PHYTOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF METHANOLIC EXTRACT OF ARDISIA HUMILIS VAHL (MYRSINACEAE)

Amina Khatun1*, Mahmudur Rahman2, Sumaiya Kabir1, Md Nahid Akter4, Sadia Afreen Chowdhury4
1Senior Lecturer, Phytochemistry and Pharmacology Research Laboratory, Department of Pharmacy, Manarat International University, 1/B, Zoo road, Mirpur-1, Dhaka-1216, Bangladesh
2Mahmudur Rahman: Assistant Professor, Faculty of Health Sciences, Department of Pharmacy, Northern University Bangladesh, 24, Mirpur Road, Globe Centre, Dhaka-1205, Bangladesh
3Department of Pharmacy, Manarat International University, 1/B, Zoo road, Mirpur-1, Dhaka-1216, Bangladesh
4Pharmacy Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh
5Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh

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*Corresponding author
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ABSTRACT

Ardisia humilis Vahl. (Family: Myrsinaceae) has been traditionally used by the folklore medicinal practitioners of Bangladesh to treat cancer, heart diseases and liver poisoning where cytotoxic, thrombolytic and antioxidant medications are implicated. Besides, some other species of Ardisia were reported to have incriminated properties and chemical constituents. In this study, the crude methanolic extract of the A. humilis was evaluated for its possible cytotoxic, thrombolytic and antioxidant activities in different methods to justify some of its folklore use. Cytotoxic property of the extract was determined against brine shrimp nauplii. The thrombolytic activity was evaluated using the streptokinase. The antioxidant activity was measured using free radical scavenging activity with 2,2-diphenyl-1-picyrhydrazyl (DPPH) method. The extract showed significant cytotoxic effect in brine shrimp lethality bioassay where it showed the value of LC50 and LC90 2.26 µg/ml and 7.13 µg/ml after 24 hours respectively. The standard cytotoxic drug vincristine sulphate showed LC50 and LC90 of 0.81µg/ml and 6.33µg/ml after 24 hour respectively. The study gave a significant indication of the use of the plant extract as a potential source for cytotoxic compounds. The extract showed moderate thrombolytic activity of 33.33% clot lysis where the standard streptokinase showed that of 84%. In DPPH free radical scavenging test, IC50 value for the methanolic crude extract was found fairly significant (4.305 µg/ml) while compared to the IC50 value of the reference standards ascorbic acid (2.8 µg/ml). The obtained results tend to suggest the probable cytotoxic, thrombolytic and antioxidant activities of the methanolic extract of A. humilis justify its use in folkloric remedies and those activities of other species of Ardisia.

Keywords Ardisia humilis, Cytotoxic, Antioxidant, DPPH free-radical scavenging, Thrombolytic, Streptokinase

INTRODUCTION

Ardisia humilis sensu F.B.I, non Vahl (A. humilis) (Family: Myrsinaceae), synonym Ardisia solanacea Roxb. locally known as Bara salla, Banzam, wild berry in English, is an erect, branched, glabrous shrub, under favorable conditions reaching up to 20 ft in height native to warm temperate to tropical regions of southeastern Asia like in Bangladesh, India. The plant is credited with stimulant and carminative properties1-2. The plant is used in the treatment of diarrhoea, bruise, dysmenorrheoa, gout, mental disorder, rheumatic arthritis, rheumatism, skin sore and vertigo3. Roots are used in fever, diarrhea and rheumatism and it has antibacterial activity4. Other species of the Ardisia have been reported for their cytotoxic, thrombolytic, antiinflammatory and antioxidant activities5-12. Different constituents like beta amyrin, resorcinol analogs, ardisipusilloside I, volatile oil, alkyl benzoquinones alkyl phenols and ardisiphenols have been isolated from other species of Ardisia5,7-9,13.

MATERIALS AND METHODS

Collection and identification of plant material

The plant A. humilis was collected from Satkhira, Bangladesh in December 2011. The species was confirmed by Sardar Nasir Uddin, Principle Scientific officer, Bangladesh National Herbarium, Mirpur, Dhaka and voucher specimen of the plant has been deposited and preserved in the library of the same institution for further collection and reference (Accession number-DACB-15693).

Preparation of methanolic extract

The collected plant parts were separated from undesirable materials and then were washed with water and shade-dried for ten days. The dried plant materials were ground into a coarse powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powdered sample was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced. About 300 g of powered material was taken in a clean, flat-bottomed glass container and soaked in 1000 ml of methanol. The container along with its contents was sealed and kept for a period of 10 days with occasional shaking or stirring6. The whole mixture then underwent a coarse filtration by cotton. It was then filtered through whatman filter paper (Bibby RE200, Sterilin Ltd., UK). The filtrate was concentrated under air and dried. It rendered a 15.4 g concentrate (5.13.0%) of greenish black color and was designated as crude methanol extract.
Test for different chemical groups
The crude methanolic extract was tested for its different chemical groups as alkaloids, flavonoids, gums, reducing sugars, saponins, steroids and tannins using standard protocol\textsuperscript{14-15}. \(10\%\) (w/v) solution of the extract in methanol was used for each of the above test.

Test for cytotoxic activity
The cytotoxicity assay was performed on brine shrimp nauplii using method of Mayer et al\textsuperscript{15-16}. Brine shrimp nauplii were obtained by hatching brine shrimp eggs (Carolina Biological Supply Company, Burlington, NC, USA) in artificial sea-water (3.8% NaCl solution) for 22 hrs. Artificial sea water was prepared by dissolving 20 g of NaCl (Sodium Chloride Crystal GR, Merck Ltd., Mumbai, India) and 18 g of table salt in one liter of distilled water and was filtered off to get a clear solution. Dissolution of 30 mg of compound was performed in 3ml artificial sea water containing 20% Dimethyl sulfoxide (DMSO, \(>99.9\%\), BioReagent, for molecular biology; Sigma-Aldrich, India) to give concentration of 10 \(\mu g/\mu l\). From this solution 10, 20, 40, 80, 160 and 320 \(\mu l\) were transferred to each 10 ml vial and using artificial sea water volume was adjusted to 10ml by artificial sea water to give concentrations of compound of 10, 20, 40, 80, 160 and 320 \(\mu g/ml\) respectively. Brine shrimp nauplii were grown in these solutions and observed their mortality for 24 h. The resulting data were transformed to probit analysis software (Ldp Line software, USA)\textsuperscript{17, 18} for determination of LC\(_{50}\) values of the extract. Artificial sea-water medium containing DMSO used for the analysis was employed as negative control. Vincristine sulfate (Techno Drugs Ltd, Bangladesh) was used as standard in this assay.

In vitro thrombolytic activity
5 ml of phosphate buffered saline (PBS) was added to the commercially available lyophilized streptokinase vial (15, 00,000 I.U.) and mixed properly. This suspension was used as a stock from which appropriate dilutions were made to observe the thrombolytic activity\textsuperscript{19}. In brief, 2 ml venous blood drawn from healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37\(^\circ\)C for 45 minutes. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each microcentrifuge tube containing pre-weighed clot, 100 \(\mu l\) of methanolic extract (10 mg/ml) of was added. As a positive control, 100 \(\mu l\) of streptokinase and as a negative non thrombolytic control, 100 \(\mu l\) of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37\(^\circ\)C for 90 minutes and observed for clot lyses. After incubation, released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lyses was expressed as percentage of clot lyses.

Test for antioxidant activity
The antioxidant activity of plant extract and the standard antioxidant were assessed on the basis of radical scavenging effect of the stable DPPH [2,2-diphenyl-1-picrylhydrazyl; Nacali tesque, Kyoto, Japan] free radical\textsuperscript{20}. Stock solution (10 mg/mL) of the methanolic extract of \textit{A. humilis} was prepared in respective solvent systems from which serial dilutions were carried out to obtain concentrations of 1, 5, 10, 50, 100, 500 \(\mu g/mL\). In this assay, an equal amount of sample solution was added to an equal amount of 0.1 mM methanolic DPPH solution, vortex and allowed to stand at the dark place at 25\(^\circ\)C for 30 min for the reaction to occur. After 30 min of incubation period, the absorbance was read against a blank at 517 nm with a double beam Analykjenya UV/Visible spectrophotometer (Model Shimadzu, UV-1800, Japan). The radical scavenging activity was expressed as the inhibition percentage (\%) and calculated as per the equation:

\[
I(\%) = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100
\]

Where \(A_{blank}\) is the absorbance of the control reaction (containing all reagents except the test compound), and \(A_{sample}\) is the absorbance of the test compound with all reagents. \(IC_{50}\) value is the concentration of sample required to scavenge 50% DPPH free radical and was calculated from the plot of inhibition (%) against extract concentration. All the tests were carried out in triplicate and average of the absorptions was noted. Ascorbic acid was used as positive control standard for this study.

Statistical analysis
Data were presented as mean \pm S.D. Statistical differences between control and treated groups were tested by Student’s \(t\)-test. The differences were considered significant at \(P<0.05\).

| Table 1: Results of phytochemical screening of \textit{Ardisia humilis} extracts |
|---------------------------------|---------------------------------|----------------|
| Phytoconstituents               | Methanolic extract of \textit{A. humilis} |
| Alkaloids                       | \(+\)                            |                |
| Carbohydrates                   | \(+\)                            |                |
| Flavonoids                      | \(\_\)                          |                |
| Gums                            | \(\_\)                          |                |
| Saponins                        | \(\_\)                          |                |
| Steroids                        | \(\_\)                          |                |
| Tannins                         | \(\_\)                          |                |

\(+:\) Positive result; \(\_\): Negative result;

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<th>Table 2: Brine shrimp lethality bioassay of \textit{A. humilis}</th>
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<td>Sample</td>
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<tr>
<td>Methanolic extract of \textit{A. humilis}</td>
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<td>Vincristine sulfate</td>
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Results of different chemical tests on the methanolic extract of Ardisia humilis showed the presence of alkaloids, carbohydrates, steroids and tannins (Table 1).

**Antioxidant activity**

Table 4 shows the antioxidant activity of the methanolic extract of A. humilis measured by DPPH assay. The positive control ascorbic acid showed an IC50 of 2.8±0.01 µg/ml. The methanolic extract showed significant antioxidant activity with an IC50 of 4.305±0.275 µg/ml. This indicates that the extract may contain penta cyclic triterpenoid compounds that possess important antithrombotic active constituents and provided information for the exploitation and utilization of triterpenoid compounds as thrombin inhibitors for the treatment of thrombotic disease.

**DISCUSSION**

The methanolic extract of Ardisia humilis demonstrated the presence of alkaloids, carbohydrates, steroids and tannins as secondary metabolites with potential biological activities. Several species of Ardisia were reported for their free phenolic content, beta amyrin type pentacyclic triterpenoid compounds and antioxidant activities. Presence of these compounds and activities may be due to the presence of tannins and other phenolic compounds. Further bioassay guided approaches may be taken to elucidate the responsible compounds. Brine shrimp lethality bioassay is an easy and straightforward screening method for predicting important pharmacological activities like enzyme inhibition, ion channel interference, antimicrobial and cytotoxic activity. Several species of Ardisia were reported for suppressor of skin tumor, inhibitor of tumor induced angiogenesis and cytotoxic activities. In the present study the extract showed LC50 at a low concentration indicating that the extract is significantly potent. Ideally, any agent useful in the treatment of cancer should not be toxic to normal cell. However, in reality, anticancer agents are often toxic to normal cells, particularly towards rapidly growing cells. It is necessary to test this extract against various cancer cell lines as well as normal cell lines to justify the potential to further investigate this plant for anticancer activity. Further investigation is required to find the responsible compound(s) for the cytotoxic activity observed for Ardisia humilis.

The thrombolytic bioassay result suggested that the extract may contain penta cyclic triterpenoid compounds that possess important antithrombotic active constituents and provided information for the exploitation and utilization of triterpenoid compounds as thrombin inhibitors for the treatment of thrombotic disease.

Antioxidant activity of plants is one of the great virtues which can be exploited to prevent various diseases including cancer. Daily ingestion of antioxidant constituents with our diet can significantly reduce the chance of cancer by fighting against free radicals. In the present study the extract showed significant antioxidant activity and was comparable to that of ascorbic acid, used as the positive control in this assay. Antioxidant compounds cannot be treated as therapeutic agent for cancer treatment since antioxidants prevent the development of cancer through a protective manner while anticancer drugs directly affects the cellular function of cancerous cell. However, there are reports that antioxidants like tea phenolics can directly inhibit cancer cells.

Present study is based on the report of preliminary phytochemical and biological screening of A. humilis extract. The results are quite promising; support the use of this plant in traditional medicine and demand further investigation. Advanced studies including LC-MS can be carried out to get a bigger picture of the chemical constituents present in the plant. Screening methods applying various cell lines or bacterial enzymes can be carried out to find the underlying mechanism for the observed biological activities. On the basis of the results from above studies, bioassay guided approach can be undertaken to isolate and identify the active...
component(s).

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