World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 5.088

PHYTOCHEMICAL SCREENING AND POTENTIAL BACTERICIDAL EFFICACY OF *ANNONA SQUAMOSA* LEAF EXTRACTS

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Article Received on 25/01/2018 **Article Revised on 15/02/2018** **Article Accepted on 08/03/2018**

ABSTRACT

Plants have been one of the important sources of medicines since the beginning of human civilization. Extensive biological research was carried out on *Annona squamosa* because of the presence of valuable bioactive compounds in various parts of the plant, which are traditionally used for the treatment of many ailments. This work has been carried out to evaluate the antibacterial activity of *Annona squamosa* leaves. A broad range of solvents including ethanol, acetone, methanol and distilled water have been used for the extraction of bioactive compounds from plant leaves. The antibacterial activity of leaf extracts of *Annona squamosa* was investigated against gram positive and gram negative bacterial pathogens such as *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* and *Escherichia coli* by agar well diffusion method. From this study, it is clearly indicated that *Annona squamosa* leaves possess the capabilities of being a good alternative agent for bactericidal infections.

KEYWORDS: *Annona squamosa,* Antibacterial activity, GC-MS analysis, Phytochemical analysis, Inhibitory zone.

INTRODUCTION

The frequency of life threatening infections caused by pathogenic microorganisms has increased worldwide leading to the cause of morbidity and mortality in immune compromised patients.[1] This increase has been attributed to indiscriminate use of broad spectrum antibiotics, immunosuppressive agent, intravenous catheters, organ transplantation and ongoing epidemics of HIV infection.[2] The increasing prevalence of bacterial resistance has made an important public health issue in the modern world.[3] Therefore, there is an urgent need to discover an alternative, more active, broad spectrum, and safer antimicrobial agents. Many Infectious diseases have been known to be treated with herbal remedies throughout the history of mankind.[4] Recently, there is a growing request for plant based medicines, health products, pharmaceuticals, cosmetics and food supplements.[5] Bioactive compounds has vital role in therapeutic remedies in many developing countries. According to World Health Organization, 80% of the world's population presently uses herbal medicine for some aspect of primary health care.[6]

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.[7] The presence of bioactive compounds such as glycosides, flavonoids, proanthocyanidins, tannins, mono and sequiterpenoids, phenylpropanoids, triterpenoids, resins, lignans, alkaloids, furocoumarines and naphthodianthrones in plants makes them a safe choice for application in the food preservation process.[8]

Among the various medicinal plants in practice *Annona squamosa* is a naturally occurring plant, traditionally used to treat various ailments including cancer. In the traditional literature, it is found that *Annona squamosa* leaves were used as folk medicine for the treatment of wound in different parts of the world.[9] Moreover, it is also used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, hemorrhage, antibacterial infection, dysuria, fever and ulcer. It also has antifertility, antitumor and abortifacient properties.[10] It contains several constituent belonging to category alkaloids, glycosides, flavonoids, terpenoids, steroids and fruit pulp contain important nutrients.[11] In recent times, screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic use. Thus, the present study was conducted to investigate the

effects of different extracting solvents of *Annona squamosa* leaves on the gram-negative and gram-positive bacterial pathogens.

MATERIALS AND METHODS

Collection and Identification of Plant

Healthy, disease free, mature leaves of *Annona squamosa* were collected from the region of Pollachi, Coimbatore District, Tamil Nadu. The collected plant material was identified and authenticated by Botanical Survey of India, Tamil Nadu Agricultural University, Tamil Nadu, where the voucher sample was preserved.

Preparation of Leaf Material

Leaves of selected plant were plucked and washed thoroughly with running tap water. It was again washed with sterile distilled water to remove dirt prior to drying process. The leaves were dried in shade at room temperature for a week to remove the moisture content and powdered using mixer grinder. Finally, powdered sample was stored at room temperature for further studies.

Preparation of Plant Extracts

2.5 g of powdered sample was taken in air tight bottles. To this, 50 ml of different solvents such as ethanol, methanol, acetone and distilled water was added. After 2 days, the contents were stirred well thoroughly and filtered using Whatmann No.1 filter paper. The filtrate was collected and stored in sterile bottle at 4°C until further use. For antibacterial studies, each extract was prepared by dissolving 250 mg in 5 ml of 10% (v/v) aqueous dimethylsulphoxide (DMSO).

Phytochemical Analysis

Freshly prepared leaf extracts were subjected to standard phytochemical analyses using standard procedure[12] in order to find out the presence of various phytoconstituents such as alkaloids, terpenoids, flavonoids, tannins, steroids, anthroquinones, saponins, resins, glycosides and phenols.

GC-MS Analysis

GC-MS analysis is a common confirmation test to separate all the components in a sample and provides a representative spectral output. The sample is injected into injection port of the GC device. The GC instrument vaporizes the sample and then separates and analyzes the various component ideally produces a specific spectral peak that may be recorded on a paper chart or electronically. The retention time can help to differentiate between some compounds. The size of the peak is proportional to the quantity of the corresponding substances in the specimen analyzed. The analysis was carried out on the model: SHIMADZU QP-2010 Plus. Helium was used as the carrier gas, with a rate flow of 2 ml/min in the split mode (20:1). An aliquot of 2µl of sample was injected with temperature at 280ºC. GC oven temperature started at 70ºC and holding for 8 min and it was raised to 220ºC holding for 7 min at the rate of 10ºC/min. holding was allowed at 280ºC for 7 min with program rate of 10ºC/min. Iron source temperature was maintained at 300ºC. The detector was operated scan mode from with interval of 0.50 sec.

Antibacterial Studies of Leaf Extracts

Bacterial culture

The following gram positive and gram negative bacterial pathogens namely *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi,* and *Escherichia coli,* were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C. Overnight culture in the nutrient broth was used for the present experimental study.

Assay to evaluate antibacterial activity of leaf extracts

The antibacterial activity of leaf extract was evaluated using agar-well diffusion method.[13] Pure cultures of each bacterial strain were sub cultured in nutrient broth on a rotary shaker at 200 rpm for 24 hours at 37°C. For preparing Mueller Hinton agar (MHA) plates, the MHA medium was boiled to dissolve completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 30 minutes. After sterilization, 20 ml of MHA media was poured into the sterile petri plates and kept at room temperature for solidification. Then, each strain was swabbed uniformly onto the individual Mueller Hinton agar plates using sterile cotton swabs. Wells of 6 mm diameter were made on Mueller Hinton agar plates using sterile cork borer and 50 µl of plant extracts were poured into each well on all plates. The plates were incubated overnight at 37°C and the results were observed by the presence of bacterial growth inhibition zone around the sample loaded well and their diameters (mm) were measured using measuring scale. The assay was performed in triplicates.

RESULTS AND DISCUSSION

Collection and Identification of Plant

Healthy, disease free, mature leaves of *Annona squamosa* were collected from the region of Pollachi, Coimbatore District, Tamil Nadu.

Preparation of Leaf Material

Fresh leaves of *Annona squamosa* were collected and washed thoroughly with running tap water. It was again washed with sterile distilled water to remove dirt prior to drying process. Afterwards the leaves were dried in shade at room temperature for a week to remove the moisture content and then powdered using mixer grinder. Finally, powdered sample was stored at room temperature for further studies.

Preparation of Leaf Extracts

The yield of leaf extraction mainly depends on solvents, time and temperature of extraction as well as the chemical nature of the sample. Under the same time and temperature conditions, the solvent used and the chemical property of the sample are the two most

important factors. The recommended effective extracting solvents are aqueous mixtures of methanol, ethanol and acetone. The leaf extracts of *Annona squamosa* was prepared with four different solvents such as ethanol, methanol, acetone and distilled water.

Phytochemical Analysis

The results of the phytochemical composition of the leaf extracts of *Annona squamosa* is given in Table 1. The results of phytochemical studies showed that all tested extracts (ethanol, methanol acetone and aqueous) contains the presence of alkaloids, terpenoids, flavonoids, tannins, steroids, anthraquinones, saponins, resins, glycosides and phenols. *Annona squamosa* leaf extracts has various phytochemical constituents like carbohydrates, protein, fats, alkaloids, terpenoids, flavonoids, saponins, tannin and glycosides.[13]

Table 1: Qualitative phytochemical analysis of *Annona squamosa* leaf extract.

Compounds	Tests Adopted	Ethanol Extract	Methanol Extract	Acetone Extract	Aqueous Extract
Alkaloids	Mayer's Test	+	+	+	+
Terpenoids	Salkowski Test	+	+	+	+
Flavonoids	Sodium hydroxide test	_	+	+	+
Tannins	Lead Acetate Test	+	+	+	+
Steroids	Chloroform Test	+	_	_	_
Anthraquinones	Free Anthraquinones	+	+	+	+
Saponins	Foam Test	+	+	+	+
Resins	Sodium hydroxide	+	+	+	+
Glycosides	Keller Killiani's test	+	+	+	+
Phenols	Ferric chloride test	+	+	+	+

+ = present; - = absent

GC-MS Analysis

GC-MS plays an important role in the analysis of unknown component of plant. GC technique involves the separation of volatile components in the test sample using capillary column. The compounds of test sample are evaporated in the injection port of the GC equipment and segregated in the column. The elusion from the column is based on the boiling point of the compounds present in the sample. The time at which each compound is eluted from the column of the GC equipment is known as retention time (RT). GC-MS chromatogram of the ethanol extract of *Annona squamosa* showed 49 peaks indicating the presence of 49 phytochemical constituents. GC-MS chromatograph of plant extracts of *Annona squamosa* is shown in Figure 1.

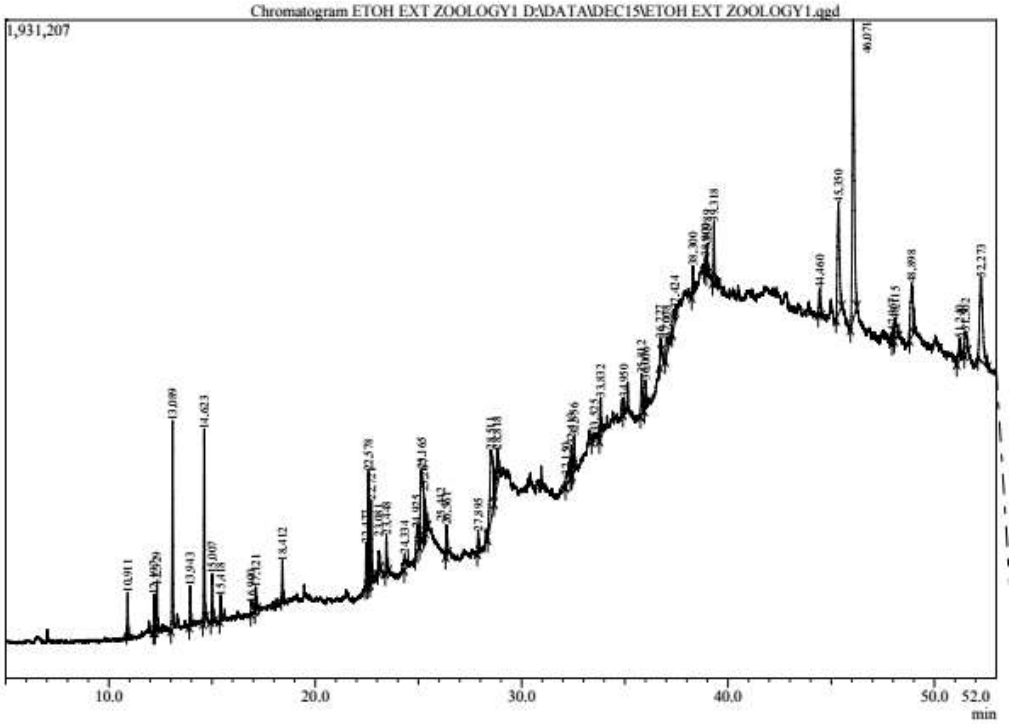

Fig. 1: GC-MS chromatograph of plant extracts of *Annona squamosal.*

Among that the major phytocomponents with their retention time (RT), molecular formula, molecular weight and the concentration of the major compounds are mentioned in the Table 2. The prevailing compounds present in the leaf of the plant *Annona squamosa* are Alpha- Tocopherol- beta- D- mannoside (4.69%), dl-alpha-Tocopherol (6.46%), Oleic acid (2.99%), n-Hexadecanoic acid (3%) and Caryophyllene (8.74%). Using Dr. Duke's phytochemical and ethnobotanical database the activity of the phytocomponents was identified. It is mentioned in the Table 3.

Table 2: Total ionic chromatogram showing the major compounds of ethanol extract of *Annona squamosa* leaf extract.

S. No	RT	Name of the Compound	Molecular Formula	Molecular Weight	Composition (%)
1	46.07	Alpha- Tocopherol- beta- D- mannoside	$C_{35}H_{60}O_7$	592	12.58
2	45.35	Alpha- Tocopherol	$C_{29}H_{50}O_2$	430	4.69
3	28.51	Oleic acid	$C_{18}H_{34}O_2$	282	2.99
4	25.16	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	3.00
5	13.08	Caryophyllene	$C_{15}H_{24}$	204	8.74

Table 3: Activities of major phytocompounds identified in the *Annona squamosal.*

Sl. No.	Compound Name	Activity*
1	Alpha- Tocopherol- beta- D- mannoside	Antitumour, anti-inflammatory, antibacteria
2	Alpha- Tocopherol	Antianginal, antiarthritic, antiasthmatic, antiatherosclerotic, anticancer, anticataract, antidiabetic, antidementia
3	Oleic acid	anti- inflammatory, antiandrogenic, cancer preventive, dermatitigenic, insectifuge, antibacterial, anaemiagenic
4	n-Hexadecanoic acid	Antioxidant, nematicide, pesticide, flavor, lubricant and antiandrogenic.
5	Caryophyllene	Allergenic, analgesic, antiacne, antibacterial, antiedemic, antidermatic, antifeedent, anti-inflammatory, antitumour, antispasmodic, antiulcer

**Dr. Duke's phytochemical and ethnobotanical database*

Antibacterial Studies of *Annona squamosa* Leaf Extracts

The antibacterial activity of various leaf extracts of *Annona squamosa* was investigated against gram positive and gram negative pathogens such as *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* and *Escherichia coli* by agar well diffusion method. Padhi *et al*. (2011)[14] investigated the antibacterial activity of *Annona squamosa* leaves extracts. They used methanol extract of *Annona squamosa* stem barks to check the efficacy of antibacterial activity against *Bacillus* coagulants and *Escherichia coli* using agar disc diffusion method. Successful prediction of bioactive compounds from plant material is largely dependent on the type of solvent used in the extraction procedure.[15] Waterman and Mole (1994)[16] reported that solvent such as aqueous mixtures of methanol, ethanol and acetone are the most recommended and effective one. *Annona squamosa* leaf extracts showed varying degrees of antibacterial activities against the selected pathogens (Table 4). The antibacterial activity of ethanol extract of *Annona squamosa* against tested pathogens showed maximum zone of inhibition (22.33 ± 1.70 mm) against *Salmonella typhi*. The minimum inhibitory zone (11.00 ± 0.82 mm) was observed against *Staphylococcus aureus.* They noticed the inhibition zones of 15.5 mm, 16.5 mm, and 12mm for 100% acetone leaf extract against *Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa*. The antibacterial activity of methanol extracts of *Annona squamosa* exhibited maximum zone of inhibition of about 16.00 ± 1.63 mm against *Salmonella typhi* followed by inhibitory zone of about 15.67 ± 0.47 mm and 12.67 ± 1.25 mm against *Bacillus subtilis* and *Escherichia coli* respectively. Similarly, the acetone extract of *Annona squamosa* showed greatest susceptibility towards *Salmonella typhi* with a zone of inhibition 17.67 ± 2.05 mm whereas the lowest susceptibility (11.00 ± 1.41 mm and 11.00 ± 0.82 mm) was noticed against both *Bacillus subtilis* and *Escherichia coli.* While methanol as well as acetone extract of *Annona squamosa* showed no inhibitory zone against *Staphylococcus aureus.* Aqueous extract of *Annona squamosa* demonstrated that it was found to have no activity against all tested organisms except *salmonella typhi* (15.67 ± 1.70 mm). Among the different leaf extracts of *Annona squamosa* used, ethanolic extract showed greatest antibacterial effect pathogenic strains.

Table 4: Inhibition zones of various extracts of *Annona squamosa* leaves against gram positive and gram negative bacterial pathogens.

Bacterial pathogens	Zone of inhibition (mm)			
	Ethanol	Methanol	Acetone	Aqueous
Staphylococcus aureus (G+)	11.00 ± 0.82	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Bacillus subtilis (G+)	15.33 ± 1.25	15.67 ± 0.47	11.00 ± 1.41	6.00 ± 0.00
Salmonella typhi (G-)	22.33 ± 1.70	16.00 ± 1.63	17.67 ± 2.05	15.67 ± 1.70
Escherichia coli (G-)	16.33 ± 0.94	12.67 ± 1.25	11.00 ± 0.82	6.00 ± 0.00

"G+" gram positive bacteria "G-" gram negative bacteria.

Results on antibacterial activity of various extracts of *Annona squamosa* revealed that gram negative bacterial pathogens were highly suppressed by all leaf extracts tested compared to gram positive bacterial pathogens. The highly resistance of the gram negative bacteria could be attributed to its cell wall structure. Moreover, gram negative bacteria have an effective permeability barrier, comprised of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of extruding the plant extract. Whereas gram positive bacteria have a mesh-like peptidoglycan layer which is more accessible to permeation by the extracts. The antibacterial activity of the plant extracts might be attributed to the presence of bioactive compounds such as tannins, phenolic compounds, polyphenols and flavonoids.[17] Among these bioactive compounds, phenolics were the most important active compounds against bacteria.[18]

As this plant has numerous medicinal applications, this work has been carried out to evaluate the antibacterial activity of *Annona squamosa* leaves. There are several solvents has been reported for the extraction of bioactive compounds from plant materials. Successful prediction of bioactive compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The extraction yield mainly depended on solvents, time and temperature of extraction as well as the chemical nature of the samples. Phenolic were the most important active compounds against bacteria. Khan and Joshi (2015)[19] observed that *Annona squamosa* was found to be a good source of antimicrobial compounds. They noticed the inhibition zones of 15.5 mm, 16.5 mm, and 12mm for 100% acetone leaf extract against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* 100% chloroform leaf extract gave zone of 17.0 mm, 15.5 mm, 15.5 mm against the respective pathogens. Various solvent extracts of *Annona squamosa* leaf inhibited the different strains of the bacteria like *Vibrio algin/lyticus, Staphylococcus aureus, Bacillus subtilis* and *Staphylococcus epidermidis.* The aqueous extract of the leaves inhibit the pathogenicity of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli.*[20]

Antibacterial studies revealed that highest inhibition zone was observed by the methanol extract which were followed by petroleum ether and chloroform extracts of *Annona squamosa.*[21] From the current study, it was observed that *Annona squamosa* inhibited the growth of all the bacteria tested. This suggests that the plant extract showed broad spectrum activity against the selected pathogens. The antibacterial effect of *Annona squamosa* was due to the phytochemical constituents present in it. It was also rich in various phytonutrients such as flavonoids, phenolic compounds, tannins, saponins, terpenoids, cardiac glycosides and alkaloids. Extracts from *Annona squamosa* contain phytochemicals which offer an enormous potential for biocontrol of these pathogens and source of antimicrobial agents of therapeutic importance. Hence, the plant *Annona squamosa* can be of immense use in phytomedicine and can be included in health care system. The phytochemicals obtained from the *Annona squamosa* can be used in the treatment of infectious diseases.

CONCLUSION

The frequency of life threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immune compromised patients in developing countries like our India. Results on bactericidal effect revealed that leaf extracts of *Annona squamosa* showed varying degrees of antibacterial property against the selected pathogens used. In conclusion, the present findings clearly indicated that *Annona squamosa* leaves possess the capabilities of being a good candidate in the search for a natural antibacterial agent against diseases caused by both gram positive and gram negative pathogenic strains.

ACKNOWLEDGEMENTS

The authors are grateful to the management of PSG College of Arts and Science, Coimbatore for their encouragement and providing facilities for this work.

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