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Phytochemical analysis and antimicrobial activities of *Annona squamosa* (L) leaf extracts

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Abstract
Plants have been one of the important sources of medicines since the beginning of human civilization. There is a growing demand for plant based medicines, health products, pharmaceuticals, food supplements and cosmetics. *Annona squamosa* (L) is a multipurpose tree with edible fruits and is a source one of the medicinal and industrial products. It is used as an antioxidant, anti-diabetics, hepato protective, cytotoxic, genetoxic, anti-tumour, anti-lice agent etc. This article intends to provide an overview of the chemical constituents present in the crude leaf extracts of *Annona squamosa* (L) with special emphasis on their pharmacological actions. Qualitative phytochemical screening was carried out using the crude leaf extracts in three different solvents such as water, alcohol and chloroform. Phytochemical analysis of the extracts revealed the presence of glycosides, alkaloids, oils, saponins and flavanoids. A comparative antimicrobial activity of dried leaf extracts of *Annona squamosa* (L) were evaluated against two gram negative bacterial strains namely *Escherichia coli* and *Pseudomonas aeroginosa* and two clinical fungal pathogens namely *Candida albicans* and *Aspergillus niger* by agar cup method. The leaf extracts of *Annona squamosa* (L) was found to have high antibacterial activity than anti-fungal activity. The results suggest that the leaves are a rich source of valuable primary and secondary metabolites exhibiting the antimicrobial activity.

Keywords: Antimicrobial, Phytochemical screening, *Annona squamosa,* Agar cup method

1. Introduction
Since ancient times, people have been exploring the nature particularly plants in search of new drugs which has resulted in the use of large number of medicinal plants with curative properties to treat various diseases (Verpoorte, 1998) [18]. According to WHO survey, 80% populations living in the developing countries rely exclusively on traditional medicine for their primary health care needs of which most involve the use of plant extracts (Sandhya *et al*., 2006) [14]. The studies of plants continue principally for the discovery of novel secondary metabolites or phytochemicals which are the non-essential nutrients derived from plants exhibiting a number of protective functions for human consumers.

Annona squamosa (L), belonging to the family *Annonaceae* is a small ever green tree commonly found in India and originates from West Indies and South America. Different parts of *Annona squamosa* (L) are used in folkloric medicine for the treatment of various diseases (Suresh *et al*., 2006) [15]. It is mainly grown in gardens for its fruits and ornamental value. This plant is commonly called custared apple in english, sharifa in hindi and sitaphalam in telungu in india (Raj Sobiya *et al*., 2009) [12].

It is considered beneficial for cardiac disease, diabetes hyperthyroidism and cancer. The root is considered as a drastic purgative (Raj Sobiya *et al*., 2009) [12]. An infusion of the leaves is considered efficacious in prolapsusani of children. The crushed leaves are sniffed to overcome hysteria and fainting spells. A leaf decoction was taken in the case of dysentery (S. Gajalakshmi *et al.,* 2011) [13]. Leaves are used as poultice over boils and ulcers. The ripe fruits of this plant are applied to malignant tumors to hasten suppuration. The dried unripe fruit powder is used to destroy vermin. The seeds are acrid and poisonous. Powdered seeds serve as fish poison and insecticides. A paste of seed powder has been applied to the head to kill lice. It is also used for destroying worm in the wound of cattles (Parvin *et al.,* 2003) [7].

Phytochemical screening is a method which exposes or reveals certain components or properties readily available in plants for bio-activity or ethno-medical applications. Plant based antimicrobials has enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (lwu *et al*., 1999) [5].

Thus it is anticipated that phytochemicals with adequate antibacterial efficiency can be used for the treatment of bacterial infections (Balandrin *et al*., 1985) [2]. Antioxidants and antimicrobial properties of various extracts from many plants have recently been of great interest in both research and in food industry, because of their possible use as natural additives to replace synthetic antioxidants and antimicrobials with natural ones (Deba *et al.,* 2008) [4]. Thus medicinal plants play an important role in the development of newer drugs because of their effectiveness, less side effects and relatively low cost when compared with synthetic drugs (Raj *et al.,* 2011) [11]. The present study aims in exploring the phytochemical constituents, antibacterial and antifungal properties of the crude leaf extracts of *Annona squamosa* (L).

2. Materials and Methods

2.1 Collection and Extraction of Plant Materials.

The fully matured fresh leaves of *Annona squamosa* (L) were collected from Kattakada area in Thiruvananthapuram district. The leaves were washed thoroughly, shade dried and finely powered. The dried powdered leaves were extracted with three different solvents such as water, acetone and chloroform. For aqueous extraction, ten grams of the powdered leaves was mixed with 100ml distilled water, boiled for two hours and filtered. Whereas acetone and chloroform extracts were prepared by mixing ten grams of powdered leaf samples with 100ml of each solvent separately in mechanical shaker for 48 hours at room temperature. Extracts were filtered, concentrated, dried and were stored in the refrigerator at 4 °C for future use.

2.2 Phytochemical Analysis

The prepared plant extracts were analysed for the presence of alkaloids, glycosides, saponins, proteins, aminoacids, fixed oils, phenolic compounds, tannins, flavonoids, gum and mucilages etc. (Raman, 2006) [10].

2.3 Preparation of Plant Extract for Antimicrobial Analysis

The collected leaves were washed thoroughly in tap water, shade dried and finely powered. Ten grams of powered leaf samples were mixed with 100ml of ethanol and kept in mechanical shaker for 48 hours at room temperature. Extracts were then filtered, concentrated and dried. The extracted powder was dissolved in 10% dimethyl sulfoxide (DMSO) and stored in refrigerator at 4 ^{0}C.

2.4 Antibacterial Activity

Antibacterial activity was carried out against two selected gram negative pathogens (such as *Escherichia coli and Pseudomonas aeroginosa*). The strains used for the present study were obtained from Biogenix Research centre, Valiyavila, Trivandrum. In order to access the biological significance and ability of the plant part, the minimal inhibitory activity was determined by Agar cup method.

Petriplates containing 20ml of Muller Hinton medium were seeded each with 24hr old culture of bacterial strains such as *E. coli and P. aeroginosa*. Wells of approximately 10mm diameter was bored using a well cutter and 25 µl, 50 µl and 100µl of the extracts were added to the well from a stock concentration of 0.1g/1ml. The plates were then incubated at 37 °C for 24 hours. Antibacterial activity was assayed by measuring the diameter of the inhibition zone in millimeters formed around the wells (NCCLS, 1993) [19]. Gentamycin (standard antibacterial agent, concentration: 20mg/ml) was used as a positive control.

2.5 Antifungal Activity

Antifungal activity was also determined by Agar cup method. Potato Dextrose agar plates were prepared and overnight grown isolates of fungi such as *Candida albicans* and *Aspergillus niger* were swabbed. Wells of approximately 10mm diameter was bored using a well cutter and extracts of 25 µl, 50 µl and 100 µl concentrations were added and the zones of inhibition were measured after overnight incubation which were then compared with that of standard antibiotics. Clotrimazole was used as a positive control.

3. Results and Discussion

3.1 Phytochemical Analysis

Table 1 represent the various phytochemical constituents present in the leaf extracts of *Annona squamosa* (L). The phytochemical studies of all the three extracts conclude that acetone and water extracts of leaf samples had more positive results for glycosides, oils, saponins and flavonoids.

Table 1: Phytochemical analysis of *Annona squamosa* (L) leaf extracts

Phytochemicals	Glycosides	Phytosterols	Alkaloids	Oils	Saponins	Phenols	Flavanoids
Water	+	-	-	+	+	+	+
Acetone	+	+	-	+	+	-	+
Chloroform	+	+	-	+	+	-	-

+: Present - : Absent

Preliminary phytochemical analysis revealed the presence of six compounds (Table 1) viz. flavanoids, glycosides, oils, saponins, phenolics, gum and mucilage. With acetone and chloroform extracts flavanoids, glycosides, phytosterols, oils and saponins were present but alkaloids, proteins, aminoacids, phenols and flavanoids were found to be absent. Traditionally saponins have been extensively used as detergents, pesticides as well as mollucides, in addition to their industrial application such as foaming, surface active agents etc. and also found to have beneficial health effects (Arunasalam, *et al.,* 2004) [1].

Chloroform leaf extract of the plant contain an active constituent Annotemoyin. Flavonoids isolated from aqueous extract of *Annona squamosa* (L) exhibits antimicrobial activity. The plant is reported to contain glycosides, alkaloids, saponins, flavonoids, tannins, carbohydrates, phenolic compounds and phytosterols by previous workers.

Patel *et al* (2008) [8] also reported the presence of 4-(2-nitro-ethyl 1)-1-6-((6-o-β-Dxylopyranosy1- β-D-glucopyranosyl)-oxy) benzene, Anonaine, Benzyltetrahydroisoquinoline, Borneol, Camphene, Camphor, car-3-ene, Carvone, β-Caryphyllene, Eugenol, Farnesol, Geraniol, 16-Hetriacontanone, Hexacontanol, Higemamine, Isocorydine, Limonine, Linalool acetate, Methylheptenone, p-(hydroxybenzyl)-6,7-(2- hydroxy,4-hydro)isoquinoline, n-Octacosanol, a- Pinene, b-Pinene, Rutin, Stigmasterol, β-Sitosterol, Thymol, n-Triacontanol etc and absence of alkaloids, proteins, amino acids etc. in the leaf extract of Annona squamosa (L).

3.2 Anti-Bacterial Activity

Antibacterial activity of *Annona squamosa* (L) (leaf ethanol extract with DMSO) was assayed *in vitro* by agar cup method against clinical isolates of *E. coli* and *P. aeroginosa.* The given table shows the microbial growth inhibition of ethanolic leaf extracts of *Annona squamosa* (L) among the varying concentration of leaf extracts, higher concentration exhibited maximum antibacterial activity against the two clinical isolates of *E. coli* and *P. aeroginosa.* Table 2 shows the zone of inhibition formed by the extracts against the bacterial strains on Muller Hinton agar.

Table 2: Zone diameter of inhibition of ethanolic leaf extract of *Annona squamosa* (L)

Test organisms	Zone of inhibition in mm			Positive Control
	Concentration of leaf extracts			
	25	50	100	
E. Coli	Nil	11	17	30
P. aeroginosa	Nil	Nil	15	30

The sequence of antibacterial activity of leaf extract against *E. coli* exhibited no activity in 25µl but produced 11mm and 17 mm zones of inhibition in 50µl and 100µl concentrations respectively (Table 2). Whereas the sequence of antibacterial activity of plant extract against *P. aeroginosa* showed no activity in both 25µl and 50µl respectively but produced a 15mm inhibition zone in 100µl concentration (Table 2). Antibacterial activity was expressed at varying degrees with the difference in concentration. Higher concentration of the leaf extract shows highest antibacterial activity. The result obtained might be considered sufficient for further studies for isolation and identification of active principle and for the evaluation of possible antimicrobial activity of other extracts from other parts of *Annona squamosa* (L).

It was supported by Cosentino *et al*., (1999) [3] and stated that the extracts from other parts of *Annona squamosa* (L) are used against microbial infections due to the presence of secondary metabolites such as phenols, essential oils, terpenoids, alkaloids and flavanoids. Flavanoids were present in *Annona squamosa* (L) which was earlier studied by Kotkar *et al.,* (2001) [6] and reported that flavanoids expose strong antibacterial activity.

3.3 Antifungal Activity

The antifungal activity of *Annona squamosa* (L) (leaf ethanol extract) was assayed *in vitro* by agar cup method against clinical isolates of *C. albicans* and *A. niger*. The given table shows the microbial growth inhibition of ethanolic leaf extracts of *Annona squamosa* (L).

The sequence of antifungal activity of leaf extract against *C. albicans* was 11mm in 100µl, but no activity was found in 25µl and 50µl concentrations respectively (Table 3). *Annona squamosa* (L) leaf extract was found to have no inhibitory activity against *A. niger* at all the three different concentrations.

Table 3: Zone diameter of inhibition of ethanolic leaf extract of *Annona squamosa* (L)

Test organisms	Zone of inhibition in mm			Positive Control
	Concentration of leaf extracts			
	25	50	100	
C. albicans	Nil	Nil	11	25
A. niger	Nil	Nil	Nil	25

In the present study the plant exhibited better antibacterial activity than antifungal activity. In literature it has been indicated that the antibacterial activity is exhibited by the different chemical agents present in the extract including essential oils, flavanoids, terpenoids and other compounds of phenolic nature or free hydroxyl group which are classified as active antimicrobial compounds. These findings can form the basis of further studies to isolate active phytocemicals, elucidate them against wider range of bacterial strains with the goal to find new therapeutic principles.

The present study reveals that the leaf extracts of *Annona squamosa* (L) were active against *E. coli* and *P. aeroginosa* than fungi. Anti fungal activity were found to be negligible when compared to antibacterial activity. The results of the study supports to a certain degree, the usage of traditional medicinal plants in human and animal disease therapy and reinforce the concept that ethno botanical approach to screening plants as potential sources of bioactive substances is successful (Valsaraj *et al*., 1997) [16]. The aqueous extract generally exhibits a high degree of antibacterial activity which seems to confirm the traditional therapeutic claims of this plant (Perumal samy *et al*., 1998) [9]. These results suggest the presence of either good antibacterial potency or high concentration of an active principle in the extract.

4. Summary and Conclusion

Medicinal plants were the potent source of human health due to the active phytochemical compounds that is responsible for its various pharmacological activities. On the basis of the results obtained in the present study conclude that *Annona squamosa* (L) are rich in phytochemical constituents and showed antimicrobial properties. The results of the phytochemical screening of the leaf extracts of samples varied, while some of the components were present, some were absent. It was observed that most of the components were present in aqueous extracts.

In *Annona squamosa* (L) glycosides, oils and saponins were present in all the three solvents. The presence of various secondary metabolites such as glycosides, phytosterols, alkaloids, oils, saponins, phenols and flavanoids confirmed the antimicrobial efficacy against selected pathogens.

The present study highlights the possible use of *Annona squamosa* (L) leaf extracts as a source of antioxidants and as

antibacterial agents that can be used to prevent enteric diseases. The study showed that the results of extraction yield, total phenolic and flavonoid compounds and bioactivity tests varied depending on the type of solvent being used. The study revealed that the leaves of *Annona squamosa* (L) contain a considerable quantity of phenolic - flavonoid compounds that were found to be the major contributor for their antioxidant and antibacterial activities. Hence it can be concluded that the leaves of *Annona squamosa* (L) would direct to the establishment of some compounds that could be used to invent new and more potent anti-microbial drugs of natural origin. Future research should be addressed on the application of using *Annona squamosa* (L) leaves as natural remedier and to protect against the enteric diseases.

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