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Research Article

Phytochemical Screening and *In Vitro* Bioactivities of the Extracts of Aerial Part of *Evolvulus nummularius*

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ABSTRACT
Objective: To investigate in vitro bioactivities of methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous extracts of *E. nummularius* and its phytochemical analysis. Methods: The antioxidant activity was determined by measuring the scavenging activity of DPPH radicals and H_2O_2. Cytotoxic activity was tested against hep G2 cell lines using MTT assay. Antimicrobial activity was assessed using the disc diffusion assays. The haemolytic activity was determined using agar diffusion techniques on blood agar plate, thrombolytic activity by clot disruption. Standard phytochemical screening tests for saponins, tannins, terepenoids, flavonoids, alkaloids and glycosides were also conducted. Results: Among the different extracts tested, ethyl acetate extract of *E. nummularius* showed potential in vitro bioactivities and phytochemical constituents. Conclusions: Further work will be carried out to find biologically active compounds that may serve as leads in the development of new pharmaceuticals.

Keywords: *Evolvulus nummularius*, antioxidant, antimicrobial, haemolytic, thrombolytic, cytotoxicity.

INTRODUCTION

In recent years the use of plants in the management and treatment of diseases has gained considerable importance. Plants and fruits are considered as one of the main sources of biologically active compounds. An estimate of the world Health Organization (WHO) states that around 85-90% of the world's population consumes trational herbal medicines [1]. *Evolvulus nummularius* (*E. nummularius*) belongs to the family Convolvulaceae (Morning-glory). *E. nummularius* is a low growing species with creeping stem and rounded leaves which are widely distributed in tropical and sub-tropical areas of India, Nepal, Bhutan, America and Africa. Pharmacological studies have demonstrated that *E. nummularius* known to possess antihelminthic activity [2], wound healing activity [3], poor sedative and anticonvulsant properties [4]. The methanolic extract of *E. nummularius* is reported to have antibacterial and antioxidant activities [5]. Not much work has been carried out on in vitro bioactivities of crude extract of *E. nummularius*. So the present investigations were carried out to study the phytoconstituents and in vitro antioxidant, thrombolytic, haemolytic, cytotoxic and antimicrobial activities of methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous extracts of *E. nummularius*.

MATERIALS AND METHODS

Plant Collection and identification

The whole plants of *E. nummularius* were collected from the Anna University campus, Tamilnadu in India. The plant material was identified and authenticated by plant taxonomist, Dr. G. V. S. Murthy, Botanical Survey of India, Southern Regional Centre, Coimbatore.

Preparation of Extract

Fresh plant material was washed under running tap water and then the leaves were removed carefully. The whole plants were air dried and then homogenized to fine powder. 200 g of powder was successively extracted with six different solvents (methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous extracts). The extraction process was carried out using Soxhlet apparatus for 72 hrs. The yield was found to be 8, 11, 9, 9.5, 8, 5 w/v, respectively.

Phytochemical screening

The freshly prepared crude extracts of *E. nummularius* were qualitatively tested for the presence of Alkaloids (Mayer's test), Flavonoids (NaOH or Alkaline reagent test), Carbohydrates (Molish's test, Fehlings's test and Benedict's test), Glycosides (Borntragar's test), Saponins (foam test), Fixed oils (spot test), Proteins (Millon's test), Tannins ($Fecl_3$ test), Terpenoids (Salkowski test), Cardinolides ($Fecl_3$ test), Anthraquinones (Ammonia test), Phenols ($Fecl_3$ test, Lead acetate test) [6-9].

DPPH radical scavenging activity

Free radical scavenging activity of *E. nummularius* extracts, based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Braca et al [10]. Leaves extract

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Table 1: Results of phytochemical screening of *E. nummularius* extracts

Phytochemical tests	Methanol	Ethyl acetate	Aqueous	Ethanol	Chloroform	Hexane
Alkaloids	+	+	-	-	+	+
Flavonoids	+	+	-	+	+	+
Carbohydrates	+	+	+	-	+	+
Glycosides	-	+	-	+	+	+
Saponins	+	+	-	-	-	+
Fixed oils	+	+	-	-	+	-
Proteins	+	+	+	-	-	-
Tannins	-	+	-	+	+	+
Terepenoids	-	+	+	+	+	+
Cardinolides	+	+	+	-	+	+
Anthraquinones	+	+	+	+	+	+
Phenolic compounds	+	+	+	+	+	+

+ : indicate presence; - : indicate absence

Table 2: DPPH radical scavenging activity of *E. nummularius* extracts with different concentration

Percentage of DPPH scavenging activity						
Conc. (µg/mL)	Methanol	Ethyl acetate	Aqueous	Ethanol	Chloroform	Hexane
20	31±0.2	33±0.4	10±0.1	27±0.02	24±0.01	30±0.03
40	37±0.2	44±0.2	31±0.2	32±0.17	31±0.21	38±0.02
60	50±0.2	58±0.0	29±0.3	39±0.11	40±0.17	47±0.12
80	57±0.1	67±0.0	48±0.6	49±0.2	51±0.13	53±0.17
100	65±0.4	78±0.7	56±0.2	62±0.11	67±0.21	60±0.21
IC_{50}	66	54	85	77	73	72

Values are expressed as mean±SD (n=3)

Table 3: H_2O_2 radical scavenging activity of *E. nummularius* extracts with different concentration

Percentage of H_2O_2 scavenging activity						
Conc. (µg/mL)	Methanol	Ethyl acetate	Aqueous	Ethanol	Chloroform	Hexane
20	2±0.2	11±0.4	2±0.1	7±0.12	4±0.01	4±0.17
40	13±0.2	21±0.2	9±0.2	19±0.13	11±0.21	15±0.28
60	28±0.2	35±0.0	17±0.3	26±0..1	17±0.22	28±0.27
80	34±0.1	46±0.0	31±0.6	34±0.21	28±0.28	33±0.16
100	45±0.4	56±0.7	39±0.2	40±0.31	42±0.4	43±0.23
IC_{50}	111	88	131	120	130	115

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

Table 4: Haemolytic activity of *E. nummularius* extracts at different concentration

Extract	Zones of haemolysis (mm)				
	Concentration of extract (mg/mL)				
	20	40	60	80	100
Methanol	3±0.001	6±0.021	6±0.018	7±0.003	7±0.023
Ethyl acetate	6±0.011	8±0.012	8±0.017	8±0.018	8±0.35
Aqueous	4±0.01	4±0.003	6±0.017	6±0.024	6±0.25
Ethanol	6±0.02	6±0.02	6±0.23	7±0.01	7±0.42
Chloroform	6±0.05	6±0.23	7±0.01	7±0.24	8±0.11
Hexane	6±0.21	6±0.32	7±0.17	7±0.33	8±0.18

Values are represented as mean±SD of three observations in each experimental set.

was added to 3 mL of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 mins and the percentage inhibition activity was calculated by using the equation: % scavenging activity = [(A_0-

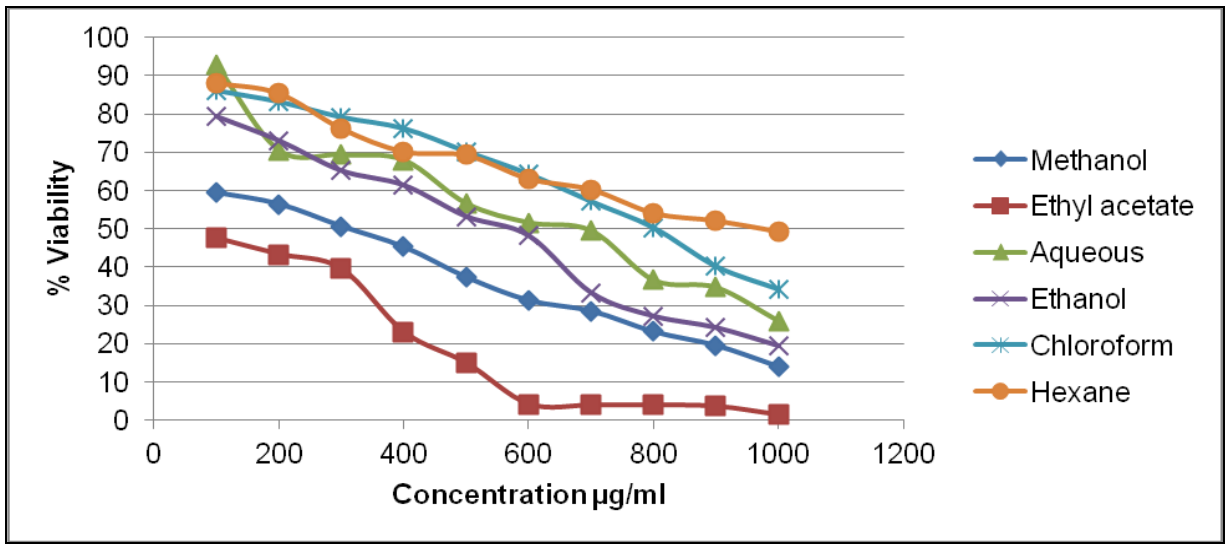

Figure 1: Cytotoxic activity of *E. nummularius* on Hep G2 cell line

Table 5: Effect of *E. nummularius* extracts on *in vitro* clot lysis

Extract	% of clot lysis				
	Concentration of crude extract (mg/mL)				
	20	40	60	80	100
Methanol	25±0.02	35±0.001	37±0.001	50±0.017	62±0.13
Ethyl acetate	16±0.017	38±0.028	50±0.012	66±0.14	69±0.18
Aqueous	12±0.02	18±0,02	23±0.001	45±0.067	53±0.032
Ethanol	26±0.01	35±0.03	41±0.02	53±0.12	58+0.18
Chloroform	22±0.03	28±0.12	35±0.41	43±0.11	47±0.14
Hexane	20±0.18	26±0.15	33±0.32	39±0.16	44±0.12

Values are represented as mean±SD of three observations in each experimental set.

Table 6: Antimicrobial activity of *E. nummularius* extracts (zone of inhibition in mm)

Microbial strains	Name of the extracts					
	Methanol	Ethyl acetate	Aqueous	Ethanol	Chloroform	Hexane
S. maltophilia	6±0.023	14±0.002	2±0.003	6±0.11	4±0.033	7±0.12
P. aeruginosa	14±0.01	16±0.003	8±0.017	3±0.17	9±0.12	8±0.03
P. aeruginosa	6±0.011	9±0.022	3±0.013	4±0.12	5±0.11	8±0.31
C. bacter	8±0.001	14±0.032	8±0.003	3±0.14	7±0.014	12±0.21
P. mirabilis	8±0.002	16±0.001	6±0.001	6±0.11	7±0.02	10±0.1
S. aureus	10±0.21	12±0.13	4±0.21	5±0.23	8±0.23	8±0.23
C. albicans	6±0.021	13±0.002	4±0.012	6±0.16	5±0.03	8±0.2
R. solani	7±0.18	12±0.17	2±0.11	3±0.21	2±0.11	6±0.12
A. fumigates	3±0.13	14±0.18	3±0.21	2±0.19	4±0.22	6±0.11

The results are expressed as mean±SD of three consecutive experiments.

$A_1)/A_0]$ x 100. Where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. Lower the absorbance, the higher is the free radical scavenging activity [11]. The curves were prepared and the IC_{50} value was calculated using linear regression analysis.

Hydrogen peroxide (H_2O_2) scavenging capacity

The ability of the extracts to scavenge H_2O_2 was determined according to method described by Ruch *et al* [12]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extract of selected concentrations (20-100 µg/mL) in distilled water were added to H_2O_2 solution (0.6 mL, 40 mM). The absorbance of H_2O_2 at 230 nm was determined after 10 mins against a blank solution containing phosphate buffer without H_2O_2. Ascorbic acid was used as a standard antioxidant. The % of H_2O_2 scavenging of both extracts and standard compounds were calculated. % scavenged $[H_2O_2]$ = $[(A_o-A_1)/A_o]$ x 100 where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract. The antioxidant activity of the extract was expressed in IC_{50} values.

In vitro cytotoxicity assay

The Hep G2 (human hepatocellular carcinoma) cell line was used to determine the cytotoxicity of the plants that showed a promising antimicrobial activity. The

cytotoxicity assays were performed according to the micro culture MTT method [13]. The cells were harvested (4.5-5.0 x 10^4 cells/well) and inoculated in 96 well microtiter plates. The cells were washed with phosphate buffered saline (PBS) and the cultured cells were then inoculated with *E. nummularius* extracts. After 72 hrs incubation, the medium is aspirated. 10 µL of MTT solution (5 mg/mL in PBS, pH 7.2) is added to each well and the plates are incubated for 4 hrs at 37 °C. After incubation, 100 µL of DMSO was added to the wells followed by gentle shaking to solubilise the formazan dye for 15 min. Absorbance was read at 570 nm and surviving cell fraction was calculated. The inhibition of cell viability was calculated by means of the formula: % inhibition = (1-absorbance of treated cells/absorbance of untreated cells) x100.

Haemolytic assay

The haemolytic activity of the extract was determined using agar diffusion technique on blood agar plate [14]. Blood agar was prepared and well measuring 5 mm were made on the agar using cork borer. The wells were filled with 20 µL of different concentration of plant extracts solution. The plates were then incubated at 37 ºC for 5 hrs.

Thrombolytic assay

Whole blood (6 mL) was collected from the healthy volunteers. Blood sample (1 mL) was distributed in pre weighed sterile micro centrifuge tubes and incubated at 37 ºC for 90 mins for clot formation. After clot formation, the serum was completely aspirated without disturbing the clot and the tubes were again weighed to determine the clot weight (clot weight = weight of the tube containing clot – weight of the empty tube). To the each Eppendorf tube containing pre weighed clot, 20 µL, 40 µL, 60 µL, 80 µL and 100 µL of extracts were added. 50 µL of sterile distilled water and streptokinase was used as a negative and positive control. All the tubes were incubated at 37 ºC for 18 hrs and observed for clot lysis. The fluid obtained after the incubation was removed carefully and the tubes were weighed again to observe the difference in weight after clot disruption [15].

Antibacterial activity

The test pathogens used for screening the efficacy of plant extracts were *Stenotrophomonas maltophilia* (*S maltophilia*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumonia* (*K. pneumoniae*), *Citrobacter* (*C. bacter*), *Proteus mirabilis* (*P. mirabilis*), *Staphylococcus aureus* (*S. aureus*), *Candida albicans* (*C. albicans*), *Rhizoctonia solani* (*R. solani*), and *Aspergillus fumigates* (*A. fumigates*).

The antimicrobial assay was carried out using agar well diffusion method. Cefotaxime and nystatin are used as reference drugs and the corresponding solvents (methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous) are used as positive controls. About 20 mL of Muller-Hinton agar medium for bacteria and potato dextrose agar for fungus was poured in the sterilized Petri dishes and allowed to solidify. The agar medium was spread with 24 hrs cultured of microbial strains by a sterilized rod. Wells of 6 mm in diameter were made in the culture medium using sterile cork borers. About 20 µL of the plant extracts (100 mg/mL) was added to the wells. Plates were then incubated at 37 °C for 24 hrs. Antimicrobial activity was evaluated by measuring the inhibition zone diameters in mm formed around the well. The assay was carried out in triplicates.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. The results were processed using Microsoft Excel 2007.

RESULTS

Phytochemical screening

Phytochemical evaluation of the various extracts of *E. nummularius* were done for the presence of Alkaloids, Flavonoids, Carbohydrates, Glycosides, Tannins, Terepenoids, Cardinolides, Anthraquinones, Phenolic compounds. The results are presented in Table 1.

DPPH radical inhibition activity

DPPH scavenging activities of methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous extracts of *E. nummularius* was shown in Table 2. Ascorbic acid used as a standard test solution. The IC_{50} value was 66, 54, 85, 77, 73, 72 and 42 µg/mL (methanol, ethyl acetate, chloroform, hexane, ethanol, aqueous extracts and ascorbic acids).

Hydrogen peroxide scavenging activity

The ability of *E. nummularius* extracts to scavenge hydrogen peroxide was shown in Table 3 and compared with that of ascorbic acids as standard and the highest IC_{50} was estimated as 131 µg.

Cytotoxicity

The results of cytotoxic activity of methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous extracts of *E. nummularius* on Hep G2 were represented in Figure 1.

Haemolytic activity

Haemolytic activities of *E. nummularius* (methanol, ethyl acetate, chloroform, hexane, ethanol and aqueous extracts) with different concentration were screened against normal human erythrocytes. Haemolytic activity of extracts is expressed in diameter of the zone of haemolysis (mm). Result indicated that methanol extract of *E. nummularius* (at dose 20 mg/mL) possess minimum haemolytic activity (3±0.001%) where as ethyl acetate extract (at dose 100 mg/mL) possess highest haemolytic activity (8±0.35) Haemolytic percentage was found to be increasing with increase in dose (Table 4).

Thrombolytic activity

Table 5 shows the thrombolytic activity of the *E. nummularius* extracts in various concentrations. The *in vitro* thrombolytic activity study revealed that methanol, ethyl acetate, aqueous, ethanol, chloroform, and hexane extract showed 62%, 69%, 53%, 58%, 47% and 44% clot lysis respectively for 100 mg/mL and compared with the negative control.

Antimicrobial activity

The antibacterial activity of plants is related to their zone of inhibition against the some of the pathogenic organisms. The ethyl acetate extraction of *E.*

nummularius exhibited potent antimicrobial activity towards all the microbes. The zones of inhibition values are presented in Table 6.

DISCUSSION

Medicinal plants were of great importance to the health of individuals and communities [16]. Phytochemical analysis conducted on the plant leaves extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities [17]. Analysis of the plant extracts revealed the presence of phytochemicals, such as proteins, carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, terepenoids and alkaloids. Several studies have described the antioxidant properties of different parts of various medicinal plants which are rich in phenolic compounds [18, 19]. In this present study, preliminary phytochemical analysis revealed a large amount of proteins, carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, terepenoids and alkaloids present in ethyl acetate extract of *E. nummularius* among all the extracts.

Based on the scavenging capacity of the free radicals (DPPH, H_2O_2), the highest antioxidant activity was found in ethyl acetate extract of *E. nummularius.* This is due to the presence of rich flavonoids, which are polyphenolic compounds [20, 21] in the ethyl acetate extract.

The antimicrobial activities of the six extracts were evaluated against gram positive, gram negative and fungi. The microbial studies of the extracts showed the most promising antimicrobial properties indicating the potential for the discovery of novel drug from plants. Extracts contain phenols and terepenoids were shown to be more efficient in the antibacterial efficacy than the other extracts. The order of the antimicrobial efficacy is ethyl acetate extract> Methanol extract> Hexane extract> Chloroform extract> aqueous extract> Ethanol extract. The result clearly shows that phenols, alkaloids and flavonoids which were abundantly found in ethyl acetate, methanol and hexane extracts were responsible for the antimicrobial activity of *E. nummularius*.

Since any compound or extract can exert antimicrobial and antioxidant activity as a result of its toxic effects on the cells, determination of the toxic effect of antimicrobial and antioxidant agents on host cell is mandatory [22]. For this purpose, cytotoxicity assay was performed. The present study observed that the ethyl acetate of *E. nummularius* inhibits the proliferation of Hep G2 cell line. The cytotoxicity effect was highest with increase in concentration. The cytotoxicity was concentration dependent and cell line specific. This clearly indicates the presence of potent bioactive principles in the extracts that might be useful as antiproliferative and antitumor agents [23]. Although the mechanisms of the action have not been elucidated, it was understood that the extract contains flavonoids and antioxidant polyphenolic compounds [24]. These compounds are known to scavenge the formation of free radicals, and have great potential in ameliorating cancer cells.

The ethyl acetate extract of *E. nummularius* shows maximum haemolytic activity compare to all the other solvent extracts. The activity of the extract to lyse the blood cell can be linked with bioactive components. It is well documented that flavonoids and the polyphenolic compounds which showed potential beneficial effects on human health and posses antiviral, anti-inflammatory, antitumor, antihaemolytic and antioxidative activity [25]. The zone of haemolysis was directly proportional to concentration of the extract. In this study, Ethyl acetate extract was found to provide the maximum thrombolytic activity. The phytochemical constituents present in the ethyl acetate extract of *E. nummularius* could participate for its clot lysis activity [26].

CONCLUSION

Based on our findings we conclude that *E. nummularius* have significant amount of phytochemical compounds. Ethyl acetate extract of *E. nummularius* has shown higher *in vitro* antioxidant, antibacterial activity, cytotoxicity, haemolytic activity, thrombolytic activity compared to other extracts. Further work will be carried out to find biologically active compounds like phenolics, flavonoids and alkaloids etc., of pharmaceutical importance through LC-MS/MS and NMR studies.

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