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Research Article

Evaluation of Antioxidant, Cytotoxic and Antimicrobial Potentials of *Anthocephalus Cadamba* (Roxb.) Leaves

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ABSTRACT
Considering the immense use in folk medicine, we persuaded to analyse the antioxidant, cytotoxic and antimicrobial properties as well as *in vitro* α-amylase inhibitory activity of methanolic extract of leaves of *Anthocephalus cadamba* (Roxb.)Miq (MELA). Total phenolics and flavonoids content of MELA were determined using colorimetric assay whereas its antioxidant properties were assessed by evaluating DPPH and ABTS scavenging abilities. Starch-iodine test was in preference to assess α-amylase inhibitory potential. In addition, cytotoxicity and antimicrobial activities were evaluated by brine shrimp lethality assay and disc diffusion methods. In this study, MELA contained high amount of polyphenolic compounds and showed remarkable antioxidant potential in all assays with IC_{50} value of 53.44 µg/mL and 22.02 µg/mL for DPPH and ABTS, respectively. Besides MELA inhibited α-amylase enzyme in a dose dependent manner and brine shrimp mortality rate was increased with increasing concentration. Additionally, the highest zone of inhibition was observed against both Gram-positive and Gram-negative bacteria at the concentration of 900 µg/µL. These findings revealed the importance of further investigation in isolating its active constituents.

KEYWORDS: *Anthocephalus cadamba*; leaves, antioxidant; antimicrobial; cytotoxic

INTRODUCTION

Diverse chemical structures and versatile pharmacological functions of secondary metabolites from natural sources are promising perspective in phytomedicine [1]. Plant kingdom is considered as an enormous source of potential drugs for healing as well as for curing many diseases caused by oxidative stress [2]. The underlying cause of oxidative stress is known to be reactive oxygen species (ROS) including free radicals such as superoxide radical anion, hydroxyl radicals, singlet oxygen, hydrogen peroxide and nitric oxide which are continuously produced in human cells [3,4]. These are the chemical entities capable to damage membrane lipids, proteins, enzymes and DNA [5]. In this sense, natural products have been attracting

scientific interest due to their antioxidant and chemopreventive properties. It is well-known that one of the main characteristics responsible for the antioxidant activity of a plant extract is its high content of phenolic compound and its ability to scavenge free radicals, which can play a part in the protection against the harmful action of ROS. Phenolic compounds also exhibit a wide range of other biological effects, such as anti-viral, antibacterial, and anti-tumor [6-10].

Anthocephalus cadamba (Roxb.)Miq, belongs to the family of Rubiaceae, is commonly known as Kadam in Indian subcontinent including Bangladesh. Various parts of this plant are used as folk medicine in the treatment of tumor, inflammation, fever, hematological diseases, skin diseases, leprosy and hypoglycemic agent [11]. Until now, scientific bioactivity determination studies have revealed the antimalarial [12], antihepatotoxic [13], anti-inflammatory [14] and antidiabetic [15] activities of *A. cadamba*. But no scientific evidences on the antioxidant, cytotoxic and antimicrobial properties of *A. cadamba* leaves have been available till now. Based on this fact, this study is an attempt to quantitively determines phytochemicals of *A. cadamba* leaves and to investigate its antioxidant, cytotoxicity, α-amylase inhibitory and antibacterial potentials.

MATERIALS AND METHODS

Plant materials

Leaves of *A. cadamba* were selected as plant samples to evaluate their phytochemical quantities and their antioxidant potentials. The plant samples were collected from campus area of University of Rajshahi. The taxonomic authentication was confirmed in department of Botany, University of Rajshahi, Bangladesh. The fresh leaves were dried in shade for a week and then crushed into powder in a mechanical blender.

Extraction

The powdered plant materials (50g/300 mL) were extracted with methanol at room temperature for 72 hours. The solution was filtered using Whatman filter paper No. 1 and residual solvent was evaporated completely. The crude methanolic extract of leaves of *A. cadamba* was (designated as MELA) weighed (3g) and kept in glass vial at 4◦ C for further use.

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•), Potassium persulfate, AlCl3, Folin-Ciocalteus's phenol reagent, sodium carbonate and Methanol were purchased from Sigma-Aldrich, Germany. Catechin, gallic acid were obtained from Wako pure chemicals Ltd, Japan; ascorbic acid were obtained from E-Merck, Germany.

Determination of total phenolics and total flavonoids content

The total phenolics content of MELA was determined using Folin-Ciocalteu reagent method [16]. The absorbance was measured at 725 nm. A calibration curve was constructed for Gallic acid that showed linearity in the range of 25-400 µg/mL concentration and results was expressed as mg of Gallic acid equivalent per gm of dried extract.

The total flavonoids content of MELA was estimated by an $AlCl_3$ colorimetric assay described by Dewanto et al. [17], using Catechin as a reference standard (1 mg/mL). The absorbance was read at 510 nm. The total flavonoid content was calculated from the standard curve (25-400µg/mL) and expressed in terms of catechin equivalent per gram of dried extract. Both assays were repeated three times

DPPH and ABTS radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging activity of MELA was assayed according to the method described by Mohsen and Ammar [18] with minor modification. 1mL different concentration of MELA in methanol was added to 3 mL of 0.1 mM DPPH solution. After incubating for 30 minutes at room temperature in dark, the discoloration of reaction mixture was measured spectrophotometrically at 517 nm using methanol as control. Ascorbic acid was used as a positive control.

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•+) radical scavenging activity was determined following the procedure described by Cai et al. [19]. ABTS•+ solution (3 mL) was added to 1 mL of MELA with various concentrations and mixed vigorously. The absorbance was measured at 734 nm after standing for 6 minute. Ascorbic acid was used as a positive control. All samples were analyzed in triplicate for both assays.

Percentage of radical scavenging was calculated using the following equation

scavenging effect(%) = $[(A_{control} - A_{sample}) / A_{control})] \times 100$

where $A_{control}$ is the absorbance of control (without test sample) and A_{sample} is the absorbance of extractives/standard. The IC_{50} value represents the effective concentration of the extract that caused 50% of the neutralization of the radical. Percent (%) of inhibition was plotted against concentration and IC_{50} was calculated from the nonlinear regression curve.

α-Amylase inhibition assay

Screening for α-amylase inhibitory potential of MELA was carried out according to Xiao et al. [20] with slight modification based on the starch-iodine test. Different concentrations of 300 µL MELA (1mg/mL) were added to 200 µL of α-amylase solution following addition of 500 µL starch solution. After incubation at 37°C for 1 hour, 500 µL HCl and 500 µL of iodine reagent were added. The absorbance was read at 620 nm. This test was performed in triplicate for MELA. The results were expressed as % inhibition and calculated using the following formula:

% Inhibition of α-amylase activity = $[(A_S - A_{B1})/(A_{B2} - A_{B1})] \times 100$

Where A_S is the absorbance of MELA, A_{B1} is the absorbance of blank with α-Amylase solution and A_{B2} is the absorbance of blank without α-Amylase solution.

Brine shrimp lethality bioassay

For the determination of cytotoxic property of MELA, brine shrimp lethality bioassay was carried out according to the method proposed by Meyer et al. [21]. In a nutshell, 10 mg of extract was dissolved in 1% DMSO with 10 ml of distilled water. Five doses (25, 50, 100, 200 and 400 µg/mL) of MELA were added to the sea water (5 mL) containing 10 living nauplii. After 24-hours of incubation, the percentage of mortality of the nauplii was calculated for each doses and the LC_{50} value was determined using probit analysis.

Antibacterial assay

Disc Diffusion Method was used to explore the antibacterial activity of MELA [22]. Eight pathogenic bacteria, including Gram-positive (*Bacillus cereus, Streptococcus mutans, Staphylococcus aureus, Bacillus subtilis*) and Gram-negative (*Shigella brodie, Shigella Soniia, Escherichia coli, Shigella dysentery*) were selected for the antibacterial activity test. Azithromycin disc (15 µg/disc) was used as positive control, whereas blank disc saturated with methanol was used as negative control. Sample discs were impregnated with MELA at the concentration of 300 µg/µL, 600 µg/µL and 900 µg/µL. Finally, the plates were incubated at 37^0C for 24 h in an incubator and afterwards, diameter of the zone of inhibition surrounding each disc was recorded. All determinations were carried out in triplicate

Statistical analysis

The data was subjected to one-tailed paired *t*-test and the significance of the difference between means was calculated using SPSS software of 15 version. Values are expressed as mean of independent samples analyzed ± standard deviation (SD).

RESULTS

Total phenolics and flavonoids content

The total phenolics and flavonoids content of MELA were 228.893±3.48 mg of GA equivalent/g of dry extract and 187.655±2.38 mg of Catechin equivalent/g of dry extract, respectively (Figure 1). The result clearly indicates the higher polyphenolic content in MELA.

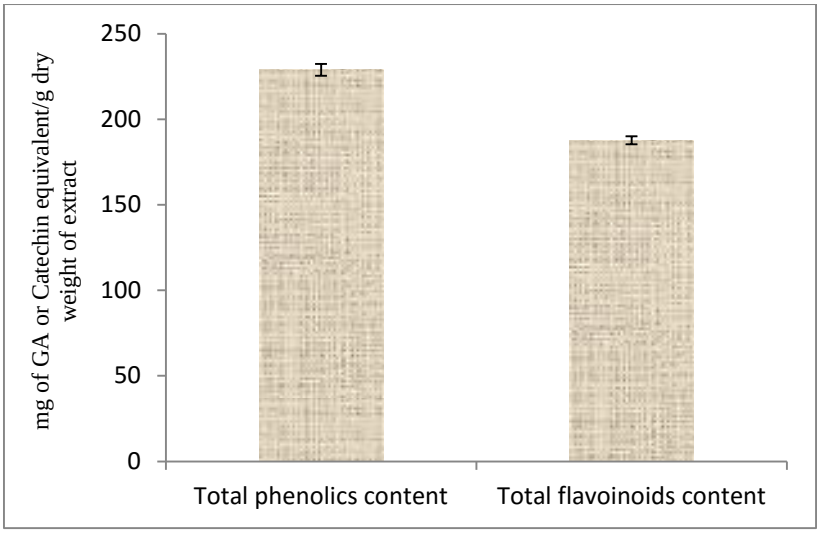

Fig. 1: Total Phenolics and total flavonoids content of MELA

Antioxidant properties

MELA showed moderate scavenging potentiality in comparison to ascorbic acid against free radicals investigated namely DPPH and ABTS. In each assay, significant changes were observed with increasing concentrations of extract. The IC_{50}values of MELA were 53.44 µg/mL and 22.02 µg/mL for DPPH and ABTS, respectively, whereas ascorbic acid exhibited potent scavenging activity in all the above mentioned assays (Table1).

Table 1: Antioxidant properties of MELA

Sample	DPPH• scavenging activity			*P value	ABTS•+ scavenging capacity			tP value
	Concentration (µg/mL)	Scavenging effect (%)	IC_{50} (µg/mL)		Concentration (µg/mL)	Scavenging effect (%)	IC_{50} (µg/mL)	
MElA	3.13	7.28±0.4849	53.44± 5.15	< 0.05	1.57	13.09±0.14	22.02 ± 1.69	> 0.05
	6.25	9.52±0.4214			3.13	17.02±0.53		
	12.5	12.2±0.9699			6.25	22.93±0.71		
	25	25.41±1.249			12.5	42.49±0.98		
	50	47.85±0.2967			25	60.46±0.93		
	100	90.31±1.0390			50	75.23±1.25		
Ascorbic Acid	3.13	15.36±0.84	19.03± 1.79		1.57	14.24±0.45	16.94 ± 2.17	
	6.25	24.41±0.72			3.13	18.32±0.10		
	12.5	48.18±0.60			6.25	35.81±0.94		
	25	86.31±0.41			12.5	57.50±1.84		
	50	93.43±0.42			25	73.17±0.27		
	100	97.19±0.59			50	95.86±0.40		

*Data are expressed as mean ± SD (standard Deviation) of three independent experiments. P values are determined using one-tailed paired t-test. *P: between MELA and ascorbic acid in DPPH assay and tP: between MELA and ascorbic acid in ABTS assay*

α-amylase inhibition

In starch iodine color assay, MELA inhibited α-amylase enzyme in dose dependent manner. The different concentrations of MELA and Acarbose (used as standard) exhibited inhibitory potential and produced the IC_{50} values of 1647.2 µg/mL and 73.75 µg/mL, respectively (Table 2).

Table 2: α-Amylase inhibitory potential of MELA

Sample	α-amylase enzyme inhibition			*P-value
	Concentration (µg/mL)	Percentage of inhibition (%)	IC_{50} (µg/mL)	
MELA	300	4.68 ± 0.38	1647.2 ± 35.5	< 0.001
	600	15.13 ±0.63		
	800	30.69 ± 0.53		
	1200	44.10 ± 1.23		
	2000	55.57 ± 0.69		
Acarbose	30	4.73 ± 0.21	73.75 ± 1.51	
	40	9.66 ± 0.21		
	50	22.18 ± 1.74		
	60	31.13 ± 0.41		
	70	44.97 ± 0.86		
	80	60.68 ± 1.14		

Data are expressed as mean ± SD (standard Deviation) of three independent experiments.
**P values were calculated using one-tailed paired t-test.*

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay was performed to evaluate the cytotoxic effect of MELA. MELA demonstrated moderate cytotoxic activity with LC_{50} value of 871.39 µg/mL. A positive correlation was found between brine shrimp mortality rate and concentration of extract.

L, 600 µg/µL and 900 µg/µL, with the highest value being seen for *Shigella dysentery* (Table 3).

Antibacterial activity

In comparison to Azithromycin, MELA showed moderate broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria *in vitro* study. The zone of inhibitions was observed in the range of 6.5–14.5 mm for concentrations 300 µg/µ

Table 3: Antibacterial activity of MELA

Name of bacteria	Zone of inhibition (mm)				P-value		
	MELA			Azithromycin			
	300 µg/µL	600 µg/µL	900 µg/µL	15 µg/disc	[a]P	[b]P	[c]P
Shigella brodie	8 ± 1.41	9.5 ± 0.71	11 ± 1.41	31 ± 1.41	< 0.01	< 0.01	< 0.01
Bacillus cereus	7 ± 2.03	9 ± 2.81	11 ± 2.82	33 ± 4.24	< 0.01	< 0.01	< 0.01
Shigella Soniia.	7 ± 1.40	9 ± 1.41	14 ± 1.41	32.5 ± 2.12	< 0.05	< 0.05	< 0.05
Escherichia coli	7.5 ± 0.70	10 ± 1.41	11 ± 2.82	36.5 ± 2.12	< 0.001	< 0.05	< 0.05
Shigella dysentery	7 ± 1.34	11 ± 1.41	14.5 ± 2.12	31 ± 2.82	< 0.01	< 0.01	> 0.01
Streptococcus mutans	7 ± 1.42	10 ± 2.24	14 ± 2.82	30 ± 1.41	< 0.01	< 0.01	> 0.01
Staphylococcus aureus	6.5 ± 0.71	9 ± 1.34	11.5 ± 2.12	28 ± 2.83	< 0.05	< 0.05	> 0.01
Bacillus subtilis	8 ± 2.82	10.5 ± 2.12	12 ± 2.82	30.5 ± 2.07	< 0.01	< 0.01	< 0.01

Data are presented as the mean ± SD (standard Deviation) of three independent experiments. P-values were calculated using one-tailed paired t-test.
[a]P: between MELA (300 µg/µL) and Azithromycin (15 µg/disc);
[b]P: between MELA (600 µg/µL) and Azithromycin (15 µg/disc)
[c]P: between MELA (900 µg/µL) and Azithromycin (15 µg/disc)

DISCUSSION

Phenolic and flavonoid compounds are the most biologically active secondary metabolites in plants and several reports have indicated that free radical scavenging activity of plants is greatly influenced by its phenolic and flavonoid contents [23-25]. Due to the presence of hydroxyl groups, these type of compounds act as reducing agents, hydrogen donors and singlet oxygen quenchers [26]. Therefore, phenolic and flavonoid compounds intercept free radicals by scavenging or trapping methods [27]. In this study, MELA contained a rich amount of phenolic and flavonoid compounds (Figure 1) and the presence of high

amount of polyphenols and flavonoids confer MELA to show effective antioxidative potential against DPPH and ABTS radicals (Table 1). There is a linear correlation between the total phenolic content of MELA and its DPPH and ABTS radical scavenging activities, with R^2 of 0.929 and 0.975, respectively. These results are in full agreement with previous studies, which proved that there is a linear correlation between the total phenolic content and the antioxidant capacity of some medicinal plants [28-30].

Furthermore, MELA showed relatively moderate *in vitro* α-amylase inhibitory activities (Table 2) compared to standard acarbose thereby indicating it's *in vitro* antidiabetic potentials. Relevant result was reported by Belal et al. [31]. Moreover, the brine shrimp lethality bioassay indicated the moderate cytotoxic effect of MELA with high LC_{50} value. However, the mild to moderate broad spectrum antibacterial activity of MELA (Table 3) can be attributed to its high phenolics and flavonoids content, because phenolics and flavonoids can complex with extracellular and soluble proteins as well as with bacterial cell wall [32]. These findings were consistent with the results obtained from a research where strawberry tree (*Arbutus unedo* L.) leaf extracts moderately inhibited bacteria as a dose dependent manner [33].

CONCLUSION

In conclusion, results of this study explore the presence of bioactive principles in leaves of *A. cadamba* which might be a potential source of antioxidant and antibacterial molecules. But as a limitation of this study, we did not identify the main active compounds responsible for the activities assessed here. Our prospect goal is to investigate the isolation and characterization of these lead compounds accountable for the above mentioned activity of this plant.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this review article.

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