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Medicinal properties of annona squamosa in theraupitic use

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ABSTRACT

Different organic solvent and aqueous extracts of leaf, stem bark and root bark of *Annona squamosa* L. were subjected separately to test their antibacterial activity by *in-vitro* methods like Disc diffusion method, agar well diffusion method and Minimum Inhibitory Concentration method. The extracts of Annona *squamosa* L. shows a wide spectrum of antibacterial activities. The chemical compounds present in the experimental plant are biologically active and inhibit the growth of human pathogenic bacteria. Four species of bacteria were selected for the present study. Among them one is gram negative (*Escherichia coli)* and other three are gram positive (*Bacillus subtilis*, *Bacillus megatherium*, *Bacillus cereus).* The experimental results obtained justified the folk use of this species as a cicatrizant and vulnery agent.

Introduction

Medicinal plants are an important therapeutic aid for various ailments. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century. (Zaika LL. 1975) Antimicrobial resistance has become a global problem. Strategies to improve the current situation include research in finding new and innovative antimicrobials (Freeman C.D., 1997). Antibiotics and the chemotherapeutic agents have been of value in controlling many infections but they depend on judicious use to minimize the incidence of resistant forms (Danso Anthony Appiah, et al., 2002). The use of medicinal plants for the treatment of various diseases is an old age practice in most countries and it still offers an enourmous potential source of new anti- infective agents (Irobi, 1993). As a result, different remedies evolved in different regions of the world as communications got improved (Lino and Deogracious, 2006). The scientific literature is full of reports of antimicrobial activity of plants and their secondary metabolites (Erdemeier et al., 1996, Hassan and Ahmed, 1996; Darokan et al., 1999; Cutter, 2000; Babu et al., 2002) and scientific evaluation of these plants remains an area of intense investigations.

Natural antimicrobials can be derived from plants, animal tissues, or microorganisms. (Gordon MC, David JN., 2001).The shortcomings of the drugs available today, propel the discovery of new pharmacotherapeutic agents in medicinal plants. (Cordell GA. 1993) Preparations from plants were the original therapeutic interventions used by man to control diseases in humans and livestock. As a result, different remedies tended to develop in different parts of the world. In some instances, related plants were used over wide geographical regions as a result of communication or importation of plant materials of high repute (Waller PJ, et al., 2001). The utilization of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO, 1996).The role of medicinal plants, both as potential antimicrobial crude drugs as well as a source for natural compounds that act as new anti-infection agents . (J.L.Ríos and M.C.Recio., 2005).

Annona squamosa L., the plant of Annonaceae family, also known as custard apple, is commonly found in deciduous forests, also cultivated in wild in various parts of India. It is a native of West Indies; now cultivated throughout India and other tropical countries. Literatures of many research works prove that every parts of *A. squamosa* possess medicinal property (Atique A et al., 1985, Rao VSN, 1979, Rathore DS, 1990 Ranjan V, 1999). *A. squamosa* L. is a well known edible tropical fruit and its seeds exhibit insecticidal and abortifacient properties (Chopra R N, et al., 1956). *A. Squamosa* consists of a variety of compounds *e.g.* amino acids (Rao S V, et al., 1955) monoterpenes (Fransworth N R, et al 1974), sesquiterpenes (Oliveros-Belardo L, et al., 1975), kaurene (Bholmann F and Rao N, et al 1973), steroids (Behari M and Sharma R K, et al 1986) *etc*.

Annonaceae seems to be one of the least chemically as well as pharmacologically known families compared with its large size. (Beena Joy and P. Remani, 2007) The biochemical studies of this plant had mentioned that *A. squamosa* contains flavonoids which expose strong antibacterial activity (Kotkar HM, et al., 200l). Volatile compound of this plant were also studied for its antibacterial activity (Chavan MJ,et al., 2006).

Leaves are used as poultice over boils and ulcers and also to kill lice. Leaf infusion is efficacious in prolapsus of children. Bruised leaves with salt make a cataplasm to induce suppuration. They are applied for extraction of guinea-worms. Leaves contain the presence of some phytochemicals in *Annona squamosa* which are responsible for antimicrobial activity. (Jayshree D. Patel and Vipin Kumar, 2008) Due this uniqueness of leaves property in curing of different ailments, this part was selected for the study. Literature survey showed that chief phytoconstituent of this plant is anonaine. Present phytochemical analysis of the *Annona squamosa* displayed presence of five known compounds namely Linalool, Borneol, Eugenol, Farnesol, and Geraniol. The Annonaceous acetogenins are a new class of natural compounds, whose potent biological

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activity and special structures have attracted considerable attention. (B.V.V.Pardhasaradhi, 2005) Numerous Annonaceous acetogenins have been shown to possess cytotoxic, pesticidal, antimalarial, cell growth inhibitory, antiparasitic and antimicrobial activities (Oberlies *et al* 1997; Chih *et al* 2001). Bullatacin is one such compound that possessed antitumoural and pesticidal activity *in vivo* (Ahmmadsahib *et al* 1993). Acetogenins isolated from the Annonaceae have been evaluated for both their cytotoxic activity in multiple ovarian cancer cell lines and their antitumor effects in a murine ovarian teratocarcinoma model *in vivo* (Rupprecht et al. 1990, Holschneider et al. 1994).

The aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral and antitumor agents (Chung et al. 1995, Vlietinck et al. 1995). Furthermore, the selection of crude plant extracts for screening programs has the potential of being more successful in its initial steps than the screening of pure compounds isolated from natural products (Kusumoto et al. 1995, Cordell 1995).

Plant description:

Annona squamosa L., is a member of Annonaceae family, a woody tropical to subtropical family of general ranalean affinity. (M. Arif Hayat, 1963).

Classification:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Magnoliidae
Order	Magnoliales
Family	Annonaceae
Genus	Annona
Species	Annona squamosa **(**Discover Life**)**

Habit: A small deciduous tree/shrub, 3-6m in height with a diameter to 20cm which is widely distributed in tropical and some subtropical regions of the world. The original distribution of A.squamosa was probably in westindies. (M. Arif Hayat, 1963) and has been naturalized throughout India in plains as well as on hills.(Kirtikar K R and Basu B R, 1984, The wealth of India; Raw Materials, 1984)

Bark & Branches: The bark is smooth with very shallow longitudinal fissures, visible leaf scars and is coloured grey; twigs are light brown/grey and lenticellate. (Baumer 1995 et al.,)

Leaves: are simple, alternate (distichous arrangement), thin and dull green, oblong elliptic to narrowly elliptic (7- 17x3-6cm) and petiolate with prominent main and side veins on the abaxial surface. (Baumer 1995 et al.,)

Inflorescences: These arise as few flowered clusters. (Baumer 1995 et al.,)

Flowers: consist of 3 sepals (1mm); 3 oblong thick petals up to 3cm long which are channelled inside, green/white in colour and purple at the base; and 3 inner reduced (sometimes absent) petals. (Baumer 1995 et al.,)

Fruits: are globose to heart-shaped, 5-10cm in diameter, with many rounded protuberances (tuberculate) so that the outside of the fruit appears segmented.

- They are greenish yellow when ripe, edible, with a sweet aromatic yellow white pulp. The numerous seeds are dark brown to black, shiny and smooth in texture.
- Flowers April to September; fruits May to September. (Baumer 1995 et al.,)

Propagation: 2,500-3,000Seeds/kg; they stay viable for about a year.

- It is an easy species to propagate. The seeds can be planted with no pre-treatment. Seedlings are ready for planting out after 6 months.
- Alternatively direct sowing can be practised; plant about three seeds, water and thin to one plant. (Baumer 1995, Burkhill, 1985)
- determine the antibacterial activity of leaf, stem bark and root bark extracts of *Annona squamosa* plant which have many pharmacological actions, by different methods like Disc diffusion method, Agar well diffusion method and Minimum Inhibitory Concentration method.

Materials And Methods

Fresh leaves stem bark and root bark of Annona squamosa were collected from the department of Biotechnology, Acharya Nagarjuna University, Nagarjuna nagar, Guntur. They are cleaned weighed and powdered by using domestic motar and pistle.

Extraction procedures:

The water extract is prepared by taking this 10g of powder and it was subjected to boiling along with 100 ml doubled distilled water in a 500 ml flask till the total volume becomes one fourth. The water extract was filtered through a Whatman No. 1 filter paper, cooled and transferred to screw capped glass vials.

The organic solvent extracts is prepared by using 10g of powdered plant material and extracted with solvents of different polarities (methanol, ethanol and petroleum ether) by cold maceration. The extracts were filtered through Whatman No. 1 filter paper into screw capped vials. These plant extracts were kept in a refrigerator (4-10^{0}c) for experimental use.

Preparation of bacterial inoculums:

The strains viz. Gram negative *Escherichia coli*, and Gram positive strains of *Bacillus megatherium*, B.*cereus* and *B.subtilis* were maintained on freshly prepared nutrient agar slants and stored at 4^{0}c for further use. Bacterial suspensions were prepared by mixing the loop-full of inoculum in 3ml sterile distilled water separately under aseptic conditions and incubated at 37^{0}c for 24 hours. All this was carried out under aseptic conditions.

Antimicrobial Screening:

The Antibacterial activity of plant extracts was assayed by Disc Diffusion method (Bauer et al,. 1996). In this method nutrient agar media was poured in petriplates and the test suspensions were uniformly spread out. The discs with dimensions of 5mm diameter were immersed into the crude plant extract and incubated for 24hours at 37^{0}c. These discs were placed in plates and zone of inhibition was observed.

In the Agar well Diffusion method (Neda mimica – Dukic et al.,2003) the standard inoculums of the test bacterial standard strains i.e. of *E.coli*, *B.megatherium*, B. *cereus* and *B. subtilis* were inoculated by pour plate method on Mueller Hinton Agar (MHA). Then, 5 mm diameter wells were bored in the MHA by scooping out with a sterile cork borer. Plant extracts of 0.5 ml were introduced into each well and allowed to stand for 1 hour at room temperature to diffuse and incubated at 37º C for 24 hours. The Inhibition Zone Diameter (IZD) was observed.

The Antibacterial activity can also be demonstrated by Minimum Inhibitory Concentration (MIC). In this method the 0.1ml of inoculums of bacterial strains and different concentrations of plant extract like 0.6, o.8 and 1.0ml were added to the aliquots. The solutions were made up to 1ml with the help of distilled water and were incubated at for 2-4 hours.

These are plated on to nutrient agar medium and the plates were incubated at 37^0c for 24hours. The zone diameter is measured.

Results And Discussion

Fresh leaves stem bark and root bark of Annona squamosa were collected are cleaned weighed and powdered by using domestic motar and pestle. The quantitative data was given in Table 1.

Table 1. Quantitative data

Plant part	Fresh weight (g)	Powder weight (g)	Quantity	Material concentration (mg/disc)
Leaves	50	15	10	0.43
Stem bark	50	12	10	0.81
Root bark	50	15	10	0.34

The results for antimicrobial screening are deduced by measuring the diameters of zones of inhibition which are shown in the following Tables (2 - 10). The activities of the water and methanolic extracts are moderately similar and concentration dependent and no activity were recorded at the lower concentrations of the extracts. In extraction results, percentage yield was found to be increased in accordance with the increasing polarity of the solvents. Highest yield was noted in water followed by methanol, chloroform and petroleum ether extracts.

Antibacterial Activity by Disc Diffusion Method:

The Antibacterial activity of plant extracts (stem, leaf & root bark) was assayed by Disc Diffusion method (Table 2-4). Stem bark petroleum ether extract was studied for its antibacterial activity; and conclude its high efficacy against *Bacillus* species. While the leaf and root extracts of petroleum ether showed good response towards the *E.coli.* The water extracts of *Annona squamosa* showed little activity against *E. coli* compared to methanol. Of all the strains E. coli was least affected by the extracts. The ethanol extracts of *A. squamosa* showed the least antibacterial activity against the given strains.

Table 2: Antibacterial activity – Disc diffusion method of Stem bark (0.81g/disc)

S No.	Test organisms	Zone of inhibition				Control (mm)
		Water extract (mm)	Methanol (mm)	Ethanol (mm)	Petroleum ether (mm)	
1.	E.coli	9	12	4	6	-
2.	B.subtilis	7	10	2	12	-
3.	B.megatherium	8	9	6	7	-
4.	B.cereus	14	8	5	6	-

Values are inhibition zones in millimeters, - shows no inhibition

Table 3: Antibacterial activity – Disc diffusion method of leaf (0.43 mg/disc)

S No.	Test organisms	Zone of inhibition				
		Water extract mm	Methanol mm	Ethanol mm	Petroleum ether mm	Control mm
1.	E.coli	9	10	5	12	-
2.	B. subtilis	10	14	2	8	-
3.	B.megatherium	11	8	6	10	-
4.	B.cereus	8	12	3	9	-

Values are inhibition zones in millimeters, - shows no inhibition

Table 4: Antibacterial activity- Disc diffusion method of Root bark(0.34mg/disc)

S No.	Test organisms	Water extract (mm)	Methanol (mm)	Ethanol (mm)	Petroleum ether (mm)	Control (mm)
1.	E.coli	8	11	6	7	-
2.	B.subtilis	14	7	4	9	-
3.	B.megatherium	7	8	5	10	-
4.	B.cereus	9	10	2	8	-

Values are inhibition zones in millimeters, - shows no inhibition

Antibacterial Activity by Well Diffusion Method:

The Antibacterial activity of plant extracts (stem, leaf & root bark) was assayed by Well Diffusion method (Table 5-7). Gram negative bacteria were found to be more resistant to the plant extracts than Gram positive bacteria, another results of the antimicrobial activities of the extracts was determined by measuring the diameters of zones of inhibition by using Well diffusion Method. Water extracts of leaf and stem bark showed the good response towards the *bacillus* species. The activity of the extract, against the tested pathogens significantly increased with increase in concentration of plant extract.

Table 5: Antibacterial activity – Agar Well diffusion method of Root bark

S No.	Test organisms	Water extract (mm)	Methanol (mm)	Ethanol (mm)	Petroleum ether (mm)	Control (mm)
1.	E.coli	9	8	7	7	-
2.	B.subtilis	5	7	6	10	-
3.	B.megatherium	9	16	5	8	-
4.	B.cereus	7	9	3	8	-

Values are inhibition zones in millimeters, - shows no inhibition

Table 6: Antibacterial activity – Agar Well diffusion method of leaf

SNo.	Test organisms	Zone of inhibition				
		Plant extract (mm)	Methanol (mm)	Ethanol (mm)	Petroleum ether (mm)	Control (mm)
1.	E.coli	9	6	7	5	-
2.	B. subtilis	7	13	4	8	-
3.	B.megatherium	13	7	6	16	-
4.	B.cereus	9	8	5	9	-

Values are inhibition zones in millimeters, - shows no inhibition

Table 7. Antibacterial activity – Agar Well diffusion method of Stem bark

S No.	Test organisms	Zone of inhibition				Control (mm)
		Plant extract (mm)	Methanol (mm)	Ethanol (mm)	Petroleum ether (mm)	
1.	E.coli	7	5	5	8	-
2.	B.subtilis	18	6	6	9	-
3.	B.megatherium	16	8	4	7	-
4.	B.cereus	9	6	2	13	-

Values are inhibition zones in millimeters, - shows no inhibition

Antibacterial Activity by Minimum Inhibitory Concentration (MIC):

The Antibacterial activity of plant extracts (stem, leaf & root bark) was assayed by minimum inhibitory concentration and the results are given in Table 8-10. Another result of the antimicrobial activities of the extracts was determined by measuring the minimum inhibitory concentrations (MIC). The MIC data for the organisms are also variable, and concentration dependent similar to the data in Table 2-4. The methanolic extract produced larger zones of inhibitions and lower MICs than aqueous extracts

Table 8: Antibacterial activity – Minimum Inhibitory concentration (Stem bark)

S No.	Extract	Colony formation							
		E. coli		*B.subtilis*		*B.megatherium*		*B.cereus*	
		PE	M	PE	M	PE	M	PE	M
1.	0.2	+	+	+	+	+	+	+	+
2.	0.4	+	+	+	+	+	+	+	+
3.	0.6	+	+	+	+	+	+	+	+
4.	0.8	+	+	+	+	+	+	+	+
5.	1.0	-	-	-	-	-	-	-	-
6.	1.2	-	-	-	-	-	-	-	-

No growth; + Growth; PE – Plant Extract; M – Methanol extract

Table 9: Antibacterial activity – Minimum Inhibitory concentration (Leaf extract)

S No.	Extract	Colony formation							
		E. coli		*B.subtilis*		*B.megatherium*		*B.cereus*	
		PE	M	PE	M	PE	M	PE	M
1.	0.2	+	+	+	+	+	+	+	+
2.	0.4	+	+	+	+	+	+	+	+
3.	0.6	+	+	+	+	+	+	+	+
4.	0.8	+	+	+	+	+	+	+	+
5.	1.0	-	-	-	-	-	-	-	-
6.	1.2	-	-	-	-	-	-	-	-

No growth; + Growth; PE – Plant Extract; M – Methanol extract

Table 10: Antibacterial activity – Minimum Inhibitory concentration (Root bark)

S No.	Extract	Colony formation							
		E. coli		*B.subtilis*		*B.megatherium*		*B.cereus*	
		PE	M	PE	M	PE	M	PE	M
1.	0.2	+	+	+	+	+	+	+	+
2.	0.4	+	+	+	+	+	+	+	+
3.	0.6	+	+	+	+	+	+	+	+
4.	0.8	+	+	+	+	+	+	+	+
5.	1.0	-	-	-	-	-	-	-	-
6.	1.2	-	-	-	-	-	-	-	-

No growth; + Growth; PE – Plant Extract; M – Methanol extract

Conclusion

All extracts of plant showed antibacterial activity. This could justify their use in treatment of microbial infections in man and livestock. Water extracts showed lesser activity compared to organic extracts like methanol on bacteria. This may be as a result of the methanol to extract the active ingredients. The methanolic extract produced larger zones of inhibitions and lower MICs than aqueous extracts probably because not all the bioactive components have been extracted in water. Aqueous extracts showed less activity than ethanol 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.

The lack of susceptibility of the bacteria to the plant extracts against *Annona squamosa* could be attributed to the fact that, unlike conventional pharmaceutical products which are usually prepared from synthetic materials by means of reproducible manufacturing techniques and procedures, herbal medicinal products are prepared from materials of plant origin which may be subjected to contamination and deterioration. The storage of extracts may require special condition of humidity or temperature or protection from light. The plant extracts might contain little of the active ingredient. The extracts which were inactive *in-vitro* may have properties similar to pro-drugs which are administered in an inactive form; their metabolites could be active *in-vivo*.

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