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Characterization of phytochemicals and determination of antioxidant, antimicrobial and cytotoxic properties of the medicinal plant *Ardisia solanacea*

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Abstract

The present study was conducted to detect possible phytochemicals and evaluate antioxidant, antimicrobial and cytotoxic activities of the extract of *Ardisia solanacea*. Phytochemical screening was carried out using the standard test methods of different chemical group. For investigating the antioxidant activity, two complementary test methods namely DPPH free radical scavenging assay and total phenolic content determination were carried out. For the evaluation of *in vitro* antimicrobial activity, disc diffusion method was used. Evaluation of cytotoxic activity was done using the brine shrimplethality bioassay. In DPPH free radical scavenging test, the petroleum ether soluble fraction showed the highest free radical scavenging activity with IC_{50} value 40.04 µg/ml. while compared to that of the reference standards ascorbic acid. *Ardisia solanacea* was also found as a good source of total phenolic contents. Moreover, the extracts revealed moderate antimicrobial activity at the concentration of 400 µg/disc. In cytotoxic activity test, the petroleum ether soluble fraction showed significant cytotoxic potential (LC_{50} value of 0.703 µg/ml) among all the fractions comparing with that of standard vincristine (0.544 µg/ml). Therefore, further studies are suggested to determine the active compounds responsible for the pharmacological activities of the plant extracts.

Keywords: Phytochemicals; Antioxidant; Antimicrobial; Cytotoxic

1. Introduction

Nature provides different remedies for human being from its plants, animals and other sources to treat all ailments of mankind [1,2]. Medicinal plants are important contributors among all natural sources [3]. They always play an important role in the development of health in mankind [4]. Accordingto World Health Organization (WHO), about 80% of the world population still uses medicinal plants for different medical purposes [2]. Even in recent time, people are still taking traditional medicine for their primary health care [3]. It has been examined that fruits and herbs containing phyto-constituents and non-nutritive may protect human from different types of diseases for their biological activities

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[3]. It is now fully believed that phytochemicals obtained from the medicinal plants serve as active molecules in the modern medicines [4]. *Ardisia solanacea* isa species of the genus *Ardisia.* Almost 500 species of *Ardisia* are found throughout tropical and subtropical regions of the world. Several of those species have been used as ornamental plants, medicines and food [5]. *Ardisia solanacea* (*A. solanacea*) is locally known as Banzam, wild berry in English. This plant has stimulant and carminative properties [6]. The plant is applied in the treatment of diarrhea, dysmenorrhea, gout, mental disorder, rheumatic arthritis, skin sore and vertigo. Roots have antibacterial activity. Other species of the *Ardisia* have been reported for their cytotoxic, thrombolytic and antioxidant properties [6].

Antioxidants are molecules which are capable of preventing the oxidation of other molecules. Natural antioxidants are more beneficial for human body than synthetic antioxidants. Plants are rich sources of natural antioxidants [7]. Antimicrobial assay procedures provide a valid measure of antibiotic activity [8]. Antibiotics are important weapons to eliminate bacterial infections.[9] Discdiffusion is a simple and convenient method of antimicrobial activity [8, 9, 10].

Now a day, available anti-tumor drugs have been associated withserious side effects. If any significant cytotoxic effect exerting herbal medicine can be obtained which is locallyavailable and relatively cheap then it will be very helpful in the treatment of cancer [11,12,13].

According to our knowledge, no significant study is presently available that document the phytochemical and pharmacological properties of *A. solanacea.* So, in this study, our main goal was to evaluate the phytochemical and pharmacological properties of *A. solanacea* to validate its use in folkloric treatments.

2. Material and methods

2.1 Plant materials

For this present investigation, the *A. solanacea* were collected from Chittagong in December 2011. After collection, the taxonomic identification of the plant was carried out with the help of taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (Accession no. 38305). The voucher specimen was also deposited there for future reference.

2.2 Chemicals and Reference drug

For performing these experiments , 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Trichloro acetic acid (TCA), L-Ascorbic acid, Butylated Hydroxy Anisole (BHA), Gallic acid, Folin-ciocalteu phenol reagent, phosphate buffer (pH6.6), distilled water, streptokinase (30000 IU and 15000 IU) of analytical grade (Merck, Darmstadt, Germany) wereused. All the chemicals and reagents were purchased fromSigma Chemical Co. Ltd, (St. Louis, MO, USA) and E. Merck (Germany).

2.3 Extraction of plant materials

For methanolic extraction 400 gm of air dried and powdered sample were submerged in 2500 ml of 80% methanol (Merck KGaA, Darmstadt, Germany) in clean, sterilized and flat-bottomed glass container. The containerand its contents was sealed and kept for maceration for 20days with occasional shaking and stirring. At the end of 20th day, the whole mixture was filtered using filter cloth and Whatman® filter paper (Sargent-Welch, USA). The resultant filtrate was then allowed to evaporate in water bath maintained 45^{o} C to dryness and thus a greenish blackcolored semisolid extract was obtained (yield 25 gms). This gummy concentrate was designated as crude extract of methanol.

2.4 Solvent-solvent partitioning

Solvent-solvent partitioning was done using the protocol designed by Kupchan and here this protocol is slightly modified [12]. The crude extract (5 gm) was dissolved in 10% aqueous methanol. It was extracted with Petroleum Ether, then with carbon tetrachloride and finally with Chloroform.

2.5 Phytochemical screening

Small quantity of freshly prepared methanolic extract anddifferent fractions of barks of *A. solanacea* were subjectedto preliminary phytochemical analysis for the detection ofphytochemicals such as alkaloids with Mayer's and Hager's reagent, Carbohydrates with Benedict's test and Fehling's test, glycosides with Legal's test and Modified Borntrager's test, phytosterols with Salkowski's test and Libermann Burchard's test, proteins with xanthoproteic test, flavonoids with alkaline reagent test and lead acetate test, tannins with gelatin test, saponins with Froth test andfoam test, phenols with ferric chloride test [13,14].

2.6 Antioxidant activity

There are various well known methods, which are followed to determine the antioxidant properties. Among them, two complementary test methods namely total phenolic content determination and DPPH free radicalscavenging assay methods were used for investigating theantioxidant activity of *A. solanacea*.

2.6.1 Total phenolic content determination

The amount of total phenolics in extracts was determined with the Folin-Ciocalteu reagent [4]. Here, gallic acid was used as a standard and the amount of total phenolics were expressed as mg/g of gallic acid equivalents (GAE). Concentration of 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml of gallic acid and concentration of 2 µg/ml of plant extract were prepared in methanol and 0.5 ml of eachsample were placed into test tubes and mixed carefully with 2.5 ml of a 10- fold dilute Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The test tubes werecovered with para-film and allowed to stand for almost 30 min at room temperature before the absorbance was readat 760 nm spectrophotometrically (UV-1800, Shimadzu,Japan). All determinations were performed nicely intriplicate [4]. Thus, total phenolic content was determined asmg of gallic acid equivalent per gram using the equationobtained from a standard gallic acid calibration curve.

2.6.2 DPPH scavenging activity

DPPH radical serves as the oxidizing radical to be reduced by the antioxidant (AH) and as theindicator for the reaction. The stable DPPH radical-scavenging activity was measured using the modified method described by Gupta[14,15]. In this assay, 2 ml of 0.2 mµ methanolic DPPH solutions was added to 2 ml of extract solution at different concentrations and the contents were stirred vigorously for 15 seconds. Then the solutions were allowed to stand at dark placeat room temperature for 30 min for reaction to occur. After 30 min, absorbance was measuredagainst a blank at 517 nm with a double beam UV/Visible spectrophotometer. The percentageof DPPH radical-scavenging activity of each plant extract was calculated as

$$DPPH\ radical - scavenging\ activity\ (\%I): \{(A_0 - A)/A0\} \times 100$$

Where,

A_0 is the absorbance of the control solution (containing all reagents except plant extracts);A is the absorbance of the DPPH solution containing plant extract.

The DPPH radical-scavenging activity (%) was plotted against the plant extract concentration to determine the concentration of extract necessary to decrease DPPH radical-scavenging by 50% (called IC_{50}). The IC_{50} value of each extract was estimated by sigmoid non-linear regression.

These values were changed to antiradical activity, defined as $1/EC_{50}$, since this parameterincreases with antioxidant activity. All determinations were performed in triplicate.

2.7 Antimicrobial activity

2.7.1 Test Organisms

Two strains of Gram-positive (Staphylococcus aureus, Bacillus subtilis), three strains of Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi), and two strains of fungi (Candida albicans, Aspergillus niger) were used to evaluate the antimicrobialactivity. The organisms were subcultured properly in nutrient broth and nutrient agar. They were collected fromthe, Chittagong Veterinary and Animal SciencesUniversity (Department of Microbiology), Bangladesh.

For the evaluation of antimicrobial activity, disc diffusion method is widely acceptable.[3] In this classical method, antibiotics were diffused from a reliable source through thenutrient agar and a concentration gradient was created. Dried, sterilized filter paper discs (6 mm diameter, HI- Media, China) containing the test samples of known amounts (400 µg/disc) were placed on nutrient agar medium consistently seeded with the test microorganisms. Standard antibiotic of ciprofloxacin (5 µg/disc) and blank discs were used as positive and negative control. For the maximum diffusion of the test materials to the surroundingmedia, these plates were kept at low temperature (4 °C) for24 h**.** Then the plates were incubated at 37 °C for about 24h to allow optimum growth of the organisms. The test materials with antimicrobial property inhibited microbial growth in plates and thereby yielded a clear, distinct zone defined as zone of

inhibition. The antimicrobial activity ofthe test sample was then determined nicely by measuring the zone of inhibition expressed in millimeter [3].

2.8 Cytotoxic activity

The cytotoxic property of the extract was determined using brine shrimp lethality test [15]. The investigation was done on*artemia salina* (brine shrimp). *Artemis Salina leach (*brineshrimp eggs) collected from pet shops was used as the test organism. One spoon of cyst was hatched for about 48 hrsin saline water, prepared by dissolving 20 g pure NaCl and18 g normal edible NaCl into 1 L water. The hatched cyst in turn became living nauplii. Different concentrations of the extract were prepared using dimethyl sulfoxide (DMSO) as solvent. For the test, different concentrations of plant extract prepared were added to test tubes, each containing 10 shrimps in saline water. Here, vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get a primary concentration of 40 µg/ml from which serial dilutions were made using DMSO to get 20 µg/ml, 10µg/ml, 5µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml and 0.78125 µg/ml solution from the extract. Then the positive control solutions were added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

2.8.1 Counting of Nauplii

After 24 hours, the vials were inspected by using a magnifying glass and the number of survived nauplii in each vial was counted consciously. From this result, the percent (%) of lethality of the brine shrimp nauplii was calculated nicely for each concentration.

2.9 Statistical Analysis

The results are expressed as mean ± SEM. Statisticalcomparisons were made using one-way ANOVA withDunnett t test. Significance was set at $p < 0.05$. Dose dependencies were determined by the regressioncoefficient (r).

3. Results

3.1 Phytochemical screening

The phytochemical screening of methanol extracts of *A. solanacea* showed varied results. The results are shown inTable 1.

Table 1 Presence of Phytochemicals in *Ardisia solanacea*

S. No.	Phytochemicals	Test	Extract	Result
1	Alkaloids	Wagner's test	Methanolic	+
		Hager's test	Methanolic	+
2	Carbohydrates	Hager's test	Methanolic	+
		Benedict's test	Methanolic	+
		Fehling's test	Methanolic	+
3	Glycosides	Legal's test	Methanolic	_
4	Saponin	Froth Test	Methanolic	+
5	Phytosterols	Libermann-Burchard's test	Methanolic	+
6	Phenol	Ferric Chloride Test	Methanolic	+
7	Tannins	Gelatin Test	Methanolic	+
8	Flavonoids	Alkaline reagent test	Methanolic	+
		Lead acetate test	Methanolic	+
9	Proteins and aminoacids	Xanthoproteic Test	Methanolic	+
		Ninhydrin Test	Methanolic	+
10	Terpenes	Copper acetate Test	Methanolic	_

Key: (+) = Present and (−) = Absent

3.2 Total phenolic content determination

The methanolic crude extract of *A. solanacea* and itsdifferent soluble fractions i.e. Petroleum ether, chloroformand carbon tetra chloride were subjected to total phenolic content determination. Based on the absorbance values of the extract solution, the colorimetric analysis of the total phenolic of the extracts were determined and compared with the standard solutions of gallic acid equivalents (Table 2). Total phenolic content of the samples are expressed as mg of gallic acid equivalent (GAE)/ gm of dry extract. *A. solanacea* was found as a good source of total phenolic contents where crude methanolic, petroleumether, carbon tetra chloride and chloroform extract showedtotal phenolic contents of 58.35 µg of GAE/mg, 69.41 µg of GAE/mg, 37.41µg of GAE/mg and 10.82 µg of GAE/mg extracts respectively.

Table 2 Total Phenolic Content of *A. solanacea* crude extracts and its fractions

Ardisia solanacea	**Concentration (µg/ml)**	**Absorbance at 760 nm**	**Gallic Acid Equivalent (µg/mg)**
Methanolic Crude Extract	250	0.301	58.353
Petroleum ether fraction of Crude Extract	250	0.348	69.412
Carbon Tetrachloride soluble fraction of Crude Extract	250	0.212	37.412
Chloroform soluble fraction of Crude Extract	250	0.099	10.824

3.2.1 DPPH scavenging activity

DPPH free radical scavenging activity of crude extracts of *A. solanacea and* their different soluble fractions were found to be increased with the increase of concentration ofthe extract (Table 3).

Table 3 Comparative DPPH radical scavenging activity of different extracts of A. solanacea and standards of Ascorbic Acid (AA)

Concentration (µg/ml)	**Methanolic Crude Extract**	**Carbon tetra Chloride Extract**	**Petroleum Ether Extract**	**Chlorofo mExtract**	**Aquous Extract**	**Ascorbic Acid (Standard)**
500	78.94	66.44	84.92	66.93	64.55	97.43
250	76.12	64.61	80.60	62.57	58.97	83.99
125	70.84	59.08	75.56	51.13	46.22	72.41
62.5	62.48	54.11	67.27	38.56	37.92	65.49
31.25	46.85	39.18	54.71	20.19	23.44	59.25
15.625	20.09	15.42	25.96	12.73	20.47	56.81
7.813	12.73	11.45	13.97	10.80	14.34	54.39
3.90	6.76	8.18	10.34	7.06	11.60	49.49
1.953	3.07	7.65	7.72	6.01	7.78	41.79
IC_{50} (µg/ml)	52.11	91.34	40.04	157.39	174.13	14.64

Different partitions of methanolic extract of *A. solanacea* were subjected to free radical scavenging activity. Here, ascorbic acid was used as reference standard. In this investigation, the petroleum ether soluble fraction showed the highest free radical scavenging activity with IC_{50} value 40.04 µg/ml. At the same time the methanolic crude extract, Carbon tetrachloride, aqueous and chloroform soluble fractions also exhibited moderate antioxidant potential having IC_{50}value 52.11 µg/ml, 91.34 µg/ml, 174.13 µg/ml and 157.39 µg/ml respectively.

3.3 Antimicrobial activity

The antimicrobial potency of the test agents are measuredby their activity to prevent the growth of the microorganisms (at concentrations of 400 µg/disc.) surrounding the discs which gives clear zone of inhibition.After incubation, the Antimicrobial activities of the test materials such as Methanolic crude extract of ***A. solanacea***(MAS), Petroleum ether

fraction of crude extract (PEAS),Chloroform fraction of crude extract (CAS), Carbon tetra Chloride fraction of crude extract (CTCAS) and Aqueous fraction of crude extract (AAS) were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale (Table 4).

Table 4 Antimicrobial activity of Methanolic crude extract of A. solanacea and its different fractions

Types of micro-organism		**Samples of *A. solanacea* – zone of inhibition mm**					
		PEAS	**MAS**	**CTCAS**	**CAS**	**AAS**	**Ciprofloxacin**
Gram Positive Bacteria	*Bacillus subtilis*	8.2	12.9	Nil	Nil	10.4	41.1
	Staphylococcusaureus	10.8	9.7	7.8	Nil	9.8	32.3
Gram Negative Bacteria	*Pseudomonas aeruginosa*	Nil	12.7	Nil	Nil	Nil	41.3
	Salmonella typhi	8.9	12.4	Nil	7.4	10.7	36.2
	Escherichia coli	9.4	8.4	8.8	Nil	Nil	32.5
Fungi	*Candida albicans*	Nil	10.9	8.4	Nil	10.4	34.5
	Aspergillus niger	8.1	7.0	Nil	Nil	9.9	45.7

Here, Standard antibiotic disc of ciprofloxacin (5 µg/disc) was used for the comparison. The methanolic extract exhibited moderate activity againstthe growth of the most of the test organisms (Table 4), while other soluble fractions exhibited less than moderateinhibitory activity against the microorganisms and thechloroform extract exhibited poor inhibitory activity against the microorganisms (Table 4). The zones of inhibition produced by crude methanolic extract were ranged from 7-12.9 mm.

3.4 Cytotoxic activity

The LC_{50} values of crude methanol extract, petroleum ether, chloroform, carbon tetra chloride and aqueous extract of *A. solanacea* found to be 1.856, 0.7033, 8.331, 0.062 and 0.331 µg/ml, respectively (Table 5). The positive control vincristine sulphate showed LC_{50} at aconcentration of 0.544 µg/ml.

Table 5 Summary of the cytotoxicity in Brine Shrimp bioassay

Sr. No.	**Sample Name**	**Regression Line**	**R^2**	**LC_{50} value**
1	Vincristine (Positive Control)	y = 33.22x + 58.78	R^2 = 0.958	0.544
2	*Ardisia solanacea* Methanolic extract	y = 27.58x + 42.59	R^2 = 0.860	1.856
3	*Ardisia solanacea* Petroleum etherfraction	y = 29.39x + 29.33	R^2 = 0.944	0.7033
4	*Ardisia solanacea* Carbon TetraChloride fraction	y = 14.29x + 67.16	R^2 = 0.925	0.0629
5	*Ardisia solanacea* Chloroform fraction	y = 30.60x + 21.82	R^2 = 0.972	8.33
6	*Ardisia solanacea* aqueous fraction	y = 18.52x + 58.89	R^2 = 0.929	0.3311

4. Discussion

Recently, focus on plant research has increased throughoutworld [16,17,18]. Medicinal plants containing phytochemicals showa variety of pharmacological actions in human body [19]. In this study, phytochemical screening showed the presence of different phytochemicals. The presence ofpolyphenolic compounds such as flavonoids, phenols and tannins are responsible for antioxidant activity of the plantextracts [20]. For maintaining a healthy biological system, the balance between antioxidation and oxidation isbelieved to be critical [21,22,23]. In our investigation the plant extract showed moderate antioxidant activities with an IC_{50} which were compared with the values of standard drugs used. Antioxidant activities of different extracts of the bark of *A. solanacea* found to increase with the increasing concentration. Hence, the extracts of this plant could be used for the prevention of free radical-mediated diseases [3]. Since the present study

showed the presence of different bioactive secondary metabolites such as tannins,flavonoids, saponin and alkaloids, that singly or in combination may be responsible to treat microorganisms and insects. For this reason, the plant extract contains antimicrobial activity.

This plant also revealed good cytotoxic potency. It is believed that plant extracts containing a higher concentration of bioactive compounds show cytotoxic activity. Flavonoids show anti-allergic, anti-inflammatory, antimicrobial, and anticancer activities. Few studies tend to suggest that tannins may contain significant cytotoxic and antitumor potency [21,22].

5. Conclusion

In the context of the above discussion, it can be revealed that the extracts of bark of *A. solanacea* showed moderate antioxidant activity. These extracts also possess significant antimicrobial and cytotoxic activity. However, further investigations, based on these preliminary researches are required to explore the bioactive molecules which are responsible for the extracts'activities as well as their mechanisms of action.

Compliance with ethical standards

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Disclosure of conflict of interest

There is not any conflict of interest.

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