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EVALUATION OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF A MEDICINAL PLANT *HYDROCOTYLE SIBTHORPIODES* LAM., GROWN IN NE INDIA

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ABSTRACT: The present study was conducted to evaluate the antioxidant and antibacterial activities of methanol and aqueous extracts of whole plant of *Hydrocotyle sibthorpiodes* Lam. Antioxidant activity was measured using reducing power, hydrogen peroxide and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays. The antioxidant activity was expressed as percentage (%) of DPPH and H_2O_2 radicals' inhibition and IC_{50} value. Methanolic extract of the whole plant showed highest antioxidant activity each for DPPH (85.80±0.29) as well as H_2O_2 (64.7±0.173) compared to the aqueous extract with an IC_{50} value of 50.1µg/ml and 60.2 µg/ml respectively. Antibacterial activity was tested against two pathogenic strains by agar well diffusion method. The extracts were found to exert low to moderate antibacterial activity compared to Chloramphenicol which was taken as standard.

INTRODUCTION: Reactive oxygen species (ROS) which is a result of oxidative stress (an unbalance between pro-oxidants and antioxidant mechanisms) encompasses all highly reactive, oxygen-containing molecules, including free radicals such as the hydroxyl radical, hydrogen peroxide, the superoxide anion radical, nitric oxide radical, singlet oxygen, hypochlorite radical, and various lipid peroxides [29] and these can react with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules. Antioxidants are substances that can reduce damage of the cells due to oxygen, such as that caused by free radicals. Thus, antioxidant has been defined as a substance that when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate [8].

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It is an established fact that the plants are a good source of antioxidants. Plant antioxidants are widely used in dietary supplements. Plant antioxidants have also been explored for the prevention of diseases such as cancer and heart disease. Of late, screening of antioxidant potential has been carried out on a good number of plant species including several rare and endemic species found in North East (NE) India [9-13]. The plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched chemical diversity they can provide [13]. On the other hand, with the increase in the escalation of bacterial resistance against the chemotherapeutic agents particularly multi-drug resistance, the necessity for the search of plant based anti-microbial agents to combat the disease causing pathogens is seeking more attention. Since the medicinal plants have been used as traditional treatment for numerous human pathogens, the phytoconstituents that these plants possess can be used with acceptable therapeutic index for the development of novel drugs [14].

The use of plant extracts and phytochemicals, with established antimicrobial properties, could be of great significance in preventive and/or therapeutic approaches. The most important antimicrobial compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds [15, 16].

In view of the above, the present study has been made to investigate the antioxidant potential as well as the antimicrobial activities of a traditionally used medicinal plant species *Hydrocotyle sibthorpodes* using *in vitro* methods. *Hydrocotyle sibthorpiodes* (Apiaceae family), syn *Hydrocotyle rotundifolia* Roxb., a diffuse prostrate herb, is commonly found in the plain areas of Assam and other parts of North East India [17]. In North East India, the plant was reported to be used traditionally in the treatment of rheumatic troubles, skin diseases including syphilis and liver complaints [18]. It was also reported to have vermifuse, diuretic and emetic activities. In Taiwan, different species of *Hydrocotyle* were reported to be used for treating common cold, tonsillitis, cephalitis, enteritis, dysentery, zoster, eczema, period pain, hepatitis and Jaundice [19]. It was reported that the species of *Hydrocotyle* could inhibit the growth of transplanted tumors in mice, such as hepatic carcinoma (Hep), sarcoma (S180) and uterine cervical carcinoma (U14). Studies have also confirmed its immunomodulatory effects [20]. In a recent work, antioxidant and anti-proliferative activities of ethanol and water extracts of four *Hydrocotyle* species was evaluated which showed positive results.

MATERIALS AND METHODS: DPPH (2, 2-diphenyl-1-picryl-hydrazyl) and Hydrogen Peroxide (30%) were purchased from Sigma-Aldrich (USA).The bacterial strains were identified and collected from Downtown hospital (Assam, India). All other chemicals and solvents were of HPLC grade. De-ionized water was used throughout all the experiments.

The plant samples were collected locally from Panikhaiti area of Guwahati, Assam (India) in the months of May and June, 2015. The species was authenticated with the standard herbarium specimen in the Department of Botany, Gauhati University. The collected samples were washed thoroughly and the whole plant consisting of leaves, stem and root was dried under shade for one week and grinded into fine powder. The extraction was performed in Soxhlet apparatus. Dried plant material (10g) in filter paper was placed in the thimble holder of the apparatus and extracted with methanol for 5 hours.

The aqueous extract was prepared by dissolving the dried plant material (3 g) in 100 ml of distilled water. The mixture was boiled for 15 minutes at 100 ºC in a hot water bath, cooled and filtered through Whatmann No1 filter paper. The filtrate thus obtained was used for the analysis.

***In vitro* Antioxidant Assays:**

DPPH-radical scavenging assay: The antioxidant activity of methanol and aqueous extract of the plant material was assayed according to a published method with slight modification [22]. The commercially available DPPH (2, 2-diphenyl picryl hydrazyl) is a stable free radical, which is purple in colour. The antioxidant molecule present in the test extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow in colour.

For the preparation of the coloured reaction, the sample extract with the concentration of 1000 µg/ml was prepared by dissolving 20 mg of the extract in methanol or water and the final volume was made up to 20ml (the preparation was used as a stock solution). Then different concentration like 10 µg/ml, 20µg/ml, 40 µg/ml, 80 µg/ml and 160 µg/ml were prepared by diluting with methanol or water from the stock solution. The total volume was made 1ml in all the test tubes and to this; 2ml of methanolic solution of DPPH was added thereby making the final volume 3ml. The test tubes were incubated at 37 ºC for 30 minutes. The absorbance for the degree of discoloration of purple to yellow was measured at 517 nm against methanol as blank. Ascorbic acid was used as a standard.

The DPPH radical scavenging activity of the plant extracts were calculated by the formula:

$$\%\text{inhibition} = [A_0 - A_S]/A_0 \times 100$$

A_0-Absorbance of DPPH (unreduced)
A_S-Absorbance of the sample and standard

IC_{50} is defined as the concentration of substrate that causes 50% loss of the DPPH activity (color) [23].

Hydrogen Peroxide Scavenging Assay [20]: The solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM pH 7.4). Different concentration of the plant extracts (10 µg/ml-160 µg/ml) was added to 600 µl of H_2O_2 and the final volume was made 3ml with distilled water. The concentration of hydrogen peroxide was determined by absorption at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was taken as standard. IC_{50} value was calculated.

The percentage of hydrogen peroxide scavenging is calculated as follows:

$$\%\text{inhibition} = [A_0 - A_S]/A_0 \times 100$$

A_0- Absorbance of H_2O_2 (unreduced)
A_S- Absorbance of the sample and standard

Reducing Power Assay: Substances having the reducing potential react with Potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}) which then further react with Ferric Chloride to form a Ferric –Ferrous complex which has an absorbance maximum at 700 nm.

Preparation for obtaining the coloured solution was done according to a published method with minor modification [23]. Phosphate buffer was prepared by mixing Dibasic Sodium Phosphate (37.50 ml of 0.2 M) with 62.5 ml of Monobasic Sodium Phosphate and diluted to 100ml with distilled water. Different concentration of the plant extracts were mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5ml of 10mg/ml Potassium ferricyanide. The mixture was incubated at 50 ºC for 30 minutes and an aliquot of TCA (Tri-chloro acetic acid) was added to the mixture which was centrifuged at 3000 rpm for 10 minutes. Finally 2.5ml of the upper layer was mixed with 2.5 m of distilled water and 0.5 ml of 1mg/ml Ferric chloride. The absorbance resulting solution was measured at 700 nm. Ascorbic acid was used as a standard. Increasing absorbance of the reaction mixture indicates increase in reducing power assay.

***In vitro* Antimicrobial Assay:**

Culture and Maintenance of Test Organisms: Pure culture of all the experimental organisms was obtained from Institute of Microbial Technology (IMTECH), Chandigarh. The pure cultures were maintained on nutrient agar medium. Each culture was further maintained by sub culturing regularly on the same medium and stored at 4 ºC before use in the experiments. The test organisms taken for the study were *Staphylococcus aureus* (MTCC-96), *Klebsiella pneumonia* (MTCC 432).

The antimicrobial study was done with suitable modification by Agar Well Diffusion method 25. The Muller Hinton Agar plates were prepared by swabbing 100 µl of the 8 hrs old broth culture of the test organisms with sterile cotton swabs. Wells of 6mm diameter were made with sterile cork borer and were impregnated with 50µl of the plant extracts of 1mg/ml concentration. The wells were then allowed to dry for 1 hour in the laminar air flow cabinet for diffusion of the extracts. Chloramphenicol (1mg/ml) was taken as standard (positive control) for the study and the solvents used for dissolution as negative control. The plates were incubated at 37 ºC for 24 hrs and the diameter of the inhibition zone (mm) was measured. Triplicates were maintained for each organism.

The percentage inhibition of the bacterial growth is calculated as:

$$\%\ \text{inhibition} = \frac{\text{Inhibition shown by the extract}}{\text{Inhibition shown by standard}} \times 100$$

Statistical analysis: All the assays were performed in triplicates. The data's obtained were expressed as mean ± Standard deviation and analysis were done by calculating applied Student's t- test and one –way ANOVA at 95% confident level. The P-values $\leq$ 0.05 were considered statistically significant. The results were presented as the mean ± S.D.

RESULTS AND DISCUSSIONS: The relatively stable organic radical DPPH was scavenged by the antioxidants present in the plant extracts. The decrease in the absorption maxima (517 nm) of the protonated radical is due to the formation of the reduced DPPH which was indicated by change in colour of the solution from purple to yellow after the reduction, which could be quantified by its decrease of absorbance at wavelength 517 nm. Hence, DPPH finds applications in the determination of the radical scavenging activity of plant materials [26]. In the present paper, the antioxidant activity of methanol and aqueous

extract of *Hydrocotyle sibthorpiodes* was evaluated, the results of which has been shown in **Table 1** and **Table 2**. Significant activity has been obtained for both the extracts. The methanol extract showed maximum DPPH scavenging activity compared to aqueous extract (**Table 1**) in a concentration dependent manner with an IC_{50} value of 50.1 µg/ml followed by aqueous extract and standard with 70 µg/ml and 119 µg/ml respectively. Higher % inhibition showed better antioxidant potential of the extract. Moreover, the % inhibition showed by the methanol extract for H_2O_2 scavenging activity was also found to be maximum compared to the aqueous extract and standard with an IC_{50} value of 60.2µg/ml followed by standard with 65.2µg/ml and aqueous extract with 100 µg/ml. **Table 3** depicted the reducing power of the extracts and with the increasing concentration of the both the extracts, methanol extract showed the higher reducing power.

The confirmation of the antioxidant activity of both the extracts can be correlated with the presence of different chemical constituents present in the extracts which has also been reported to be concomitant with the development of the reducing power [27].

Earlier, presence of total phenolic compounds, flavonoids and flavonols was reported in Hydrocotyle species [28] which was associated with a broad spectrum of chemical and biological activities including radical scavenging properties. It was also claimed that phenolic compounds are powerful chain breaking antioxidants [29]. The crude methanol and aqueous extracts therefore merits further experiment *in-vivo* with the purification, isolation and characterization of the active constituents which are also known to have several other properties including antimicrobial activities.

TABLE 1: % INHIBITION OF DPPH BY DIFFERENT CONCENTRATIONS OF EXTRACT AND STANDARD

Sl. No.	Concentration (µg/ml)	% inhibition		
		Methanol extract	Water extract	Standard
1	10	59.23±0.61	25.91±21.29	19.91±0.11
2	20	69.53±0.30	44.56±13.91	25.05±0.89
3	40	75.43±0.41	54.44±18.09	28.90±0.76
4	80	76.43±0.11	56.53±15.30	32.87±0.74
5	160	85.80±0.29	81.80±3.81	42.07±0.91

TABLE 2: % INHIBITION OF H_2O_2 BY DIFFERENT CONCENTRATIONS OF EXTRACTS AND STANDARD

Sl. No.	Concentration (µg/ml)	% inhibition		
		methanol extract	water extract	Standard
1	10	11.06±0.152	11.03±0.24	10.79±0.199
2	20	32.23±0.251	26.08±0.18	27.47±0.196
3	40	44.53±0.152	32.02±0.12	36.47±0.467
4	80	64.7±0.173	40.48±0.24	53.36±0.592
5	160	71.24±0.233	42.0±0.18	54.5±0.450

TABLE 3: THE REDUCING POWER OF DIFFERENT CONCENTRATION OF EXTRACTS AND STANDARD

Sl. No.	Concentration (µg/ml)	Methanol extract	Aqueous extract	Standard
1	10	0.844±0.001	0.816±0.001	0.454±0.002
2	20	0.888±0.001	0.916±0.002	0.646±0.001
3	40	0.965±0.002	0.977±0.001	0.734±0.002
4	80	1.143±0.001	0.986±0.002	0.816±0.001
5	160	1.173±0.001	1.202±0.001	1.098±0.001

TABLE 4: THE ZONE OF INHIBITION FOR TESTED ORGANISM OF DIFFERENT EXTRACT AND STANDARD AT 50µL OF 1mg/ml CONCENTRATION

Sl. No.	Concentration (µl)	Tested Organism	% inhibition				
			Methanol	Aqueous	Standard	Methanol extract	Water extract
1	50	*Staphylococcus aureus* (MTCC-96)	19.0±1.0	10.83±0.76	24.83±1.75	76.5	43.6
2	50	*Klebsiella pneumonia* (MTCC 432)	12.5±0.5	9.83±0.76	18.50±0.5	67.5	53.1

In the present investigation methanol extract of the whole plant showed the maximum inhibition (76.5% and 67.5%) against both gram positive and gram negative bacteria namely, *Staphylococcus aureus* (MTCC-96) and *Klebsiella pneumonia* (MTCC 432) **(Table 4)**. The antibacterial activities of the aqueous and methanol extracts were categorized as follows: (a) strong: for inhibition P 70 %; (b) moderate: for inhibition 50-70 %; or (c) weak: for inhibition <50 % [24].

CONCLUSION: The result obtained from the *in vitro* experiments has demonstrated considerable antioxidant and antimicrobial activity of *Hydrocotyle sibthorpiodes.* The activity showed by the plant species might be due to the synergistic effect of the active compounds. Therefore, the individual compounds responsible for such activities can be purified, isolated and characterized for their further study on combating the out coming chronicity associated with the antioxidant stress as well as the infection caused by harmful human pathogens.

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