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Studies on *in vitro* evaluation of antibacterial and antioxidant activity of *Hydrilla verticillata* (L.f.) Royle

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Abstract

The purpose of the present investigation was to evaluate the therapeutic effect of *Hydrilla verticillata* (L.f.) Royle by examining its antibacterial and antioxidant properties in *in vitro* condition. For this study the methanolic and n-hexane extracts of *Hydrilla verticillata* (L.f.) Royle was tested against 5 pathogenic bacteria such as "*Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Shigella flexneri* and *Vibrio choleriae"* by using agar well diffusion method and for antioxidant activity DPPH scavenging assay was carried out. An antibiotic Ciprofloxacin was taken as reference. The result of the study revealed that, the n-hexane extract of *H. verticillata* showed highest zone of inhibition against *Enterococcus faecalis* (20±1.41 mm) and least against *Vibrio choleriae* (12±1.63 mm) at concentration of 15 mg/mL. While, methanolic extract showed highest zone of inhibition against *Shigella flexneri* (26±0.75 mm) and the least zone of inhibition against *Klebsiella pneumoniae* (20±1.24 mm). The significant result for antioxidant activity was evaluated. The IC_{50} value of n-hexane and methanolic extracts were 36.26 and 16.84 respectively. The methanolic extract was found to be having better result than n-hexane extract. The n-hexane extract showed the presence of alkaloids, triterpenoids and polyphenolic compounds, tannin, glycoside while phytoconstituents like alkaloids, saponins, tannins, steroids, flavonoid, glycosides, triterpenoids and polyphenolic compounds were found to be present in methanolic extract of *H. verticillata.* Further study can be done on the evaluation of secondary metabolites compound analysis for therapeutic effect.

Keywords

Hydrilla verticillata; antibiotic resistant; antibacterial; antioxidant activities

Introduction

Since ancient time, it was believed that all the plants retain some pharmacological properties due to the presence of secondary metabolites such as "alkaloids, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids" etc. which should be utilized to fight against the disease-causing pathogens (1-3). Now a day's significant progress has been noticed in the field of drug discovery by naturally and synthetically with the innovation of science and technology (4). Antibiotic are considered as most promising solution for bacterial causing diseases. However, only one third of the infectious diseases known have been treated from these synthetic products (5). New therapeutics are needed to control the disease resistance bacterial borne diseases which posing an increasing therapeutic problem. Several

human pathogenic bacteria are found to exhibit antibiotic resistance due to misuse or over dose of drug (6-8). The use of herbal plants as traditional health remedies is the most common in Asia, Latin America and Africa, majority world's population has reported to have minimal side effects (9). One such example is *Shigella flexneri*, a gram-positive bacterium which causes common diseases like diarrhoea, abdominal cramp, fever and also causes serious disease like ulcer in rectum and colon. It can cause acute condition in immune compromised body and HIV infected individual. *Shigella flexneri* was found to be resistant to Azithromycin. Another example is Methicillin-resistant *Staphylococcus aureus* (MRSA), a gram-positive bacterium, has been documented as one of the most disease-causing bacteria. It causes infection in skin and soft tissues of body and also infects the lungs causing pneumonia. If it left untreated, it may transmit to blood stream called bacteremia and also to bone. *Micrococcus luteus* a gram-positive bacterium causes meningitis, septic, arthritis, cutaneous infection and bacteremia and also found in HIV positive patient. This bacterium is a rare pathogen of endocarditis (10). This bacterium is resistant to both tetracycline and erythromycin (11, 12). *Escherichia coli*, a multi-drug resistant gram-negative bacterium causes many acute diseases including bloody diarrhoea, stomach cramp and urinary tract infection (13). Now-a-days multi-drug resistant (MDR) pathogens are observed to play major role to make failure in organ transplantation. New natural therapeutics and alternative approaches are needed to control the multidrug resistant bacteria. One way to control these MDR bacteria by using antibiotic resistant inhibitors from plant extract (14, 15). Plants are known to produce a variety of compounds to protect themselves against a large number of pathogens. It is expected that plant extracts showing target sites other than those used by antibiotics might be active against drug resistant pathogens (16). The crude extracts of plants have been shown to provide better therapeutic agent than the isolated compound without any side effect (17, 18).

Hydrilla, a submerged aquatic plant, attracts the attention of many researchers due to its aggressive competitive growing nature (invasive) and contains many nutritive components. It is loaded with high level of Vitamins including rare Vitamin-B12, antioxidant and minerals (19). Although this plant is having lots of nutritional properties, it is not commonly utilized as a pharmaceutical product. Additive worth can be given to this plant if it is proven to have medicinal properties like anti-bacterial and anti-oxidant activity. In the present study, methanolic and n-hexane extracts of *Hydrilla verticillata* have been evaluated for their antimicrobial and antioxidant behaviour.

Materials and Methods

Collection and preparation of plant extract

The experimental plant sample *Hydrilla verticillata* (L.f.) Royle was collected from the water body located in the CUTM (Centurion University of Technology and Management) campus, Bhubaneswar, Odisha in the month of August, 2019. The plant specimen (*Hydrilla verticillata* (L.f.) Royle; Voucher No. BB-341) was identified by following the available literature (Saxena & Brahmam, 1994-96) and matching with the preserved specimen at Herbarium unit, IMMT-CSIR, Bhubaneswar (20). Collected plant sample washed thoroughly in running tap water then distilled water; kept them in room temperature to get them dry. Dried samples were then grinded properly to make a powder form. 15 g of powdered plant materials were successively extracted using 2 different solvents i.e. 250 mL each of n-Hexane and methanol by Soxhlet apparatus (Borosil, India) separately (21). All the extracts were evaporated at room temperature and kept in woven at 40 °C over night to evaporate all the solvent and stored in sterile glass vials at 4 °C for further use.

Preliminary phytochemical analysis

For preliminary phytochemical analysis, standard protocol was used to detect the presence of different secondary metabolites in experimental plant *Hydrilla verticillata*. 2 mL of plant extracts were taken and treated with various chemical reagents as per the test methods (22-24).

Antioxidant Assay

The *in vitro* antioxidant study was done by free radical scavenging assay (DPPH assay). In this technique, stock solution for the experiment was prepared by dissolving 50 mg of extract in 50 mL of methanol and n-hexane. Different concentration ranges from (300, 250, 200, 150, 100 and 50µg/mL) of methanolic and n-hexane extract of *H. verticillata* was prepared by diluting the stock solution. Then, from above solution 1.5 mL sample was taken and 1.5 mL of DPPH was added to it and kept them in dark condition at room temperature for 30 min for allowing them to react properly. Then, the absorbance was taken using UV spectrophotometer at 517nm. Standard stock solution was prepared by dissolving dry ascorbic acid in 10 mL of methanol and n-hexane. And 5 different aliquots were prepared such as 50, 100, 150, 200, 250 and 300 µg/mL by diluting it. The percentage of free radical scavenging was calculated based on the absorbance on extent of reduction in the colour. The IC_{50} value was calculated for the expression of antioxidant activities of the extracts (25).

The ability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ of radical scavenging activity} = [(Ac-As)/Ac] \times 100$$

Where Ac = Absorbance of control, As = Absorbance of sample.

Collection of Bacteria

The microbial strains employed in the study included "*Staphylococcus aureus* (MTCC-1430), *Escherichia coli* (MTCC-0614), *Klebsiella pneumoniae* (MTCC-109), *Shigella flexneri* (MTCC-1457), *Micrococcus luteus* (MTCC-1809)", procured from MTCC (Microbial Type Culture Collection Centre) and Gene Bank, Chandigarh, India. All the microbial strains were cultured and sub-cultured in nutrient agar slants. Then, the agar slant stored in refrigerator at 4°C for further experimental use.

Microbial culture media

The culture media for the antibacterial susceptibility analysis was done by using solid nutrient agar media applying on petridishes and the test was performed by using agar well diffusion method. To execute the antibacterial investigation, nutrients agar (28 g/L) "(HIMEDIA), REF- M001-500G, LOT-0000145979" was used for developing surface colony growth. The suspension culture for bacterial cells growth was done by preparing nutrient broth "(HIMEDIA), (REF-M088-500G, LOT- 0000154058)". The sterilization process was then followed for both nutrient solid agar media and broth culture media at 121 °C for 20 min. Sterilized cultured tube was used to make fresh media for slant preparation which was used to maintain bacterial strains.

Preparation of test plates

Sterile petridishes were kept in UV light for 20 min to avoid contamination. About 30 mL of nutrient solid media was poured in to each sterile petridishes in an aseptic condition making a depth of approximately 4 mm. The plate was kept in undisturbed condition to make them dry; then swabbing process was carried out using sterile cotton swab with 8 hrs old broth culture of each test microorganism. Then, plates were kept preserved to apply drug sample.

Anti-Bacterial Activity Assay Using Agar Well Diffusion Method

To determine the antibacterial assay of *H. verticillata* agar well diffusion method was followed (26). The wells in each of these swabbed plates were made by using sterile cork borer with 10 mm diameter and about 2 cm apart. About 50 µl of methanolic and n-hexane extract was poured with micropipette in agar well and allowed them to diffuse at room temperature for 2 hrs. without disturbed. Ciprofloxacin drug was taken as standard at concentration of 0.5 mg/mL. The inoculated plates were transferred to incubator at 37 °C for the growth of bacteria with triplicate for 18-20 hrs. The experiment was repeated thrice; the result was recorded in form of zone of inhibition in diameter in 3 different fixed directions.

Preparation of drug for antibacterial analysis

The stock solution (plant sample) for the drug assay was prepared as 500 mg/mL using dimethylsulphoxide (DMSO). Working solution of the drug was prepared by 1% of DMSO diluted with culture media and the fraction is taken as 15 mg/mL followed by 12 mg/mL, 9 mg/mL, 6 mg/mL and 3 mg/mL. "Ciprofloxacin" was taken as standard drug (Reference Antibiotic). The stock solutions of RA were prepared in 10 % DMSO to give a concentration of 0.5 mg/mL for RA. The diameter of zone of inhibition (in mm) was measured by using transparent scale.

Results and discussion

Preliminary Phytochemical analysis

The preliminary phytochemical study of the plant extracts revealed the presence of alkaloids, glycosides, flavonoids, saponins, steroids, triterpenoids, tannins and polyphenolic compounds (Table 1). The n-hexane extract showed the presence of alkaloids, triterpenoids and polyphenolic compounds, tannin, glycoside while phytoconstituents like alkaloids, saponins, tannins, steroids, flavonoid, glycosides, triterpenoids, tannins and polyphenolic compounds were found to be present in methanolic extract of *H. verticillata*. The result, thus indicated that the methanolic extract contains a greater number of phytoconstituent than the n-hexane solvent extract.

Table 1. Preliminary phytochemical analysis of *Hydrilla vericillata*

Sl.No	Phytochemicals	*Hydrilla verticillata* Extracts in solvents	
		Methanol	n-Hexane
1	Alkaloids	+ve	+ve
2	Flavonoids	+++ve	-ve
3	Saponins	++ve	-ve
4	Steroids	++ve	-ve
5	Tannins and polyphenolic compound	+++ve	++ve
6	Triterpenoids	+++ve	+ve
7	Glycosides	+ve	+ve

(+ve) indicating presence of chemical compounds , (-ve) indicating absence of chemical compounds

Natural remedies are now being center of attraction for curing any type of disease. Phenolic compounds present in medicinal plants can prevent from oxidative damage (27, 28). Earlier it was reported that the mutagenesis and carcinogenic effect could be reduced by the act of phenolic compounds, when consumed through vegetables and fruits (29). Tannin content has been considering another important phytoconstituent which can scavenge free radicals. Thus, they reduce hazardous free radicals from our body (29).

Antioxidant Assay

The result of antioxidant assay is summarized in Fig. 1. The free radical scavenging activity of both methanolic and n-hexane extract of *H. verticillata* with different

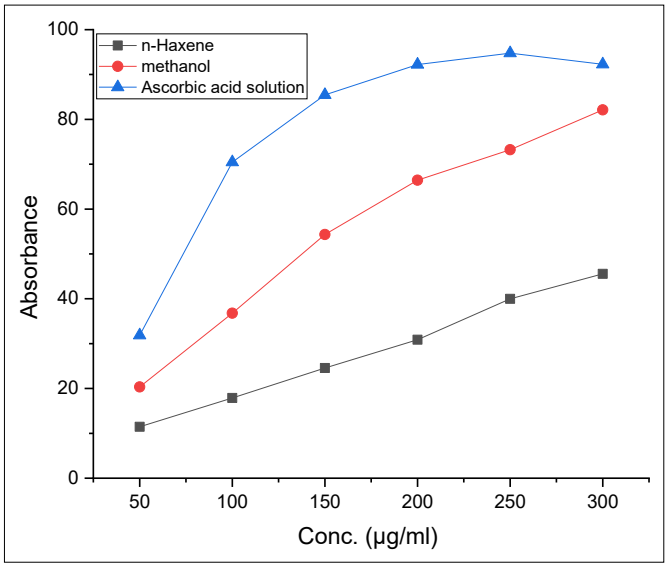

Fig. 1. Antioxidant assay of *H. verticillata* with standard Ascorbic Acid.

concentration ranges from 50 µg/mL-300µg/mL was evaluated in dose dependent manner. The effect of antioxidant on free radicals is thought to be because of their hydrogen donating capacity. In DPPH assay, the reduction capability of "1, 1-diphenyl-2-picrylhydrazyl" was determined by decrease absorbance at 517 nm. Absorbance was decreased significantly by increasing the concentration of the crude extract. Whole experiment was conducted by taking ascorbic acid as standard. From the above experiment it was found that the methanolic extract showed comparatively higher antioxidant activity than n-hexane extract. The half maximal inhibitory concentration (IC_{50}) value is widely used to calculate the antioxidant activity. The IC_{50} value of n-hexane extract, methanol extract and ascorbic acid was found 36.86, 16.71 and 12.63 respectively.

Present study was conducted to evaluate antioxidant competence of *H. verticillata* which is beneficial at both nutritional levels as well pharmaceutical sectors. Phytoconstituent like phenolic compounds, flavonoid, tannin, alkaloid and glycosides are believed to be responsible to control disease related to stress and are considered as good antioxidant substances (30). Generally, salt tolerant macrophytes are reported to have a greater number of phytochemicals and with higher capacity to scavenge oxidative damage than the normal land plants (31). *Hydrilla verticillata* was also previously proven to enhance the activity of superoxide dismutase and peroxidase after expose to saline conditions (32).

Antibacterial Analysis

The extracts of this plant subjected to antibacterial screening against 5 bacterial pathogens causing different human diseases. The results indicated that methanolic extract of *Hydrilla verticillata* exhibited highest zone of inhibition against *Shigella flexneri* (26±0.75 mm) and least against *Klebsiella pneumoniae* (20±1.24 mm) in concentration of 15 mg/mL. This extract showed moderate activity against *Micrococcus luteus* (22±0.53 mm) and *Escherichia coli* (21±1.21 mm). While n-hexane extract exhibited highest zone of inhibition on *Staphylococcus aureus* (20±1.41 mm) at concentration of 15 mg/mL of drug, least against *Micrococcus luteus* (12±1.63 mm) and moderate against *Escherichia coli* (18±0.47 mm) and *Klebsiella pneumoniae* (16±1.23 mm) (Table 2; Fig. 2 and 3). The GC-MS study revealed that the presence of "phytol and hexadecanoic acid ethyl ester" as major component in ethanolic extract of *Hydrilla* (33). This may play a major role for antimicrobial and antioxidant properties.

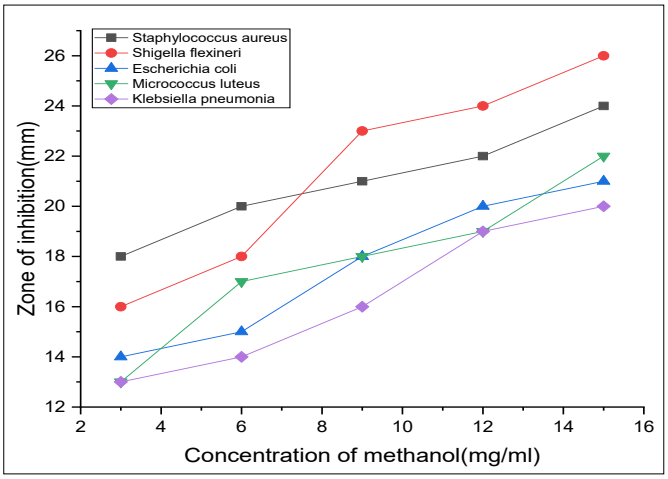

Fig. 2. Antibacterial assay of *H. verticillata* with methanolic extract.

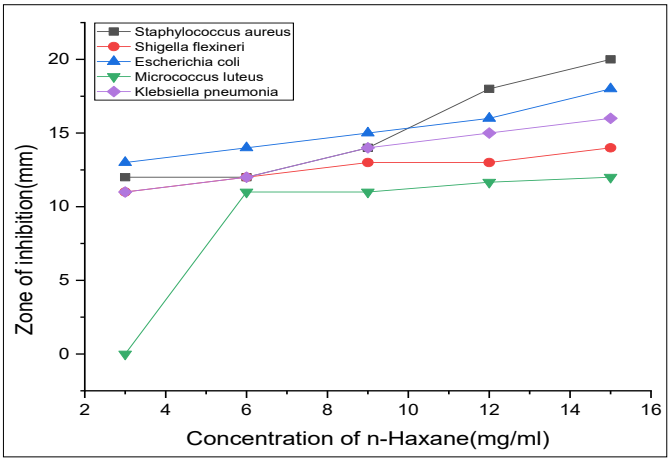

Fig. 3. Antibacterial assay of *H. verticillata* with n-Hexane extract.

Table 2. *In vitro* Antibacterial activity (zone of inhibition) of different extracts of *Hydrilla verticillata*

Test substance	Concentration	Diameter of zone of inhibition (mm)				
		Staphylococcus aureus	***Shigella flexneri***	***Escherichia coli***	***Micrococcus luteus***	***Klebsiella pneumoniae***
Ciprofloxacin 0.5mg/mL (RA)	0.5 mg/mL	37±0.21	33±0.43	36±0.63	29±0.51	32±0.76
	15 mg/mL	24±0.46	26±0.75	21±1.21	22±0.53	20±1.24
	12 mg/mL	22±0.81	24±0.61	20±0.47	19±0.47	19±0.21
Methanol extract	9 mg/mL	21±0.94	23±0.81	18±1.41	18±0.4	16±0.48
	6 mg/mL	20±0.84	18±0.82	15±1.24	17±1.69	14±1.31
	3 mg/mL	18±1.24	16±0.24	14±1.62	13±0.23	13±0.38
	15 mg/mL	20±1.41	14±0.81	18±0.47	12±1.63	16±1.23
	12 mg/mL	18±o.81	13±0.47	16±0.46	11.66±0.94	15±0.48
n-Hexane extract	9 mg/mL	14±0.52	13±0.21	15±0.51	11±0.29	14±0.61
	6 mg/mL	12±0.12	12±0.65	14±0.42	11±0.11	12±0.21
	3 mg/mL	12±0.31	11±1.21	13±0.62	---	11±0.68

Result expressed as mean ±S.D (n=3), (---) denotes no zone of inhibition·

Conclusion

Results from the present investigation revealed that methanolic extract of *Hydrilla verticillata* had a strong antibacterial effect against all the test pathogenic bacteria and also showed good results on antioxidant activity. In antibacterial study; methanolic extract showed highest zone of inhibition against *Shigella flexneri* whereas n-hexane showed good result against *Staphylococcus aureus.* In study of DPPH assay, the IC_{50} value was found to be lowest in methanolic extract than n-hexane. From these inhibitory effects of the extracts of *H. verticillata* was proven that it can be used as potential herbal drugs against all tested pathogenic organisms and also a good antioxidant product. In future, compound isolation and analysis can be examined for its specific antibacterial and anti oxidant activity. As it has great nutritional value as well pharmaceutical value, this plant can be recommended as the super food for human.

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Authors contributions

Kunja Bihari Satapathy and Bhagyeswari Behera conceived the idea. Bhagyeswari Behera performed the experiments. Kunja Bihari Satapathy and Bhagyeswari Behera analyzed the information. Both authors have made significant contributions in drafting the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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