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In vitro evaluation of antibacterial potential of *Annona squamosa* L. and *Annona reticulata* L. from Similipal Biosphere Reserve, Orissa, India

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Three different solvent extracts of leaf of *Annona squamosa* L. and *Annona reticulata* L. were studied for its antibacterial activity. Agar cup and broth dilution methods were selected to test antibacterial activity using three Gram-positive (*Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis*) and five Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa, Salmonella typhi, Vibrio alginolyticus, Vibrio cholerae*) bacteria. The screening results showed that highest inhibition was observed by the methanol extract followed by petroleum ether and aqueous extracts for both *Annona squamosa* and *Annona reticulata* leaf. *Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Vibrio alginolyticus* are the most sensitive bacterial strains among all test organisms. None of the plant extracts showed growth of inhibition against *Salmonella typhi*.

Key words: similipal biosphere reserve, antimicrobial activity, medicinal plant, phytochemical analysis

Introduction

In India, from ancient times, different parts of medicinal plants have been used to cure specific ailments. Today, there is widespread interest in drugs derived from plants. This interest primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects. Hence, there is need to screen medicinal plants for promising biological activity. Plants of the genus *Annona*, members of the Annonaceae family, are native to South and Central America. They are mostly

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small trees, and produce compound fruits. *Annona squamosa* L., known as custard apple, is commonly found in deciduous forests, also cultivated in wild in various parts of India. Literatures of many research works prove that every parts of *A. squamosa* possess medicinal property (Kirtikar and Basu, 1993). Roots are employed internally in depression of spirits and spinal diseases. Bark is known to be a powerful astringent. In Ayurveda, fruits are considered as a good tonic, enrich blood, used as expectorant, increases muscular strength, cooling, lessens burning sensation and tendency to biliousness, sedative to heart and relieves vomiting (Patel and Kumar, 2008). Due to uniqueness of leaf property in curing of different ailments, this part was selected for the study. *Annona reticulata* L. is referred as bullock's heart having a smooth skin fruit that becomes dull red when ripe. Less volatile substances such as alkaloids, diterpenoids, and acetogenins (Ogunwande *et al.,* 2006) have been identified so far from oil of various parts of the plant. However, the antibacterial potential of *Annona reticulata* leaf is not yet been explored properly. With this in mind, the present work was an attempt to perform the studies on antibacterial activity of the leaf extract of *Annona reticulata* and *Annona squamosa*.

Matherials and methods

Study area

Similipal Biosphere Reserve is located in the district of Mayurbhanj in the northern region of Orissa, between 21°08" - 21°27" N latitude and 86°04" - 86°15" E longitudes, unique habitat of mixed tropical forest (fig. 1). The ecosystem is enriched with more than 500 medicinal plants (Saxena and Brahmam, 1989).

Plant material

Leaf of *Annona reticulata* and *Annona squamosa* were collected in the month of April, 2007 from Similipal Biosphere Reserve, Mayurbhanj, Orissa and their identity were confirmed at Dept. of Botany, M. P. C. (Auto) College, Baripada. The shed dried healthy leaves were powdered separately using mechanical grinder and then were passed through sieve so that uniform powder size is maintained.

Preparation of extracts

500 g of each powdered plant material were taken in six separate conical flasks and soaked with 2 lit. of each solvent (petroleum ether, methanol and distilled water) at room temperature for 48 h. The extracts were filtered through

Buchner funnel using Whatman filter paper no.1. The filtrate were evaporated to dryness under reduced pressure and the concentrated extracts were freeze dried to remove the solvent at -2 ºC till further use.

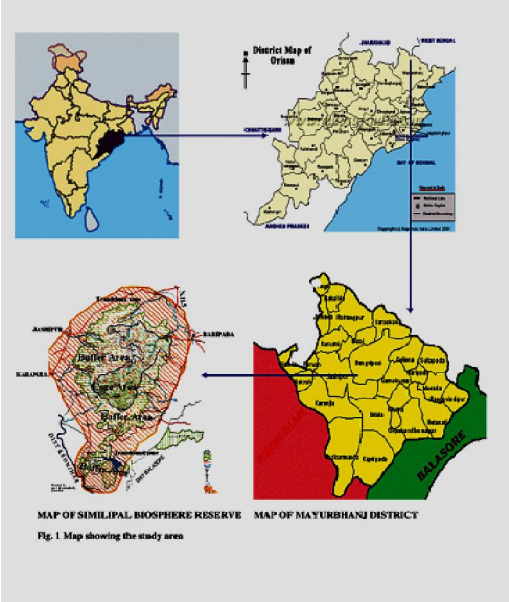

Fig.1. Map showing the study area.

Phytochemical analysis

Qualitative phytochemical analysis was carried out using method described by Trease and Evans Trease and Evans (1989). Each extract was screened for presence of alkaloids with Mayer's, Wagner's, Hager's and Dragendorff's reagents; flavonoids (NaCl and HCl); carbohydrates with Molisch's, Benedict's and Fehling's reagent; glycosides with Keller-Killiani and Borntrager's; protein and amino acids with Biuret, Xanthoproteic, Ninhydrin and Millon's reagent; tannin and phenolic compound ($FeCl_3$ and Gelatin); triterpenoid with thionylchloride; steroid and sterols with Liebermann Burchard and Salkowski's reagents and fat and fixed oil with alcoholic KOH reagents.

Antimicrobial activity

Microbial strains

The antibacterial activity was tested against *Bacillus subtilis* MTCC 7164, *Staphylococcus aureus* MTCC 1144, *Staphylococcus epidermidis* MTCC 3615, *Escherchia coli* MTCC 1098, *Salmonella typhimurium,* MTCC 3216, *Pseudomonas aeruginosa* MTCC 1034, *Vibrio cholerae* MTCC 3904 and *Vibrio alginolyticus* MTCC 4439. These bacterial strains were obtained from MTCC, Chandigarh, India (Customer no. 4853).

Agar cup method

The agar cup method was used to study the antibacterial activity of the extracts as described by Panda *et al.* (2009). Mueller-Hinton agar (MHA) (Hi-Media, India) was used as bacteriological medium. MHA plates were prepared by pouring molten media into sterile Petri plates. The plates were allowed to solidify for 5 min. Wells were prepared in seeded agar plates. 0.1% inoculums suspension was swabbed uniformly and the inoculums were allowed to dry for 5 min. The extracts were diluted in 100% DMSO. A total of 6 mm diameter wells were punched into the agar and filled with the 50 µl (20 mg/ml in DMSO) extracts, 20 µl DMSO (negative control) and 5 µl of standard antibiotic (Ciprofloxacin at concentration 10 µg/ml) were used as a positive control. The plates were incubated at 37 ºC for 24 h. After the incubation period formation of zones around the wells, confirms the antibacterial activity of the respective extracts. The same procedure was followed for each strain and extract. Each experiment was carried out in triplicates. The mean ± SD of the inhibition zone was taken for evaluating the antibacterial activity of the extracts.

Determination of minimum inhibitory concentration (MIC)

In the present experiment, extracts which showed positive result were further evaluated for determination of MIC. A broth micro-dilution technique was adopted using 96 well micro-titer plates and tetrazolium salt, 2,3,5-Triphenyltetrazolium Chloride (TTC) was carried out to determine the MIC following the methods with few modification as described by Eloff (1998). In the plate, A_1 to H_1 was the blank and consisted of MH broth only. A_3 to H_3 was having the stock solution of the test extract(s) and A_4 to H_4 till A_9 to H_9 were the wells in which the test extracts were serially diluted using MH broth. Wells A_{12} to D_{12} were control having 20µl of DMSO and E_{12} to H_{12} served as control over control. All wells were dispensed with 100µl of MH broth. 20µl of the

herbal extract was transferred from stock test solution to the first well i.e. from A_4 to H_4 containing 100µl of MH broth. 20µl of the MH broth containing herbal extract was then transferred to the next well to create serial dilutions. 100µl of the 0.5 McFarland adjusted activated culture in MH broth was then added to all the wells except the blank. 5µl of 0.5% TTC was further added to all the dilutions, blank, control and control over control. The final volume of all the wells was 205µl. The Microplate was sealed and incubated at 37°C at 130 rpm. 10µl of the broth from each culture tube exhibiting MIC and control tubes were taken aseptically and were plated on one day old MH agar plate as a point inoculum and allowed to dry for 10 min under the laminar air hood. The micro-plate was sealed and incubated at 37ºC at 130 rpm and observed for growth of the microorganism.

Results and discussion

The result of antibacterial screening by agar cup method (Table 1, Fig. 2) indicates that highest zone of inhibition was shown by the methanol extract followed by petroleum ether and aqueous extracts for both *Annona squamosa* and *Annona reticulata* leaf. Extracts of *Annona squamosa* inhibited the growth of all test strains except *Salmonella typhimurium*. Aqueous extracts showed less activity than methanol extracts possibly because i) the same active substances were present in water extracts, but in low concentrations ii) active substances were soluble in organic solvents and therefore, not present in water extracts as also suggested by de Boer *et al.* (2005). The antibacterial action of the extracts is more pronounced on Gram-positive than on Gram-negative bacteria, and these findings correlate to the observations of previous screenings of medicinal plants for antibacterial activity (Panda *et al.,* 2009). *Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Vibrio alginolyticus* were the most sensitive bacterial strains in the present experiments. Among the extracts of *A. reticulata* aqueous and methanol extracts showed significant activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Vibrio alginolyticus*. However, no extract showed any activity against *Escherchia coli* and *Pseudomonas aeruginosa*. In compared to the exracts of *Annona reticulata*, *Annona squamosa* had strong antibacterial activity.

There are several reasons that people use plants for medication. This includes improvement of health after herbal treatment, low cost of the drugs, non availability of synthetic drugs particularly in the rural areas, where available were either fake or expired drugs and in some cases the people are more accustomed to and comfortable with traditional healing (Audu, 1995). Our study showed that aqueous and methanol extracts inhibited the Gram-positive bacteria superior to Gram-negatives. Findings are also supported by other

scientist those reported that the petroleum ether extracts of *Annona squamosa*, to be active against *E. coli, P. aeruginosa, S. aureus* and *B. subtilis* while both the methanol and aqueous extracts were active against *S. epidermidis, S. aureus* and *B. subtilis*. From agar cup method results obtained that there were marked differences between the activities of the plant extract and those of the pure antibacterial drugs (ampicillin and ciprofloxacin). Such significant differences are normally present when crude (unpurified) plant extracts are compared with pure drugs that are already in clinical use (Yoder, 1982). Also the agar cup method is not always dependable for accurate assessment and comparison. This is because of the high degree of interference inherent in this method, arising from drug diffusion problems (Dickert *et al.,* 1981).

A more generally accurate method of assessment is the broth dilution technique. In this study, therefore, the broth dilution method was used in determining the activities measured as MIC. The range of MIC values for both *B. subtilis* and *V. alginolyticus* correlated well with the results obtained using the agar cup method (Table 2). The MIC values for methanol extracts against Gram-positive bacteria are lower in compared with Gram-negatives. This shows that the Gram-positive bacteria are more susceptible to the effect of the extracts from *Annona squamosa* with respect to its Gram-negative counterpart. The MBC values were higher than the MIC values of the extracts against all the tested bacteria. The lowest MICs exhibited by extracts with MBC values four or eight time of MIC, in corresponding microorganisms, highlighting their interesting antimicrobial potency. From these results, it can be observed that, most of test samples exerted a lethal effect on the test organisms. In addition to these MMC/MIC ratios lower than 4 was obtained with most of the samples, suggesting killing effects come to mind (Carbonnelle *et al.,* 1987).

Phytochemical screening of both plants has been summarized in Table 3. Evaluation of phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, tannin and phenolic compounds, steroids and sterols and triterpenoids are present in *A. squamosa* while alkaloids, flavonoids, tannin and phenolic compounds and carbohydrates are present in most of polar extracts of *A. reticulata* such as methanol and aqueous extracts as compared to non polar extract (petroleum ether). Presence of more phytochemicals in *A. squamosa* might be resulted better antibacterial activity than *A. reticulata*. The phytochemical constitute such as alkaloids, flavonoids, tannin and phenols compound have been reported to be important compounds in many other medicinal plants (Burapedjoand Bunchoo, 1995; Barnabas and Nagarajan, 1988.). Comparison of the data obtained in this study with previously published result of Rahman *et al.* (2005) is problematic. First, the composition of the plant

Table 3. Phytochemical analysis of different extracts.

Name of the phytochemicals	Name of the test	Petroleum ether extract		Methanol extract		Aqueous extract	
		As	Ar	As	Ar	As	Ar
Alkaloids	Mayer's reagent	+	-	+	+	+	+
	Dragendroff's	+	-	-	-	-	+
	Hager's reagent	+	-	+	-	-	+
	Wagner's reagent	-	-	-	-	-	-
Carbohydrates	Molisch's test	+	-	+	+	+	+
	Fehling's test	+	-	+	+	+	+
	Benedict's test	-	-	-	-	-	-
Tannin and phenolic compound	With Ferric chloride	+	-	+	-	+	+
	With lead acetate	+	-	-	-	-	-
	With gelatin solution	-	-	-	-	-	-
Glycoside	Keller-Killiani test	+	-	+	-	-	-
	Legal Test	-	-	-	-	-	-
	Borntrager's test	-	-	-	-	-	-
Proteins and amino acids	Biuret test	-	-	-	-	-	-
	Ninhydrin test	-	-	-	-	-	-
	Xanthoproteic test	-	-	-	-	-	-
Gum & mucilages	Millon's test	-	-	-	-	-	-
	Molisch's test	-	-	-	-	-	-
Flavonoids	With NaOH	+	+	+	-	+	+
	With H_2SO_4	-	-	-	-	-	-
	With Mg/HCl	-	-	-	-	-	-
Saponins	Honeycomb foam	-	-	-	-	-	-
	Foam test	-	-	-	-	-	-
Steroids and sterol	Salkowski's test	-	-	-	-	-	-
	Liberman Burchard	+	-	+	-	-	-
Triterpenoids	Thionylchloride test	+	-	+	-	-	-
Oils and fats	With filter paper	-	-	-	-	-	-
	With alkaline KOH	-	-	-	-	-	-
Vitamin C	With Indophenol's	-	-	-	-	-	-
	Sod. nitroprusside	-	-	-	-	-	-

(+) Present; (-) Absent

extracts is known to vary according to local climatic and environmental conditions (Janssen *et al.,* 1987; Sivropoulou *et al.,* 1995). Secondly the method used to assess antibacterial activity and the choice of the test organisms also varies (Janssen *et al.,* 1987). Most frequently used methods to antibacterial activity are agar diffusion techniques and broth dilution methods. The results obtained by each of these methods may differ as many factors vary between assays (Janssen *et al.,* 1987; Hili *et al.,* 1997). *In vivo* studies may be required to confirm the values of the some of the results obtained. The result of the

antibacterial activity of *Annona squamosa* leaf extracts is particularly important considering the test human pathogenic bacteria.

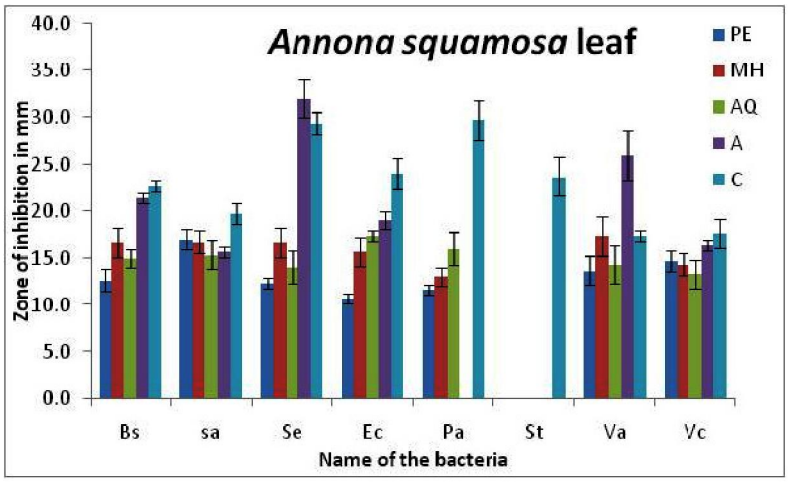

Fig. 2. Antibacterial activity of *Annona squamosa* leaf by agar cup method.

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Table 1. Antibacterial activity by agar cup method.

Bacteria	Petroleum ether		Methanol		Aqueous		Antibiotic	
	Ar	As	Ar	As	Ar	As	A	C
Bs	11.7 ± 0.58	12.7±1.15	12.7±1.15	16.7±1.53	11.7±0.58	15.0±1.00	21.3±0.58	22.7±0.58
Sa	-	17.0 ± 1.00	17.0 ± 1.0	16.6 ± 1.53	13.0 ±1.73	15.3 ±1.50	15.6 ± 1.53	19.6 ± 1.00
Se	-	12.3± 0.58	-	16.6± 1.53	12.3± 2.08	14.0± 1.73	32.0± 2.00	29.3± 1.15
Ec	-	10.6± 0.58	-	15.6± 1.53	-	17.3± 0.58	19.0± 1.00	24.0± 1.73
Pa	-	11.6±0.57	-	13.0±1.00	-	16.0±1.07	-	29.7±2.08
St	-	-	-	-	-	-	-	23.7±2.08
Va	12.7± 1.15	13.6± 1.52	12.7±1.15	17.3±2.08	12.3± 0.58	14.3± 2.08	26.0± 2.65	17.3± 0.58
Vc	-	14.6±1.15	14.7±2.08	14.3±1.15	-	13.3±1.53	16.3±0.58	17.7±1.53

All values are mean zone of inhibition± SD; (-) No zone of inhibition; Zone of inhibition including 6 mm borer; A-Ampicillin, C-Ciprofloxacin; Extract concentration (30 mg/ml); **Bs-** *Bacillus subtilis*, **Sa-** *Staphylococcus aureus*, **Se-** *Staphylococcus epidermidis*, **Ec-** *Escherichia coli*, **Pa-** *Pseudomonas aeruginosa,* **St-** *Salmonella typhi,* **Va-** *Vibrio alginolyticus*, **Vc-** *Vibrio cholerae*, **Ar-***Annona reticulata;* **As-** *Annona squamosa*

Table 2. Determination of MIC and MBC.

Name of bacteria	*Annona reticulata* (concentration in mg/ml)						*Annona squamosa* (concentration in mg/ml)						Ampicillin in mg/ml)	
	Petroleum		MeOH		Aqueous		Petroleum		MeOH		Aqueous			
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bs	5.00	<10.0	5.00	<10.0	5.00	<10.0	5.00	<10.0	1.25	5.00	2.50	10.0	0.005	0.005
Sa	-	-	2.50	<10.0	5.00	<10.0	1.25	2.50	1.25	5.00	2.50	10.0	0.005	0.005
Se	-	-	-	-	5.00	<10.0	2.50	5.00	1.25	5.00	5.00	<10.0	0.025	0.025
Ec	-	-	-	-	-	-	5.00	<10.0	2.50	10.0	2.50	10.0	0.005	0.005
Pa	-	-	-	-	-	-	5.00	<10.0	5.00	<10.0	2.50	10.0	*0.005	*0.005
Va	5.00	<10.0	5.00	<10.0	5.00	<10.0	5.00	<10.0	1.25	10.0	5.00	<10.0	0.005	0.005
Vc	-	-	5.00	<10.0	-	-	5.00	<10.0	2.50	<10.0	5.00	<10.0	0.005	0.005

Bs- *Bacillus subtilis*, **Sa-** *Staphylococcus aureus*, **Se-** *Staphylococcus epidermidis*, **Ec-** *Escherichia coli*, **Pa-** *Pseudomonas aeruginosa,* **St-** *Salmonella typhi,* **Va-** *Vibrio alginolyticus*, **Vc-** *Vibrio cholerae*, * Ciprofloxacin.