19		
	500	--	10	15	22		--
A. fumigates	125	--	--	8	10	--	21
	250		--	10	13	--	23
	500		9	12	17	--	
A.niger	125	--	--	11	15	--	22
	250	--	--	14	17	--	
	500	--	8	16	19	--	
M. purpura	125	--	--	8	14	--	23
	250	--	--	10	16	--	
	500	--	8	14	18	--	
C. albicans	125	--	--	9	13	--	20
	250	--	--	13	16	--	--
	500	--	6	15	19	--	--

Table 6: Minimum Inhibitory Concentration (in µg/ml) of Ethyl Acetate and Methanol Extracts of Aerial Part Extracts of *Euphorbia nivulea* Buch-Ham

Microorganism	Ethyl acetate extract	Methanol Extract
S. aureus	500	250
M. luteus	500	250
B. subtilis	500	125
E.coli	500	125
S. paratyphi	500	250
P. aeruginosa	500	250
K. pneumonia	250	125
A. fumigates	500	250
A. niger	500	250
M. purpura	500	250
C. albicans	250	125

DISCUSSION

The anti-microbial potentials of substances are useful tools in the control of various infections caused by micro-organisms especially fungal infections. Antibiotic resistance is a major concern and development of new agents from plants could be useful in meeting the demand for new anti-microbial agents with improved safety and efficacy (Srivastava *et al.,* 2000). Newer anti-microbials from plant extracts may be useful in many (food, dairy and pharmaceutical) industries to prevent contamination by limiting the microbial growth. In this study, it was found that the ethyl acetate and methanol extracts of aerial parts of *Euphorbia nivulea* exhibited highest anti-microbial activity than the petroleum ether and chloroform extracts. The difference in the anti-microbial efficacy could be due to the presence of variable phytochemical compounds in different extracts. The bacterial organisms found in this study to be susceptible include *E. coli*, *S. Aureus* and *B. subtilis* which have been implicated in many systemic infections such as respiratory and genitourinary tract infections. The phytochemical screening of aerial part of the plant different extracts revealed the presence of phytosterols and fats and fixed oils in petroleum ether extract, slight reaction for alkaloids in chloroform extract, gave positive results for flavonoids, phenolic acids and tannins in ethyl acetate and methanol extracts. The phytochemical screening of this plant showed the presence of flavonoids in both of the ethyl acetate and methanol extracts which have been shown to possess anti-microbial properties (Hostettman *et al*., 1995; Oboh *et al*., 1998). Flavonoids are known for their anti-inflammatory, anti-arthritic and anti-microbial properties (Trease and Evans, 1989). Therefore, the anti-microbial activities of this plant may be ascribed to the presence of flavonoids. These results support the popular ethno pharmacological use of this plant for the treatment of a scaly fungi infection. Bioassay directed fractionation of the most active extract is in progress to isolate and identify the compounds responsible for the anti-microbial activity.

CONCLUSION

Crude extracts from *Euphorbia nivulea* have medicinal applications from ancient days and very little work has been done on the biological activity and credible medicinal applications of isolated compounds of *Euphorbia nivulea*. From the study it was found that ethyl acetate and methanol extracts exhibited remarkable anti-microbial activity against the tested micro-organism. The good quality activity may be attributed to the presence of flavonoids and phenolic compounds in these extracts.

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Received:06.08.18, Accepted: 08.09.18, Published:01.10.2018

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