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In vitro Antioxidant and Antibacterial Activity of Aqueous and Methanolic Extract of *Mollugo nudicaulis* Lam. Leaves

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ABSTRACT

Objective: To investigate *invitro* antioxidant activity, antibacterial activity and phytochemical screening of the aqueous and methanolic extract of *Mollugo nudicaulis* leaves (MN). **Methods:** The total phenolic and flavonoids content was quantified by Folin-Ciocalteu and Aluminum chloride method respectively. *Invitro* antioxidant activity was carried out by DPPH, ABTS and reducing power assays and antimicrobial activity was carried out by disc diffusion method. **Results:** The phytochemical tests of *Mollugo nudicaulis* revealed the presence of alkaloid, steroids, flavonoids and reducing sugar in the both aqueous and methanolic extracts. Terpenoids were absent in both aqueous and methanolic extract of *Mollugo nudicaulis*. The total phenolics content of the methanolic and aqueous extract of leaves was 47.01 ± 0.8 and 46.4 ± 0.05 mg/100 g. The total flavonoid content was 41.3 ± 0.04 and 36.2 ± 0.01 mg/100 g respectively. The methanolic and aqueous extract of leaves showed IC_{50} values of DPPH radical scavenging as 48 and 190 μg/ml respectively. The IC_{50} values of ABTS radical scavenging for methanolic of aqueous extracts was 83 and 198.3 μg/ml of plant extract respectively. The total phenolics and flavonoids content and *invitro* antioxidant activity of methanolic extract was higher compared with aqueous extract. The methanolic extract of *Mollugo nudicaulis* used to determine antibacterial activity against bacterial species namely Pseudomonas aeruginosa, Proteus sp, Streptococcus sp, Entrobacter sp. **Conclusion:** This investigation suggests that the methanolic extracts of *Mollugo nudicaulis* possess potential antioxidant and antibacterial compounds.

1. Introduction

Free radicals are produced by exogenous and endogenous factors in the human body. The most common reactive oxygen species (ROS) includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO) and nitric oxide (NO). ROS play an important role in cell metabolism including energy production, phagocytosis and intercellular signaling. These ROS produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a role in wide variety of metabolic diseases such as DNA damage, carcinogenesis and various degenerative disorders such as cardiovascular diseases, aging and neuro-degenerative diseases, atherosclerosis and rheumatoid arthritis.

Antioxidants are the compounds which has the ability to trap free radicals. Antioxidant compounds could be either synthetic (BHA and BHT etc) or natural (plant secondary metabolites such as polyphenols and flavonoids). Antioxidants scavenge the free radicals, such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases[1, 2]. Recent decades, the interest has increased considerably in finding naturally occurring antioxidants in foods or medicinal plants to replace synthetic antioxidants, which are being restricted due to their side effects such as inflammation and carcinogenicity etc. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases. Many of the previous literatures show large number of plants that can be used against diseases, in which reactive oxygen species and free radical play important role[3]. Medicinal plants are playing an important role in both antioxidant and antimicrobial activities[4- 6]. Aromatic and medicinal plants are known to

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produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth[7, 8]. Many studies reported the activities of spices and herbs on food borne pathogenic microorganisms[9, 10]. In the recent investigation, Anjali Rawani *et al*[11] reported that the aqueous, chloroform and methanolic extracts of *Alternanthera philoxeroides*, *Plumeria obtusa*, Polyalthia cerasoides and Ixora acuminate showed inhibition against human pathogens.

Mollugo nudicaulis belongs to the family Molluginaceae which is prevalent commonly during the rainy season. *Mollugo nudicaulis* is traditionally used to treat whooping cough and jaundice. The ethanolic extract of MN shown anti-diabetic activity against alloxan induced diabetics in female albino rats[12]. The literature survey revealed that so far no scientific studies carried out on antioxidant and antimicrobial capabilities of *Mollugo nudicaulis* leaves. Hence, in the present study, we focused to evaluate the total phenolics and flavonoids content, *invitro* anti oxidant and antibacterial ability of *Mollugo nudicaulis*.

2. Materials and methods

2.1. Chemicals and Reagents

Butylated Hydroxyl Toluene (BHT), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Trichloroacetic acid (TCA), Ortho phosphoric acid, Magnesium metal strips, Dragendorff′s reagent, methanol, Gallic acid, Rutin, Chloroform, Sulphuric acid, Folin-Ciocalteu reagent, Sodium carbonate, Aluminium chloride, Potassium acetate, Phosphate buffer, Potassium ferricyanide, Hydrochloric acid, Ferric chloride, Potassium persulphate, Hydrogen peroxide, Glacial acetic acid, Ferrous ammonium sulphate. Nutrient broth and Nutrient agar was purchased from Himedia, India. All other chemicals used were of analytical grade.

2.2. Plant material

Leaves of MN were collected during February, 2010 from Bharathidasan University campus, Tamil Nadu in India. The plant material was authenticated by Department of Plant Science, Bharathidasan University, Tiruchirappalli. The leaves were picked and washed with water to remove dust particles and shade dried (25℃ ± 2 for 14 days). The dried leaves were then ground to a fine powder in a mechanical blender.

2.3. Methanolic extract

Five grams of powder was extracted with 100 ml of 99% methanol using a Soxhlet apparatus[13]. The solvent was evaporated under reduced pressure at 45 ℃ using rotary evaporator (Buchi R-210, Germany). The dried extract obtained was stored in desiccator at - 20℃ until further use.

2.4. Aqueous extract

The powdered plant material (5 g) was extracted in distilled water (250 ml; 25℃) on shaker for 48 hours. The extract was filtered through Whatman No.1 filter paper using a Buchner funnel. The filtrate of aqueous extract obtained was quickly frozen at - 50℃ and dried for 48 h using a vacuum freeze dryer (CHRIST ALPHA, German) to give a yield 6.75% of dry extract. The resulting extract was reconstituted with distilled water to give desired concentration and used for further analysis.

2.5. Phytochemical screening

The dry extract was used for the phytochemical screening of compounds which include flavonoids, alkaloids, saponins, terpenoids, steroids and reducing sugar in accordance with[14] Harborne protocol. To 1 ml of the extract, 5 ml of Benedict′s solution was added and kept in boiling water bath. Red, yellow or green precipitate indicates the presence of reducing sugars. About 0.5 g of the extract was dissolved in 3 ml of chloroform and filtered. Concentrated H2SO4 was carefully added to the filtrate to form lower layer. A reddish brown color at the interface was taken as positive for steroid ring. Two ml aliquot of the extract was treated with Dragederoff′s reagent to test the presence or absence of alkaloids. About 1 ml of alcoholic extract was diluted separately with 20 mL of distilled water and shaken in a graduated cylinder for 15 minutes. A one cm layer of foam indicated the presence of saponins. About 0.2 g of the extract was dissolved in 2 mL of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange coloration was indicative of presence of flavonoids. To 1 ml of extract, 2 ml of Tricholoroacetic acid (TCA) was added and the formation of yellow to red precipitate shows the presence of terpenoids.

2.6. Determination of total phenolics

Total phenolic content of aqueous and methanolic extracts of MN were determined by the modified Folin-Ciocalteu method[15]. 100 μL of Folin-Ciocalteu reagent and 200 μL of Na_2CO_3 (2% w/v) was added to 100 μL of plant extract solution (1 mg/ml). The resulting mixture was incubated at 45℃ with shaking at 120 rpm for 15 min. The absorbances of the samples were measured at 765 nm using uv- visible spectrophotometer. Results were expressed as milligrams of gallic acid equivalent/g of plant extract. The same procedure was used for making standard curve using gallic acid and concentration range of 0-10 μg/ml was taken. All

experiments were carried out in triplicates.

2.7. Estimation of total flavonoids

Total flavonoids content was determined using for Aluminum chloride method[16]. One ml of sample (1 mg/mL) was mixed with 3 mL of methanol, 0.2 ml of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water and kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm using UV-Visible spectrophotometer. The total flavonoid content was determined from calibration curve made by rutin as standard (0-100 μg/mL in methanol). The concentration of total flavonoids was expressed as mg of rutin equivalent/g of plant extract.

2.8. ABTS scavenging activity

The method of Re *et al*.[17] was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM potassium persulphate solution in equal amount and allowed to react for 12 h at room temperature in the dark. 1 ml of the resulting solutions was allowed to react with 1ml of the plant extract with different concentration ranging from 50 to 250 μg/mL and the reaction mixture was vortexed and absorbance was measured at 734 nm after 6 min interval. The same was done for the BHT standard of various concentrations. The percentage ABTS scavenging activity of plant extract was calculated and compared with Butylated hydroxyltoluene (BHT). The percentage of inhibition capacity of ABTS by the plant extract was calculated from the following equation;

ABTS Scavenging activity (%) = [(A control-A sample)] / (A control)] ×100

Where A control is the absorbance of ABTS + methanol; A sample is the absorbance of ABTS radical + sample (i.e. standard or extract).

2.9. DPPH scavenging activity

The scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to method reported before[18]. One ml of 0.135 mM DPPH prepared in methanolic was mixed with 1.0 ml of aqueous extract with various concentrations ranging from 50-250 μg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The percentage of inhibition activity of DPPH by the extract was calculated from the above mentioned equation.

2.10. Determination of reducing power

The reducing power was determined the following procedure described by[19, 20]. The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)6$ (1% w/v) was added to 1.0 ml of the extract dissolved in distilled water. The resulting mixture was incubated at 50℃ for 20 min, followed by the addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged at 5000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm against blank sample.

2.11. Antibacterial activity

The bacterial species used for this study are Pseudomonas aeruginosa, Proteus sp, Streptococcus sp and Entrobacter sp. The agar disc diffusion method was employed to determine the antibacterial activity of the methanolic leaf extract of MN. Disc-assay was found to be a simple, cheap and reproducible practical method[21]. The microorganisms were inoculated in the nutrient broth (10 ml) and incubated on a rotary shaker (12 hrs at 37℃). 0.2 ml of inoculum was poured into the molten Muller Hinton agar media in the petri plate. The test compound (methanolic extract of MN) of different concentrations ranging from 50, 100, 150 and 200 μg/6 mm disc was introduced into the well and the plates were incubated at 37 ℃ for 12 h. The diameters of the inhibition zones were measured in millimeters[22]. Gentamysin was used as positive reference standards to determine the sensitivity of each tested microbial species.

2.12. Statistical analysis

The experimental results were expressed as mean ± standard error of mean (SEM) of three replicates. The results were processed using Microsoft Excel 2007 and Origin 6.0.

3. Results

3.1. Phytochemical screening

The phytochemical screening (Table.1) of the aqueous extract of MN indicated the presence of alkaloids, steroids, flavonoids, and reducing sugars, whereas saponins, and terpenoids are absent in aqueous extract. The methanolic extract indicated the presence of alkaloids, steroids, flavonoids, reducing sugars and saponins, whereas terpenoids are absent. The total phenolics content of the methanolic and aqueous leaf extract was 47.01 ± 0.8 and 46.4 ± 0.05 mg/100g of gallic acid equivalent of plant extract with reference to gallic acid standard curve ($y = 0.25x+0.010$, $R^2 = 0.996$). The total flavonoid content of the methanolic and aqueous leaf extract was 41.3 ± 0.04 and 36.2 ± 0.01 mg/100g of rutin equivalent of plant extract respectively

Table 2.
Invitro Antibacterial activity of methanolic extract of *Mollugo nudicaulis*

Microorganism	Zone of inhibition (mm) Plant extract (μg/ml)				Gentamycin
	50	100	150	200	
Pseudomonas aeruginosa	10.1 ± 0.8	10.2 ± 1.1	13.5 ± 0.6	11 ± 1.3	15.2 ± 1.2
Proteus sp	4.5 ± 0.3	5.6 ± 0.2	9.3 ± 0.8	10.2 ± 0.7	14.2 ± 0.3
Streptococcus sp	12.1 ± 0.6	10.2 ± 0.5	11.8 ± 0.2	13.1 ± 0.4	19.5 ± 0.8
Enterobacter sp	12.2 ± 0.2	13.3 ± 0.3	12.4 ± 0.4	15.1 ± 0.5	17.2 ± 0.3

All results were expressed triplicate

with reference to rutin standard curve (y = 0.23x+0.018, R^2 = 0.973). These phytochemical compounds are known to be bioactive compounds and all play a role for antioxidant and antibacterial activities of MN extract.

Table 1.
phytochemical screening of *Mollugo nudicaulis*

Phytochemical Compounds	Aqueous Extract	Methanolic Extract
Alkaloids	+	+
Steroids	+	+
Flavonoids	+	+
Carbohydrates	+	+
Saponins	−	+
Terpenoids	−	−

++ = appreciable amount (positive within 1–2 mins.); + = moderate amount (positive within 2–5 mins; − = completely absent.

3.2. ABTS scavenging activity

The ABTS radical is produced by the reaction of potassium persulphate with ABTS under dark condition. The greenish blue ABTS radicals produced are spectrometrically measured at 734 nm. This method measures antioxidant capability of MN by estimating the percentage of ABTS radical scavenged based on dose dependent manner. Figure 1 depicts a steady increase in the ABTS radical scavenging capacity of *Mollugo nudicaulis* up to a concentration of 250 μg/ml. The IC50 value observed was 198.3, 190.6 and 37.6 μg/ml of AEMN (Aqueous Extract of *Mollugo nudicaulis*), MEMN (Methanolic Extract of *Mollugo nudicaulis*) and BHT respectively.

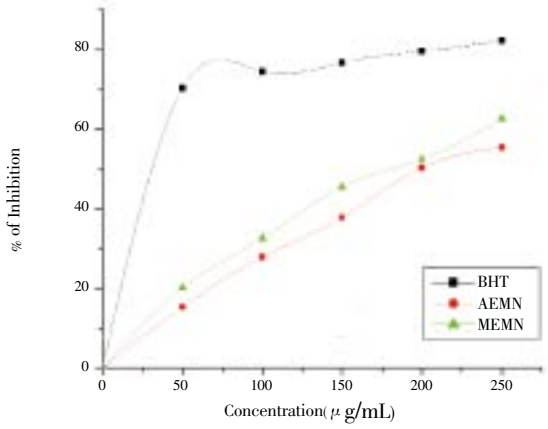

Figure 1. ABTS Radical scavenging activity of plant extract with different concentrations

3.3. DPPH radical inhibition activity

DPPH scavenging activity of aqueous and methanolic extract of MN shown in Figure 2. Commercial BHT is a reference sample. The IC50 value was 48.5, 83.4 and 20.2 μg/ml of methanolic, aqueous extract of MN and BHT respectively. Similarly Sreena *et al*[23], illustrated the dose response curve of DPPH radical scavenging activity of methanolic, ethanolic and ethyl acetate extracts of Morinda tinctoria leaves. The IC_{50} values of methanolic, ethanolic and ethyl acetate extracts of Morinda tinctoria were found to be 47 μg, 51 μg and 58 μg/ml respectively.

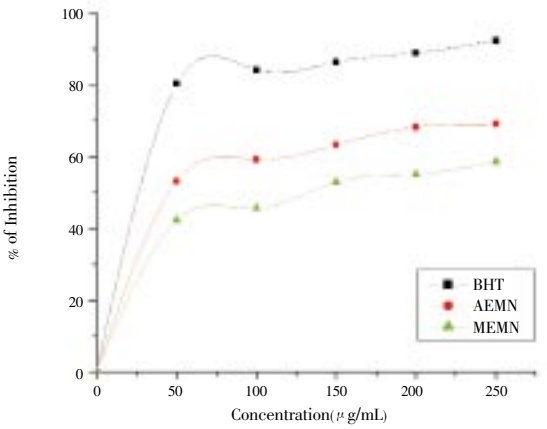

Figure 2. DPPH Radical scavenging activity of plant extract with different concentrations

3.4. Determination of reducing power

The reducing properties of antioxidants are generally associated with the presence of reductones, such as ascorbic acid and other secondary metabolites. Such reductones exert antioxidant action by breaking the free radical chain by donating hydrogen atoms; Reductones have also been reported to react with certain precursors of peroxide, thus preventing peroxide formation[24]. In the presence of antioxidants in the sample, would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the absorbance at 700 nm. In the present study, the reducing power of the methanolic extract of *Mollugo nudicaulis* was found to be steadily increased in direct proportion to

the increasing concentration of the extract (Figure 3). The reducing power of methanolic, aqueous extract of *Mollugo nudicaulis* and BHT at 250 μg/mL concentration was found to be 0.3156, 0.2982 and 0.8125 respectively.

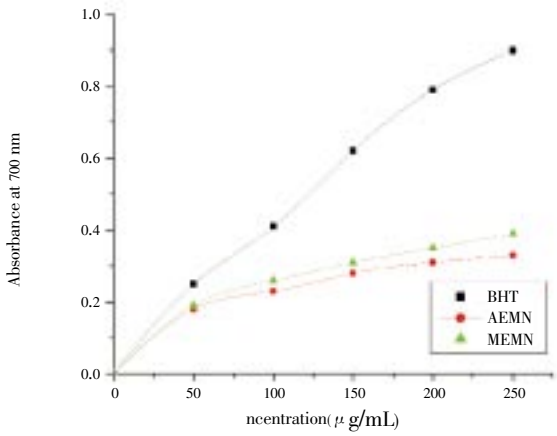

Figure 3. Reducing power activity of plant extract with different concentrations

3.5. Antibacterial activity

The antimicrobial activity of plants is related to their zone of inhibition against the some of the pathogenic organisms. The methanolic extraction of MN showed their activity against infectious bacterial species such as Pseudomonas aeruginosa, Proteus sp, Streptococcus sp and Entrobacter sp. Table.2 shows the zone of inhibition by the plant extract against Pseudomonas aeruginosa, Proteus sp, Streptococcus sp and Entrobacter sp. Gentamycin was used positive control since it is commonly used antibiotic against gram positive and gram negative bacterial sp. Methanolic extract of MN shown inhibition against all four selected pathogenic organism. The plant extract showed highest zone of inhibition to a distance of 15.1 ± 0.5 mm at 250 μg/ml concentration against Entrobacter sp. In the present study, the growth of all pathogenic bacteria was remarkably inhibited by methanolic extract of MN at 250 μg/ml concentration which was significantly similar to gentamycin.

4. Discussion

In this study, aqueous and methanolic extracts of MN were taken for analysis, methanolic extract exhibited higher antioxidant activities in all the three antioxidant assays performed (ABTS, DPPH and reducing power). The antioxidant activities of methanolic extracts from the leaves and stems of were assessed in various herbal plants in an effort to compare and validate the medicinal potential by quantifying phenolic, flavanoid contents and by antioxidant assays such as DPPH, ABTS and ferric reducing power assays[25, 26]. Our results indicate that presence of significant quantity of total phenolis and flavonoids and antioxidant activity in MN leaves. This makes us to interpret that MN leaves posses promising health beneficial phytochemicals. It is widely accepted that phenolic and flavonoids compounds may significantly contribute to overall antioxidant activities and also to antimicrobial activity. Ok-Hwan Lee and Boo-Yong Lee[27] have purified the phenolic compounds from olive leaves and tested them for antioxidant and antimicrobial activities. Methanolic extract of MN leaves showed antibacterial activity against all the four bacterial species tested such as Pseudomonas aeruginosa, Proteus sp, Streptococcus sp and Entrobacter sp. Similar studies were carried out in Merremia emarginata leaves extracts against *S. aureus*, *Staphylococcus epidermidis*, *E. coli*, and *P. aeruginosa* to test the antibacterial activity.[7]

Based on our findings we conclude that *Mollugo nudicaulis* have significant amount of total phenolics and flavonoids. Methanolic extract of *Mollugo nudicaulis* has shown higher in *invitro* antioxidant and antimicrobial activity compared to aqueous extract. 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.

Conflict of interest statement

We declare that we have no conflict of interest.

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