Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(4):372-380

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Pharmacological properties, phytochemical and GC-MS analysis of *Bauhinia acuminata* Linn.

Anju Krishna S. R., Hafza S., Poorna Chandrika G., Lekhya Priya C. and Bhaskara Rao K. V.*

Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

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ABSTRACT

The current study was designed to perform phytochemical screening, antimicrobial, antioxidant, hemolytic activities and GC-MS analysis of B. acuminata leaves and stem extracts. Antimicrobial activity for hexane, chloroform, ethyl acetate, methanol and aqueous extracts were screened by agar well diffusion method against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium and Aspergillus fumigates, Aspergillus niger. To check the antioxidant status, DPPH radical scavenging, lipid peroxidation inhibition, reducing power and total antioxidant activities were performed. Hemolytic activity was evaluated by Spectrophotometric method. Phytochemical screening showed the presence of carbohydrates, phenolic compounds, saponins, flavonoids, oils and fats in leaves and stems of all the extracts. Aqueous extract of stems and leaves (1000µg/ml) resulted in prominent antimicrobial activity against Aspergillus fumigates, Aspergillus niger with maximum zone of inhibition (19mm and 14mm) moderate zone of inhibition (10mm and 4mm) respectively. In addition, aqueous leaf and stem extracts exhibited 84% and 81% of DPPH radical scavenging (IC_{50} values: 39.75µg/ml and 42.89 µg/ml) and 41% and 64% of lipid peroxidation inhibition (IC_{50} values: 0.69 mg/ml and 1.226 mg/ml) respectively and also no hemolytic activity was observed which proves non toxicity of the extracts against human erythrocytes. Both the extracts also showed good amount of phenolics (19.99 mg GAE/g and 29.007 mg GAE/g of extract) and flavonoids (150.99 mg QE/g and 54.98 mg QE/g of extract) within them. GC-MS analysis detected hexamethyl cyclotrisiloxane and 1-methyl 3-nonyl indane in leaf and stem extracts. In future, the compound responsible for the antimicrobial and antioxidant activity is to be extracted and could be used as natural medicine.

Keywords: *Bauhinia acuminata*, antimicrobial, antioxidant, Hemolytic, GC-MS analysis.

INTRODUCTION

Oxidation is a chemical process that generates free radicals in cell that transfers electrons or hydrogen ion from a substance to an oxidizing agent. Most of the biological free radicals are highly reactive and are involved in damaging all components of cell such as proteins, lipids and mitochondrial DNA leading to increased production of chemically reactive molecules containing oxygen. Thus enhances accumulation of free radicals within cells and contribute to the aging process. Natural oxidants namely phenols in medicinal and dietary plants can prevent the oxidative damage caused by free radicals [1]. Automobile exhaust, radiation, pesticides and air pollution etc can generate free radicals [2]. Reactive free radicals are involved in the pathophysiology of various diseases like Alzheimer's, AIDS, cardiovascular diseases and ageing etc [3]. The effect of oxidative stress is due to production of free radicals which cause disruptions in normal mechanism of the immune system. Oxidative stress is thought to be important in neurodegenerative diseases. Oxidation can be inhibited by molecules known as antioxidants. Antioxidant potential activity of plants is mainly due to their phenolic compounds [4]. Since flavonoids are polyphenolic compounds involved in free radical scavenging, inhibition of hydrolytic, oxidative enzymes and anti-inflammatory action [5, 6]. Medicinal plants serves as potential sources of antioxidant activity. Hence, antioxidants

play a crucial role in termination chain reactions by removal of free radicals and inhibit other oxidation reactions. There are synthetic antioxidants commercially available that exhibit low solubility and moderate antioxidant activity. In addition they result in undesirable side effects [7, 8].

The present study was carried out in a systematic record of the relative free radical scavenging activity in selected medicinal plant species *B. acuminata. B. acuminata* is a flowering plant species native to tropical Southeastern Asia, member of the fabaceae family which is being used traditionally to cure gastrointestinal diseases, skin diseases, venereal diseases and leprosy. Different parts of this plant such as bark, leaves, stem, flowers and roots have been used in traditional medicine [9]. Though different parts of this plant were reported to possess good medicinal properties [10, 11], there is no published study particularly on the phytochemical, antioxidant, antimicrobial and hemolytic activity of *B. acuminata* leaves and stems. Hence, the focus of this study was to investigate phytochemical, antioxidant, antimicrobial and hemolytic activities in leaves and stem extracts of *B. acuminata*.

EXPERIMENTAL SECTION

Chemicals

Dimethyl sulfoxide, 1,1-Diphenyl-2-picryl hydrazyl (DPPH), quercetin, gallic acid, tert-butyl-4-hydroxy toluene (BHT), Folin Ciocalteu reagent, 0.1% ferric chloride, ethyl acetate, ninhydrin solution, Sodium carbonate, aluminum chloride, Absolute ethanol, methanol, disodium phosphate, ammonium solution, ammonium molybdate, potassiumferricyanide and trichloroaceticacid purchased from Sisco research laboratories Pvt Ltd. Mueller Hinton Agar, Nutrient Agar, Potato Dextrose Agar, Nutrient Broth were obtained from Hi- Media Pvt Ltd. All the chemicals purchased were of analytical grade.

Plant material

B. acuminata leaves and stems were collected from the natural population growing in the forest area, Kerala, India during September 2014. The identification of the specimen was done by Department of Botany, University of Kerala, Kariavattom with Voucher number: KUBH-5864.

Processing of plant

The leaves and stems of *B. acuminata* were collected and washed thoroughly in distilled water and were allowed to dry at room temperature. Dried leaves and stems were uniformly grinded using mechanical grinder to make fine powder. The powder was serially extracted in hexane, chloroform, ethyl acetate, methanol and distilled water (10% w/v) using a Soxhlet apparatus. These extracts were concentrated at 40˚C under reduced pressure (72 mbar) with a rotary evaporator and dried using lyophilizer. Dried extract was collected in air tight container for further use.

Phytochemical screening

Preliminary phytochemical screening of stems and leaves of *B. acuminata* was performed by the method developed by Harborne [12].

Antimicrobial activity

Microorganisms used

The bacterial isolates used were *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhimurium*. The fungal isolates used were *Aspergillus fumigates* and *Aspergillus niger.* All these clinical isolates were maintained on nutrient agar at 4°C.

Antibiotics used

Ciproflaxocin, Amoxicillin, Gentamycin, Cephalosporin, Chloramphenicol, *Enilconaezole* were used against *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumonia*, *S. typhi*, *A. fumigatus* and *A. niger*.

Evaluation of antimicrobial activity

Antimicrobial activity was carried out using agar well diffusion method [13]. All the bacterial test organisms were inoculated in nutrient broth and kept for overnight incubation at 37°C where as all the fungal test strains were inoculated in Potato Dextrose Agar (PDA) (pH 7.4) for 8 hours. Later, isolates were seeded on Muller Hinton Agar (MHA) plates using sterile cotton swabs and wells were bored using sterilized gel borer. Different concentrations of the extract (125-1000µg/ml) were made and poured in the well. Plates were incubated at 37°C for 24 hours.

DPPH radical scavenging activity

The method of Gunjan et al., 2010 was referred for performing DPPH radical scavenging activity. Different dilutions of plant extracts were used (10, 20, 40, 60, 80 and 100 µg/ml). One ml of DPPH solution along with 2ml of

each dilution of the extracts were mixed well and incubated at 20^{o}C for 40mins in dark condition. Using UV-Spectrophotometer absorbance of the solution was measured at 517nm by keeping methanol as blank [14].

Percentage DPPH radical scavenging activity of each extract was done by using the formula

% DPPH radical scavenging= $[(A_c - A_t) / A_c] \times 100$

Here A_c is the absorbance of the control (DPPH); A_t is the absorbance of test sample.

Reducing power activity

The reducing power assay for the extracts was performed fallowing the method of Oyaizu, 1986 [15]. One ml of different concentrations (125, 250, 500 and 1000 µg/ml) of extracts was mixed with 1ml 2M phosphate buffer at pH 6.6 and 1ml 1% potassium ferricyanide and the mixture was incubated at 50ºC for 20 min. 1ml 10% trichloroacetic acid (TCA) was added to the mixture including blank. The solution was mixed with 1ml distilled water and 0.5 ml 0.1% $FeCl_3$ and incubated at 40°C for 20 mins. Substances, which have reducing potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe^{2+}),which in turn react with ferric chloride to form ferric ferrous complex that gives a thick green to light green coloration and the absorbance was read at 700nm.

Lipid peroxidation inhibition assay

Lipid peroxidation inhibition activity was performed by the method of Mandal and Chatterjee, 1980 [16]. To 0.1 ml of extract at different concentrations (125, 250, 500 and 1000µg/ml), 2.8 ml of 10% liver homogenate, 0.1ml of $FeSO_4$ was added and mixed gently. The mixture was incubated for 30 min at 37°C. After incubation, to this mixture 2ml of 10% TCA and 2ml 0.67% TBA in 2% acetic acid was added and kept in water bath for 1hr at 100°C. After incubation, samples were centrifuged at 10000 rpm for 5 min. The supernatant was taken and the absorbance was measured in UV-Vis spectrophotometer at 535nm using distilled water as a blank.

Total phenolic content estimation

Total phenolic content analysis was performed by the method of Marja et al., 1999 [17]. To 100µl of sample at different concentrations (125, 250, 500 and 1000 µg/ml), 2.5ml of 1:10 diluted Folin Ciocalteau reagent was added and the mixture was incubated at 45°C for 15min. After incubation, absorbance was measured in UV-Vis spectrophotometer at 750 nm by using Na_2CO_3 solution as blank.

Total flavonoid content estimation

Total flavonoid content analysis was exhibited by the method described by Adedapo et al. 2009 [18]. To 1ml of extract at different concentrations (125, 250, 500 and 1000µg/ml), aluminum chloride solution was added and kept for incubation at room temperature for 1hr. The absorbance was read at 510 nm by using UV-Vis spectrophotometer and the results were expressed in mg quercitin equivalent per gram of extract.

Hemolytic activity

Hemolytic activity was performed by taking 5ml of blood from a healthy individual [19]. The heparanised blood was then centrifuged at 1500 rpm for 3mints. The supernatant was discarded and the pellet was washed three times with sterile phosphate buffer saline (pH 7.2). The cells were then re-suspended in 0.5% normal saline to make RBC cell suspension. Extract at different concentrations were taken in the test tubes (125, 250, 500 and 1000 µg/ml) and made up to 1ml by adding phosphate buffer. To this, 1ml of RBC cell suspension was added, mixed gently and incubated for 30 min at 37°C. After incubation, mixture was centrifuged at 1500 rpm for 10 min. The free hemoglobin in the supernatants was measured in UV-Vis spectrophotometer at 540 nm. Saline and distilled water were used as minimal and maximal hemolytic controls. The level of percentage hemolysis by the extracts was calculated according to the following formula:

% Hemolysis= [At – An / Ac – An] × 100

Here: At is the absorbance of test sample.
An is absorbance of the control (saline control)
Ac is the absorbance of the control (water control)

GC-MS analysis

Bioactive extract of *B. acuminata* was analysed for the compounds based on molecular weight in Gas chromatography mass spectroscopy instrument detection. Helium was used as a carrier gas at 0.1ml/min flow rate. Initially, temperature of the column was programmed at 60^0 C for 2mints ramp 10^6 C/ min to 300^0 C with a holding of 6mints. Injector temperature was maintained at 250^0C for the analysis. The identification of chemical constituents

was based on existing mass spectra data correlation those prevailed from the Wiley 8.LIB and NIST08.LIB library spectrum provided by the software in GC-MS System [20].

Statistical analysis

All the results were expressed as mean ± standard deviation of the response of three replicates per sample. Results were analyzed using Microsoft Excel 2007 and Graph Pad Prism 5.

RESULTS AND DISCUSSION

Many medicinal plants are used widely in traditional medicinal system, in which most of them are effective and are not been studied scientifically. *B. acuminata* is a shrub which is been used traditionally by many tribes and rural communities for treating a wide range of diseases. Scientifically, the leaf extracts of *B. acuminata* were reported to have cytotoxic, thrombolytic and antidiarrheal activities [10, 11]. Whereas, seeds were reported to possess antibacterial activity [21] however no studies have been made on stem extracts and antimicrobial activity of leaf extracts. Thus, this study was designed to evaluate antimicrobial and antioxidant efficacy of leaf and stem extracts of *B. acuminata*.

Yield of extract

The powdered leaves and stems were serially extracted from different polarity solvents and the dried extract was measured. The yield of the extracts was calculated in respect to the fresh mass and dry weight. Aqueous extract of stem showed maximum yield than the rest of the extracts.

Phytochemical screening

Phytochemical compounds play a vital role for the treatment of different types of diseases and still are use in both traditional and modern system of medication. It has been reported that the presence of bioactive substances in plants play an important role in prevention of colorectal carcinoma, hyper cholesterolemia, renal calculi, can control human cardiovascular diseases and also provides protection against microbial degradation of dietary proteins in the semen [22, 23]. Thus, extracts of *B. acuminata* was screened for the presence and absence of various phytochemical constituents. Extracts showed the presence of saponins, oils and fats, carbohydrates, tannins and flavonoids (table 1).

Table 1. Qualitative analysis of photochemical components of *B. acuminata* stems and leaves

Tests for	STEM				LEAVES			
	HE	CE	ME	AE	HE	CE	ME	AE
Carbohydrates	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve
Flavanoids	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve
Oils and Fats	-ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
Saponins	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
Tannins	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Proteins and amino acids	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Antimicrobial activity

Among the four extracts, methanol and aqueous extracts of leaves and stems at 1000 µg/ml resulted in prominent antimicrobial activity against *Aspergillus fumigates, Aspergillus niger* with maximum zone of inhibition (19mm and 14mm) and moderate zone of inhibition (10mm and 4mm) respectively (table 2 and figure 1). Thus showing evidence for its property to inhibit growth of *A. fumigates* and *A. niger* [24, 25].

Table 2. Antifungal activity

ZONE OF INHIBITION OF B. ACUMINATA										
Test organisms	*Leaves*					*Stems*				
	HE	*CE*	*EE*	*AE*	*NC*	*HC*	*CE*	*EE*	*AE*	*NC*
A. Niger	*Na*	*na*	*na*	*4mm*	*na*	*Na*	*na*	*Na*	*14mm*	*na*
A. Fumigatus	*Na*	*na*	*na*	*10mm*	*na*	*na*	*na*	*Na*	*19mm*	*na*

Figure 1: A. Aqueous extract of *B. acuminata* stem inhibits the growth of *A. niger*; B. Aqueous extract of *B. acuminata* stem inhibits the growth of *A. fumigates*; C. Aqueous extract of *B. acuminata* leaf inhibits the growth of *A. fumigates*; D. Aqueous extract of *B. acuminata* leaf inhibits the growth of *A. niger*

Antioxidant activity

Oxidative damage within the body occurs due to the free radical chain reactions results in many disorders and also contributes to aging process. Antioxidants through their scavenging power helps in preventing oxidation process [26-28]. Plants are natural source of antioxidants. Therefore, the stem and leaf extracts were estimated for DPPH radical scavenging activity. As the aqueous stem and leaf extracts exhibited highest DPPH scavenging activity with IC_{50} values of 42.89 and 39.75µg/ml shown in the figures 2 and 3, for analyzing the electron donating property of the extracts, reducing power assay was performed [29], aqueous stem and leaf extracts showed promising reducing power property (figure 4). Anti-Lipid peroxidation assay resulted in 65% and 41% of lipid peroxidation inhibition by aqueous stem and leaf extracts (figure 5).

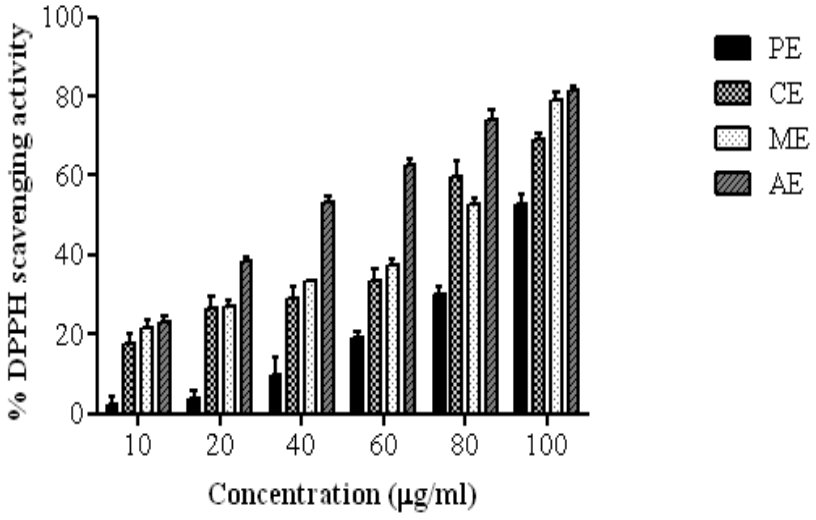

Figure 2: DPPH radical scavenging assay of *B. acuminata* stems

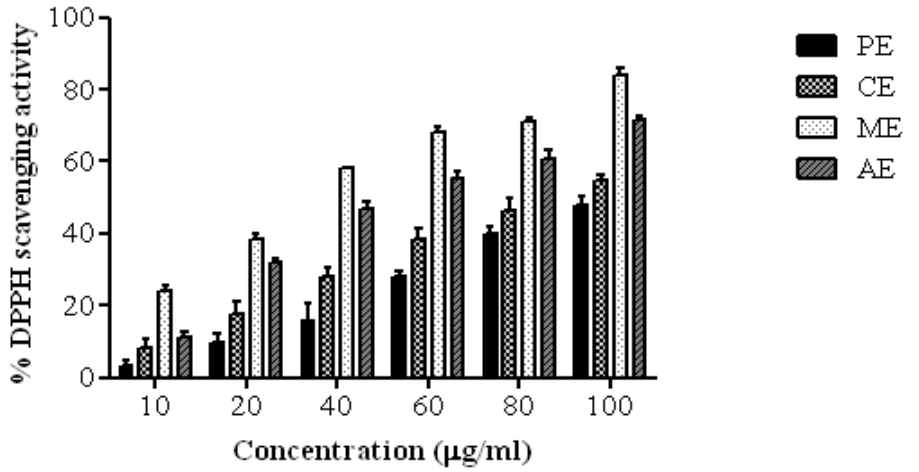

Figure 3: DPPH radical scavenging activity of *B. acuminata* leaves

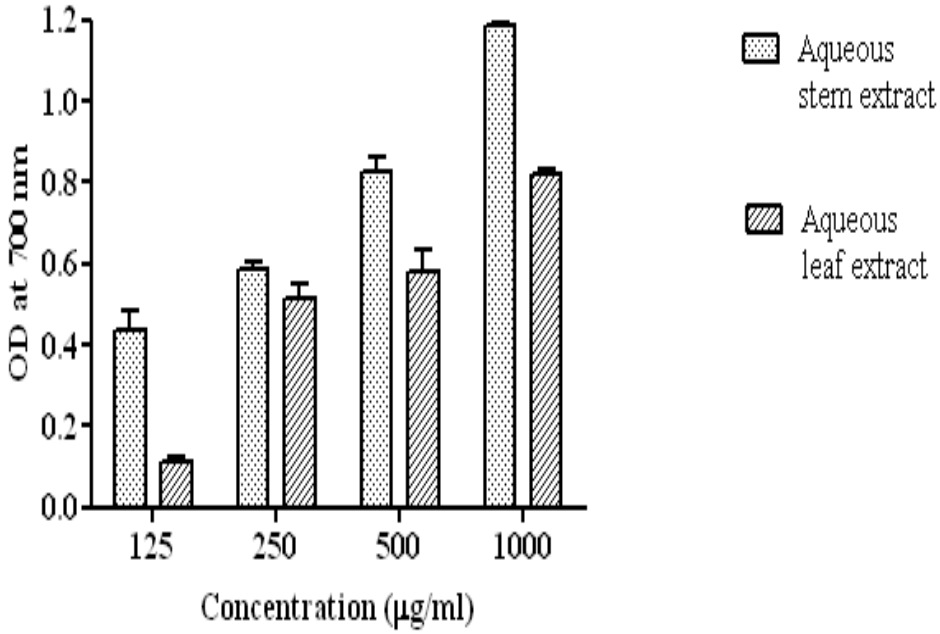

Figure 4: Reducing power assay

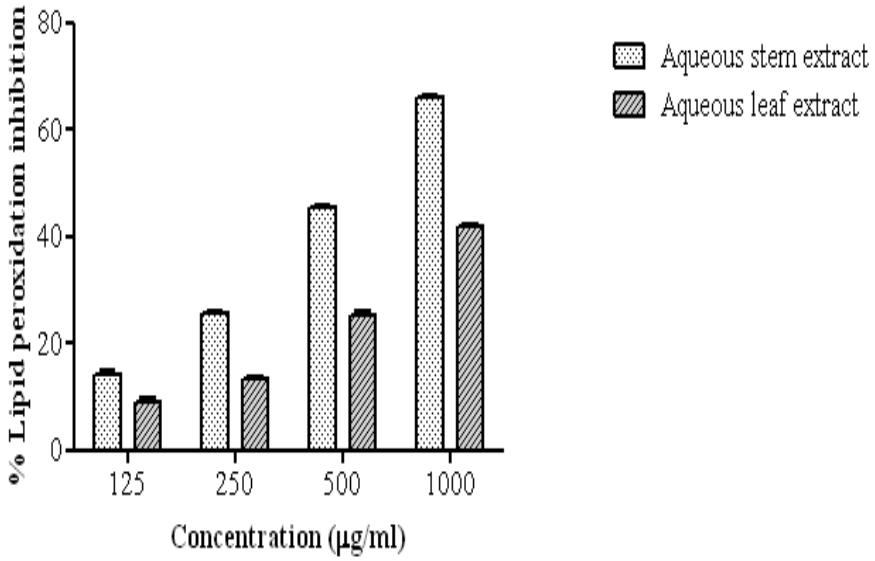

Figure 5: Anti-Lipid peroxidation assay

Quantitative estimation of phenolics and flavonoids

Antioxidant nature of the plants is because of the polyphenols within them [30-32]. Therefore, aqueous stem and leaf extracts were estimated for total phenolics and flavonoids. The total phenolic content was found to be 29.007 mg and 19.99 mg GAE/gm of the aqueous stem and leaf extracts (figure 6). Total flavonoids analysis was recognized to be 54.98mg and 150.99 mg QE/gm of the aqueous stem and leaf extracts (figure 7).

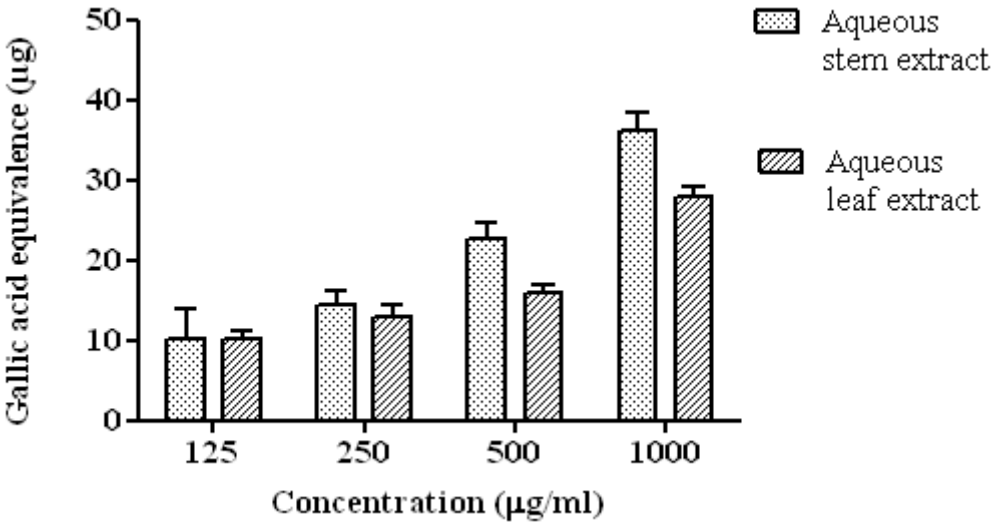

Figure 6: Total phenolic content estimation

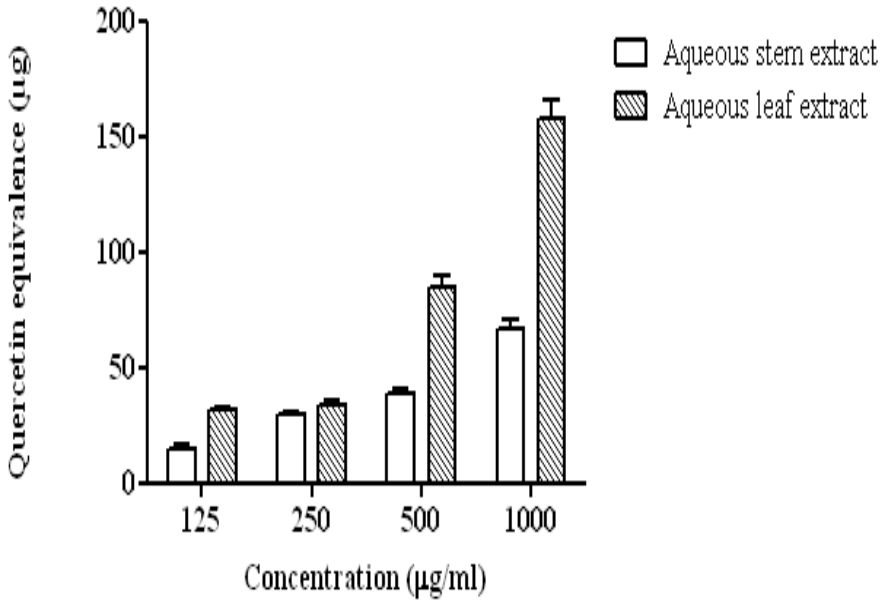

Figure 7: Total flavanoid content estimation

Hemolytic activity

Hemolytic activity was performed to measure the toxic effect of stem and leaf aqueous extracts towards human erythrocytes which showed very low hemolytic activity (figure 8). Thus, *B. acuminata* could be considered as safe for human RBC [33, 34].

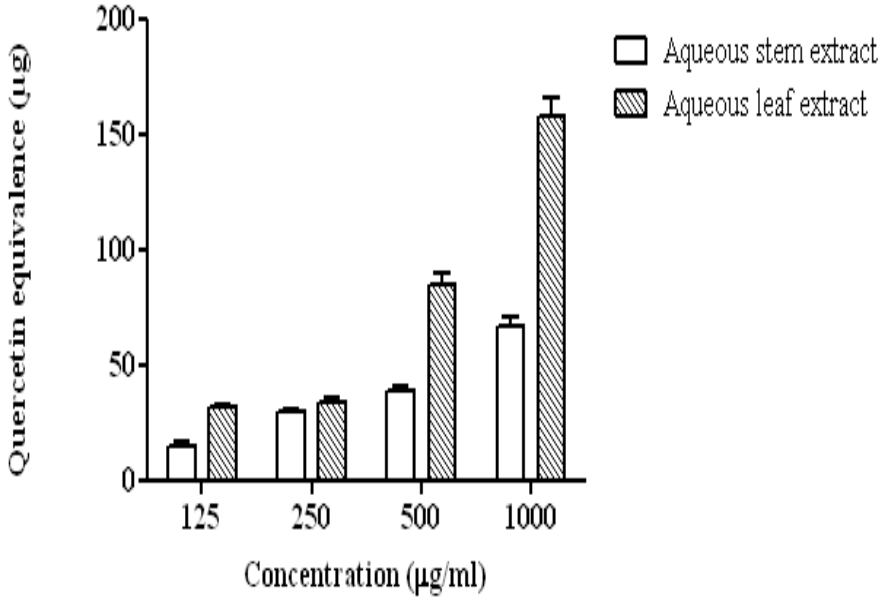

Figure 8: Hemolytic activity

GC-MS analysis

GC-MS analysis showed the presence of hexamethyl cyclotrisiloxane and 1-methyl 3-nonyl indane in leaf and stem extracts respectively (figure 9 and figure 10).

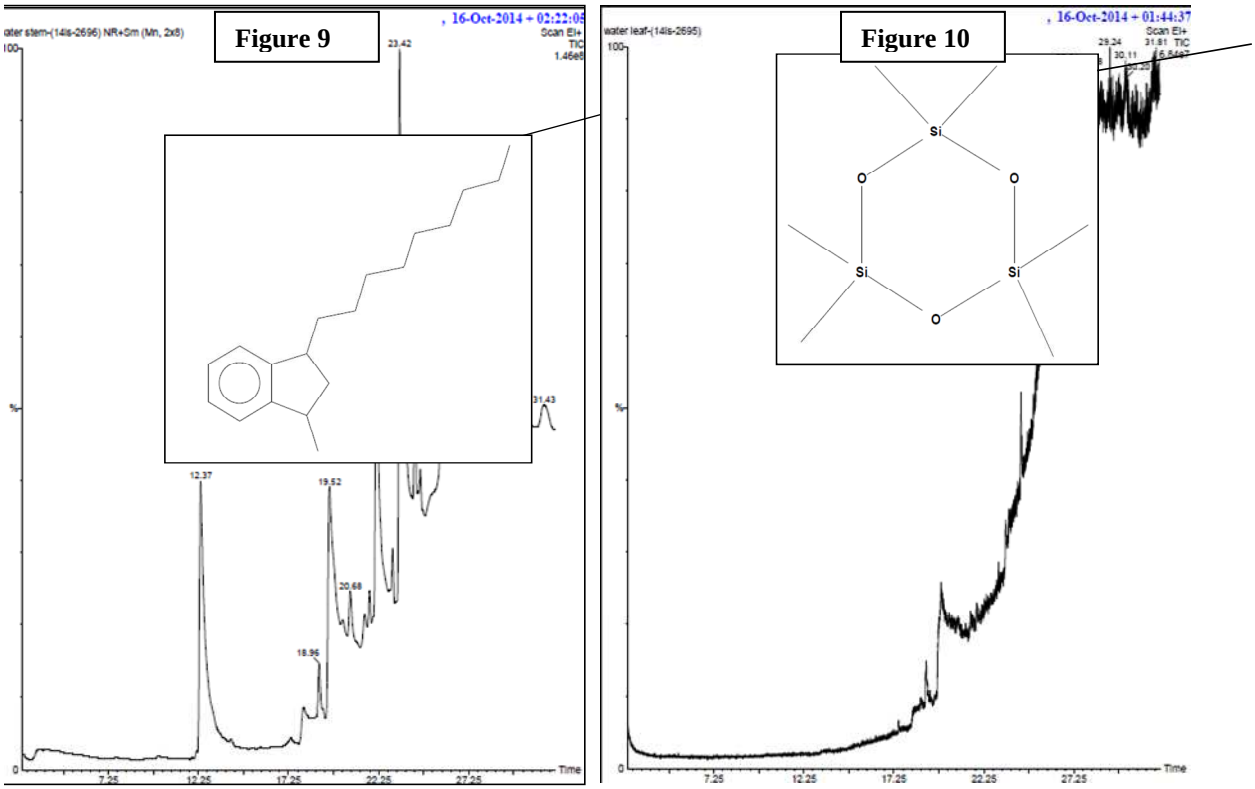

Figure 9: GC-MS analysis of aqueous stem extract identified indan,1-methyl3-nonyl; Figure 10: GC-MS analysis of aqueous-leaf extract identified cyclotrisiloxane, hexamethyl

CONCLUSION

Aqueous extract of *B. acuminata* leaves and stem showed strong antimicrobial activity. It also exhibited good antioxidant activity for various tests performed. Hemolytic activity revealed the non toxic nature of the extract. Quantitative analysis detected higher amount of phenols and flavonoids. GC-MS analysis identified two compounds. Hence, antimicrobial and antioxidant nature might be due to the presence of these compounds within the extract. Further, bioactive compound responsible for these activities is to be isolated and used as a safe antimicrobial drug.

Acknowledgement
The authors are grateful to the management and staff of VIT University, Vellore, Tamil Nadu, India for providing the facilities to complete this study successfully.

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