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Antimicrobial and Antioxidant Efficacy of Aqueous Extract of *Anthocephalus cadamba* Leaves

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Hot aqueous extract (HAE) of leaves of *Anthocephalus cadamba* (Roxb.) Miq, Rubiaceae has been screened for antimicrobial and antioxidant activity. Leaf extract was tested against some gram positive, gram negative and certain fungal strains. Leaf extract showed significant ($p < .01$) antibacterial and antifungal activity against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aureginosa* and *Aspergillus niger*. Ofloxacin and amphotericin-B were used as standard with respect to bacteria and fungi. MIC of leaf extract was 0.039 mg/ml for *S. aureus*, 0.078 mg/ml for *B. cereus* whereas, 0.156mg/ml for *E. coli, P. aureginosa* and *A. niger*. In-vitro antioxidant activity of the extract was evaluated by 2,2-diphenylhydrazyl free radical scavenging assay. Significant ($p < .01$) antioxidant activity in term of percentage inhibition of free radicals by the extract was found at 0.25 mg/10ml, 0.50 mg/10ml and 1.0 mg/10ml concentrations when compared with standards (ascorbic acid and butylated hydroxyl toluene)

Key words: *Anthocephalus cadamba,* antimicrobial activity, phytochemicals, antioxidant, DPPH free radical scavenging assay.

Anthocephalus cadamba Miq., also known as kadam, is a tropical tree species that is native to South Asia and Southeast Asia, including Indonesia [1]. In India, it is found on the slopes of evergreen forests up to 500 meters. It is commonly distributed in the sub-himalayan tract from Nepal eastwards on the lower hills of Darjeeling terai in West Bengal; in Chota Nagpur (Bihar), Orissa, and Andhra Pradesh; in damp places along large streams in the Andamans; in Karnataka and Kerala on the west coast, and at low level in wet places of the western ghats [2].

In folk medicine *Anthocephalus cadamba* has been used for the treatment of skin disease, leprosy, dysentery, blood disease, anaemia, utrine complaints, fever, colitis, menorrhagia and in improvement of semen quality [3,4]. Analgesic, anti-inflammatory, antipyretic [5], anthelmintic [6], wound healing [7], antihepatotoxic [8], diuretic, laxative[9] activity of *Anthocephalus cadamba* has been investigated. The medicinal properties are attributed to be outcome of phytochemicals present in *Anthocephalus cadamba*. Phytochemical screening of various parts of this plant revealed out the presence of saponins [10, 11], alkaloids-cadamine and isocadamine [12], quinoline [13], secoiridoid glucoside [14], triterpenes [15]. Therefore an attempt has been made to investigate antimicrobial and antioxidant efficacy of hot aqueous extract of *Anthocephalus cadamba* leaves in order to provide scientific support to the folklore claims.

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EXPERIMENTAL

Plant Material and Extract preparation

Anthocephalus cadamba leaves were collected from vrindavan, Mathura (U.P) at the month of july 2015. The leaves were authenticated

by Agharkar research institute, Pune with voucher deposition no.L-084 by Dr. A.S. Upadhye. Authenticated fresh leaves were collected in mass, washed, dried under shade and grinded to obtain coarse powder.

About 200-250 gm coarse powder of shaded dried leaves were subjected to hot aqueous extraction with triple distilled water in a Soxhlet apparatus at 100°C for 2-3 hours. The extracted solution was evaporated in a rotary evaporator under controlled temperature and reduced pressure to get a dark brownish crystals (yield: 17-21%, w/v) and was stored at 4°C for further use.

Phytochemical Screening

Preliminary phytochemicals screening was done by for qualitative analysis of secondary metabolites using standard protocols [16]. Various chemical tests were performed to detect the presence of steroids, alkaloids, flavonoids, glycosides, phenolic compounds, triterpenoids, proteins, carbohydrates. The results are shown in Table 1.

Microorganisms

Gram positive (*Bacillus cereus*: MTCC-1306 and *Staphylococcus aureus*: MTCC-9760) and Gram negative (*Escherichia coli*: MTCC-1563 and *Pseudomonas aureginosa*: MTCC-8076) were used along with two fungi (*Aspergillus niger*: MTCC-0872 and *Candida krusei*: MTCC-9215) for antimicrobial assay. These strains were identified and procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Antimicrobial activity

The agar disc diffusion method [17] was used to investigate antimicrobial activity. Nutrient agar medium (Hi Media) was used for *B. cereus*, *S. aureus*, *E. coli* and *P. auregenosa*. Potato Dextrose agar medium (Hi Media) was used for *A. niger* and *C. krusei*. One – two loopful growth was taken from fresh bacterial culture, allowed to incubate at 37°C for 6 hrs after inoculating into 5 ml of nutrient broth, which was further centrifuged at 30000 rpm for 10 minutes. After washing with normal saline bacterial pellet was further suspended in 5 ml of normal saline kept in test tube, which was further adjusted to 5 x 10^6 CFU/ml (standardized 0.5 McFarland Nephelometer) and after giving incubation at 28°C for 72 hrs in Potato Dextrose Broth, 10^4 spore/ml of fungi was taken for antimicrobial activity.

Dried and sterilized filter paper discs (5 mm diameter) were impregnated with 10 µl of HAE of *Anthocephalus cadamba* at 125 mg/ml, 250 mg/ml, 500 mg/ml and 1000 mg/ml concentrations. Discs seeded with different concentrations of HAE were placed at even distance over nutrient agar medium with test microorganisms placed on culture plates. Standard disc of ofloxacin (10 µg/discs) against bacteria and amphoterecin-B (10 µg/discs) against fungi were taken as positive control while discs impregnated with distilled water was used as negative control. The culture plates with discs were then incubated at 37°C for 24-48 hrs. The antimicrobial activity of extract at different concentrations was determined by measuring the diameter of zone of inhibition including disc with 5 mm diameter. Each experiment was performed in triplicates at each concentration.

MIC of a HAE was considered as a minimum concentration at which no growth of bacteria was observed and was determined by transferring a loop of microbial culture to nutrient broth and kept at 37°C for 26 hrs for growth. Concentration of microorganism was confirmed using UV-Vis Spectrophotometer at 660-665nm. Cell count was adjusted to 1×10^8 cells/ml by providing extra growth period in case count is less and by dilute if the growth was high. 100ml of broth was prepared and stock solution (3.75 mg/ ml) of plant extract (HAE) was prepared. 1 ml of stock was dissolved in 2ml of fresh broth represent as A in a test tube, mixed gently with the help of vertex and then dilution was prepared as follows: 1 ml of A is transferred to another 1ml of broth (B), 1 ml of B in another 1 ml of broth (C), 1ml of C in another 1 ml of broth and it is followed up to 10 test tubes by following dilution method through proper mixing with the help of vertex after each dilution and then 0.5ml of microbial culture was added in each test tube. After this test tube were incubated at 37°C for 18 hrs and observed for bacterial growth and then OD was taken at 660-665nm. Inoculate a tube containing 2ml broth with the organism and keep at 4°C in a refrigerator overnight to be used as standard for the determination of complete inhibition and take absorbance.

Antioxidant activity

DPPH Free Radical Scavenging Assay

The free radical scavenging capacity of HAE of *A.cadamba* leaves was performed with the

methodology described by [18] as elaborated by [19]. Chemicals used were 2,2-diphenyl-1-picrylhydrazyl (DPPH, make :Sigma Aldrich), ascorbic acid (Merck), butylated hydroxyl toluene (BHT) and other chemicals were of analytical grade. DPPH solution (.1mM) was prepared and various concentrations (0.25 mg/10ml, 0.50 mg/10ml, 1.00 mg/10ml and 2.00 mg/10ml) of HAE of *A. cadamba* leaves were screened for antioxidant activity. 3 ml of DPPH was mixed to 0.1 ml of HAE at different concentrations. Mixture was allowed to incubate in dark at 20°C for 40 min.

Absorbance was measured at 517 nm using UV–Vis spectrophotometer with ethanol and water (1:1) as blank. Each experiment was performed in triplicates at each concentration. [20] proposed formula to determine scavenging capacity of DPPH radical.

% inhibition of DPPH = [(Ac-At) / Ac] x100

Where Ac is the absorbance of control (DPPH) and At is the absorbance of HAE of *A. cadamba* leaves. BHT and ascorbic acid were used as standards.

Statistical Analysis

Statistical analysis of data was done using one way analysis of variance (ANOVA) using SPSS version 20.0 software and DMRT at $p < .05$ and .01 to determine significant differences among treatment means. Values are expressed as mean ± S.E.M.

RESULTS AND DISCUSSION

The yield of HAE through soxhlet extraction method was found to be 17-21% with dark brownish crystals. HAE was subjected to phytochemical screening, antimicrobial activity and antioxidant activity. The result of the phytochemical screening of HAE of *A.cadamba* leaves showed the presence of glycosides, flavonoids, fats, oils, tannins, phenolic compounds, alkaloids, carbohydrates, anthraquinones, saponins and triterpenoids as indicated in Table 1. Gums, mucilages, proteins and amino acids were found to be absent.

Extract exhibit significant ($p < .01$) antibacterial and antifungal activity against (*B. cereus*, *S. aureus* , *E. coli* and *P. aureginosa* and *A. niger)* except *C. krusei* as shown in Table no. 2, 3, 4 and 5 after 24 and 48 hrs of incubation. Among the test organism *S. aureus* was found to be most sensitive (Fig. 1) followed by *E. coli and P. aeruginosa* (Fig. 2). Dose dependent activity was observed against all test organisms except *C. krusei* (Fig.1, 2 & 3). HAE with concentration 10.0 mg/disc was found to be most effective (superscript

Table 1. Qualitative analysis of phytochemicals present in HAE of *A. cadamba* leaves

Extract	Sterols		Triterpenoids	Glycosides			Carbohydrates	Alkaloids			Flavonoids		Tannins and Phenolics		Proteins and Amino acids	Gum and Mucilage's	Fats and Oils
	Salkowski's	Liberman Burchard's	Salkowski's	Legal's	Haemolysis	Borntrager	Molisch's	Mayer's	Hager's	Wagner's	Shinoda	Alkalinereagent	5% $FeCl_3$	Bromine water	Ninhydrin	Ruthenium red	Saponification
HAE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+

[b] or [c]) with respect to other doses like 1.25 mg/disc (superscript [a]), 2.50 mg/disc (superscript [ab] or [b]) and 5.00 mg/disc (superscript [ab] or [b]or [bc]) as given in Table no. 2, 3, 4 and 5. Positive control- ofloxacin and amphoterecin-B show significant activity (superscript [c] or [d]). Zone of inhibition against bacterial and fungal strains was found to increase at 48 hrs of incubation when compared after 24 hrs of incubation. This suggested bacteriostatic and probable bacteriocidal nature of HAE. Confirmation of bacteriocidal potency of HAE can be achieved by comparing zone of inhibition after 24, 48 & 72 hrs of incubation. Present study depicts significant ($p < .01$) zone of inhibition as compared with result

Table 2. Antibacterial Activity of HAE (Disc Diffusion Method) - After 24Hrs

Name of the Organism	Zone of Inhibition (mm)				
	1.25mg/disc	2.5 mg/disc	5.0 mg/disc	10.0 mg/disc	Ofloxacin 10 µg/disc(Standard)
S. aureus	$10.00^{a}\pm1.00$	$10.67^{ab}\pm0.33$	$11.00^{ab}\pm0.58$	$12.67^{b}\pm0.33$	$32.33^{c}\pm0.88$
B. cereus	$7.33^{a}\pm0.33$	$9.67^{b}\pm0.88$	$10.33^{bc}\pm0.67$	$12.00^{c}\pm0.58$	$30.67^{d}\pm0.67$
E. coli	$4.67^{a}\pm0.33$	$9.33^{b}\pm0.33$	$10.00^{b}\pm0.58$	$10.67^{b}\pm0.33$	$31.00^{c}\pm0.58$
P. aureginosa	$5.67^{a}\pm0.33$	$8.67^{b}\pm0.33$	$8.67^{b}\pm0.33$	$9.67^{b}\pm0.33$	$33.33^{c}\pm0.67$

The values represent the mean ± SEM of triplet experiments. Statistical analysis through one way ANOVA followed by DMRT revealed that results are significant at $p < .01$.Same superscript at various concentrations of HAE with each strain indicate no significant difference between values where as variation in the superscript in increasing order of alphabets indicates proportionate difference at $p < .01$

Table 3. Antibacterial Activity of HAE (Disc Diffusion Method) - After 48Hrs

Name of the Organism	Zone of Inhibition (mm)				
	1.25mg/disc	2.5 mg/disc	5.0 mg/disc	10.0 mg/disc	Ofloxacin 10 µg/disc(Standard)
S. aureus	$10.33^{a}\pm0.67$	$11.67^{ab}\pm0.33$	$13.00^{bc}\pm0.58$	$14.67^{c}\pm0.67$	$34.00^{d}\pm1.00$
B. cereus	$8.33^{a}\pm0.33$	$11.00^{b}\pm0.58$	$12.33^{bc}\pm0.33$	$13.00^{c}\pm1.00$	$31.67^{d}\pm0.33$
E. coli	$7.33^{a}\pm0.67$	$10.33^{b}\pm0.33$	$11.33^{b}\pm0.88$	$12.00^{b}\pm1.00$	$33.00^{c}\pm0.58$
P. aureginosa	$7.67^{a}\pm0.33$	$11.33^{b}\pm0.33$	$12.67^{b}\pm0.33$	$21.33^{c}\pm0.33$	$34.67^{d}\pm0.88$

The values represent the mean ± SEM of triplet experiments. Statistical analysis through one way ANOVA followed by DMRT revealed that results are significant at $p < .01$.Same superscript at various concentrations of HAE with each strain indicate no significant difference between values where as variation in the superscript in increasing order of alphabets indicates proportionate difference at $p < .01$

Table 4. Antifungal Activity of HAE (Disc Diffusion Method)- After 24Hrs

Name of the Organism	Zone of Inhibition (mm)				
	1.25mg/disc	2.5 mg/disc	5.0 mg/disc	10.0 mg/disc	Amphotericin-B 10 µg/disc(Standard)
A. niger	$5.67^{a}\pm0.33$	$8.00^{b}\pm0.58$	$8.00^{b}\pm0.58$	$10.00^{c}\pm0.58$	$11.33^{c}\pm0.33$
C. krusei	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	$13.33^{b}\pm0.88$

The values represent the mean ± SEM of triplet experiments. Statistical analysis through one way ANOVA followed by DMRT revealed that results are significant at $p < .01$.Same superscript at various concentrations of HAE with each strain indicate no significant difference between values where as variation in the superscript in increasing order of alphabets indicates proportionate difference at $p < .01$

of previous study carried by researchers [21]. Significant antimicrobial activity in the HAE extract of *A. cadamba* leaves may be due to the synergistic effects of tannins, flavonoids, terpenoids, saponins and steroids through different mechanism. Tannins blocks cell wall synthesis by the formation of stable complexes with prolein rich protein [22]. Leakage of proteins and certain enzymes of cell occur due to saponins [23]. Terpenoids causes disruption of the cell wall of microorganism by weakening the membranous tissue [24]. Flavonoids are effective antimicrobial substances and have ability to complex with extracellular and soluble proteins and to bind with bacterial cell walls [25]. Steroids as antibacterial agents specifically bind with membrane lipids and cause leakage from liposomes [26]. MIC of HAE against bacterial and fungal strains was determined by broth dilution method and readings were taken at 660- 665 nm. MIC value of HAE against *S. aureus* was found to be low (0.039 mg/ml) in comparison to MIC value against *B. cereus* (0.078 mg/ml), *E. coli* (0.156 mg/ml), P. *aureginosa* (0.156 mg/ml) & *A. niger* (0.156 mg/ml) as shown in Table no. 6

Significant ($p < .01$) antioxidant activity in term of percentage inhibition of free radicals using DPPH free radical scavenging method was found at different concentrations of HAE when compared with ascorbic acid and BHT as shown in Table 7. Percentage inhibition was found to be dose dependent. HAE brings about 73.1% DPPH radical scavenging activity in comparisons to ascorbic acid (12.56%) and BHT (19.72%) at same concentration (.25 mg/10ml) and even more percentage inhibition with HAE was found with respect to ascorbic acid and BHT at .50 mg/10ml, 1.00mg/10ml and 2.0 mg/10ml concentration (Fig. 4). DPPH radical scavenging activity of ascorbic acid was found to be more (93.37%) when compared with HAE (86.23%) and BHT (75.88%) at 2.0 mg/10ml concentration as given in Table 7.

Present study on in-vitro antioxidant activity of HAE of *A. cadamba* leaves reveals out the fact that aqueous extract shows greater

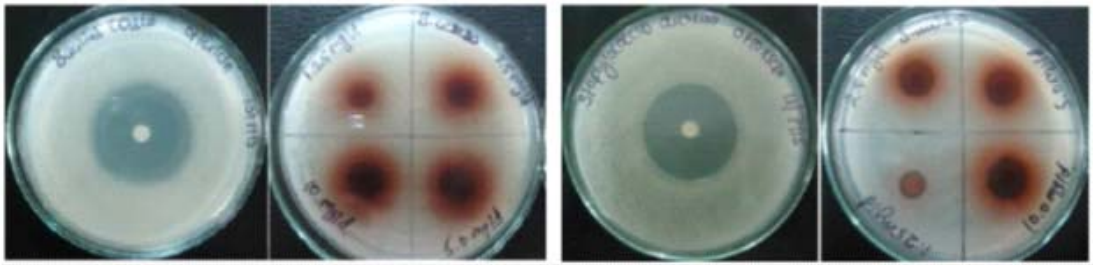
Table 5. Antifungal Activity of HAE (Disc Diffusion Method) - After 48Hrs

Name of the Organism	Zone of Inhibition (mm)				
	1.25mg/disc	2.5 mg/disc	5.0 mg/disc	10.0 mg/disc	Amphotericin-B 10 µg/disc(Standard)
A. niger	7.00^{a}±0.00	8.67^{b}±0.33	9.00^{b}±0.00	11.33^{c}±0.33	12.00^{c}±0.58
C. krusei	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	5.67^{b}±1.45

The values represent the mean ± SEM of triplet experiments. Statistical analysis through one way ANOVA followed by DMRT revealed that results are significant at $p < .01$.Same superscript at various concentrations of HAE with each strain indicate no significant difference between values where as variation in the superscript in increasing order of alphabets indicates proportionate difference at $p < .01$

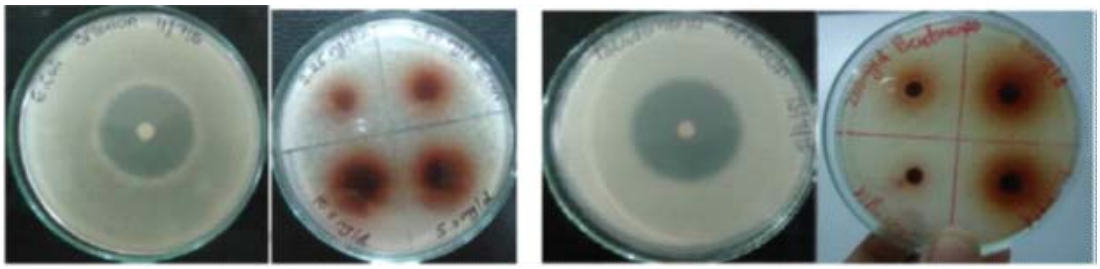
Table 6. MIC of HAE against Bacterial Strains and Fungal strains

S. No	Concentration (mg/ml)	Absorbance				
		S. aureus	*B. cereus*	*E. coli*	*P. aureginosa*	*A. niger*
1.	1.250	0.009	0.007	0.008	0.006	0.011
2.	0.625	0.007	0.009	0.010	0.008	0.010
3.	0.312	0.010	0.011	0.012	0.009	0.009
4.	0.156	0.008	0.007	0.009	0.012	0.008
5.	0.078	0.008	0.008	0.193	0.209	0.226
6.	0.039	0.012	0.213	0.247	0.257	0.278
7.	0.019	0.174	0.286	0.288	0.298	0.304
8.	0.009	0.233	0.324	0.341	0.362	0.353
9.	0.004	0.297	0.369	0.384	0.391	0.381
10.	0.002	0.316	0.403	0.422	0.443	0.416



B. cereus (+ve control) *S. aureus* (+ve control)

Fig. 1. Antibacterial activity of HAE against gram +ve bacteria (*B. cereus and S. aureus*)



E. coli (+ve control) *P. aureginosa* (+ve control)

Fig. 2. Antibacterial activity of HAE against gram -ve bacteria (*E. coli and P. aureginosa*)

A. niger (+ve control) *C. krusei* (+ve control) (No Zone of Inhibition)

Fig. 3. Antifungal activity of HAE against *A. niger* and *C. krusei*

Table 7. Antioxidant activity of HAE, Ascorbic acid and BHT using DPPH free radical scavenging method in term of percentage inhibition of free radicals

S. No.	Concentration(mg/10ml)	HAE	Ascorbic acid	BHT
1.	0.25	73.10^{c}±1.02	12.56^{a}±0.16	19.72^{b}±0.71
2.	0.50	80.12^{c}±0.82	24.31^{a}±0.97	43.25^{b}±0.64
3.	1.00	84.61^{c}±0.81	42.82^{a}±0.30	55.74^{b}±1.07
4.	2.00	86.23^{b}±1.12	93.37^{c}±0.02	75.88^{a}±0.80

The values represent the mean ± SEM of triplet experiments. Statistical analysis through one way ANOVA followed by DMRT revealed that results are significant at $p < .01$.Same superscript at various concentrations of HAE, Ascorbic acid and BHT indicate no significant difference between values where as variation in the superscript in increasing order of alphabets indicates proportionate difference at $p < .01$

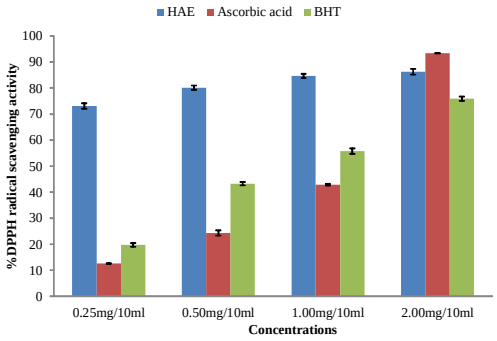


Fig. 4. DPPH radical scavenging activity of the various concentrations of HAE, Ascorbic acid and BHT

antioxidant activity as compared to ethanolic, chloroform and carbon tetrachloride extract of *A. cadamba* leaves [27]. Oxidation in cell will result in the generation of free radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3). Free radicals causes disruption of cell membranes, enzymes, fats, nucleic acids and brings about several disorders like Parkinson's disease, Alzheimer's disease and myocardial infarction [28, 29]. Antioxidant compounds counter free radicals mediate oxidative stress in the cell [30]. Presence of compounds viz. Chlorogenic acid, Catechin/ epicatechin, Kaemferol, indole alkaloids, β –sitosterol in leaves extract of *A .cadamba* may be responsible for its antioxidant activity [31].

CONCLUSION

The aqueous extract of *A. cadamba* exhibit significant antimicrobial activity which scientifically validate the use of this plant in folk medicines for the treatment of various bacterial and fungal diseases. Powerful antioxidant activity of plant extract makes it a very potent source of natural antioxidants which may be useful in pharmaceutical application. Results encouraged for further studies to evaluate possible synergistic effects among the extract components for their antimicrobial and antioxidant potential.

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