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PHYTOCHEMICAL PROFILING AND ANTIBACTERIAL ACTIVITY OF MULTIPLE SOLVENT EXTRACTS OF *ANNONA SQUAMOSA* SEEDS AGAINST FIVE CLINICALLY RELEVANT BACTERIAL STRAINS

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In recent years, plant products have been reported to be efficient anti-microbial agents without side effects. *Annona squamosa L.* (Family: Annonaceae) is traditionally used in treating diarrhoea, dysentery, colds, chills, rheumatism, and sleeplessness. It also has an anticancer function. The seed oil is used against agricultural pests.

The present study focused on investigating the antibacterial activity of hexane, petroleum ether, chloroform, and ethanol extracts of *A. squamosa* seeds.

All the extracts were screened qualitatively for chemical constituents. The antibacterial activity of seed extract was evaluated by the agar well diffusion method against five pathogenic bacteria in five different concentrations (10, 5, 2.5, 1.25, and 0.625 mg) of the extract. The zones of inhibition were measured in mm.

Alkaloids, carbohydrates, glycosides, saponins, proteins, amino acids, phenols, flavonoids, terpenoids, steroids, tannins, anthraquinones, and quinones were detected. The tested organisms were susceptible to all the extracts at different concentrations. 10 mg hexane extract showed the highest zone of inhibition in cultures of *Enterobacter cloacae* (15 mm) and *Escherichia coli* (14 mm). Petroleum ether extract at 10 mg was effective against *Escherichia coli* (13 mm) and *Klebsiella pneumoniae* (12 mm). The chloroform extract was effective against *Enterobacter cloacae* (18 mm) and *Escherichia coli* (14 mm) cultures at 10 mg concentration. The zone of inhibition formed by 10 mg ethanol extract was highest in *Escherichia coli* (18 mm) and *Staphylococcus aureus* (18 mm).

Our results confirm that *A. squamosa* seed extract has anti-bacterial efficacy due to the presence of various active principles in the extracts.

Keywords: Phytochemicals, Antibiotics, Medicinal plants, Zone of inhibition, Agar well diffusion assay.

Introduction

Infectious disease is accounted as the world's leading cause of premature death, killing almost 50,000 people every day. Mortality continues to be a major problem in many developing countries, especially among children. Infection that occurs due to many pathogenic bacteria such as *Escherichia sp., Staphylococcus sp., Streptococcus sp., Klebsiella sp., Salmonella sp., Enterobacter sp.* are the most common. The World Health Organization forecasts thirteen million deaths to these causes in 2050.[1] In addition, nosocomial infections in recent times are no exception in a hospital. In

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hospitalized patients many pathogens are potential to cause infection. Gram-positive and Gram-negative bacteria are equally responsible for the majority of nosocomial infections. Among them, *Staphylococcus*, *Escherichia, Pseudomonas,* and *Enterococci* take the leading.[2] Although infectious diseases and nosocomial infections are treated by antibiotics of either microbial origin or purely synthetic or semi-synthetic, unfortunately, the antimicrobial agent continues to present problems in modern-day medicine suggesting a significant increase in the incidents of bacterial resistance to several antibiotics.[3] In addition to this problem antibiotic is sometimes associated with adverse effects on the host which includes hypersensitivity, immune suppressant, and allergic reactions.[4] This has created immense clinical problems in the treatment of infectious diseases.[5] Therefore there is a need to search and develop an alternative antimicrobial drug for the treatment of infectious diseases.

Our approach is to screen local medicinal plants for possible antimicrobial properties. Since ancient times, herbs and their external oils have been reported for their varying degrees of antimicrobial activity. Many reports are published on the effectiveness of traditional herbs against Gram-positive and Gram-negative bacteria and as a result, plants are still a recognized core for modern medicine to treat infectious diseases.[6]

Annona squamosa, widely available in India and reported in the traditional ancient system of medicine, few of which have been established through the systematic scientific report, has been selected for the study. *A. squamosa* Linn. commonly known as custard apple "Sitapazham" in Tamil produces an edible sweet fruit.[7,8] Seeds are hard, shiny brownish-black enclosed in the flesh, ovoid, numerous, and scattered over the white pulp.[9] Traditionally the plant is praised for its astonishing medicinal property.[10,11,12,13] Seeds are believed to be anti-parasitic and anti-cancerous.[14] Several reports are available to prove its pharmacological property.[15,16,17,18,19,20] Seeds are proven antitumor,[21] insecticidal,[22] and pesticidal.[23]

Although extensive work has been carried out in the aerial parts including the leaves, fruit, bark, and roots of the plant, the review revealed scarce research in the seed, hence we sought to test the effect of *A. squamosa* on five pathogenic bacteria.

Materials and Methods

Plant collection and extraction:

A. squamosa seeds were collected from Tirunelveli District (Tamil Nadu, India). The seeds were shade-dried and powdered. Extraction was by cold maceration technique[24] in which the powdered seed was soaked in four different solvents (hexane, petroleum ether, chloroform, and ethanol) in a conical flask. The entire mixture was shaken at regular intervals to ensure thorough extraction. The stoppered container was allowed to stand 72 hr at room temperature after which the mixture was filtered and concentrated to obtain the extracts.

Phytochemical analysis:

The four extracts of *A. squamosa* seeds were subjected to preliminary phytochemical analysis by following standard methods.

1. **Alkaloids:** To 1 ml extract, 1 ml picric acid was added. Yellow colour appears indicating the presence of alkaloids.
2. **Carbohydrates:** To 1 ml extract, 2 ml Fehling solution I and II were added in equal volume and kept in a water bath. The appearance of red colour indicates the presence of carbohydrates.
3. **Glycosides:** To 1 ml extract, 1.5 ml chloroform was added and shaken well. To the separated layers, 10% ammonia was added. The appearance of pink colour indicates the presence of glycosides.
4. **Saponins:** 1 ml extract was mixed with distilled water and vigorously

shaken. The formation of foam indicates the presence of saponins.

5. **Proteins:** To 2 ml extract, 2% copper sulphate, 1 ml 95% ethanol, and excess potassium hydroxide were added and shaken well. The appearance of the pink colour ethanoic layer indicates the presence of protein.
6. **Amino acids:** To 2 ml extract, ninhydrin solution was added. Formation of purple colour confirms the presence of amino acids.
7. **Phenols:** To 1 ml extract, distilled water and ferric chloride were added. The dark green colour indicates the presence of phenols.
8. **Flavanoids:** To 2 ml extract, 3 ml dilute ammonia, and 1 ml conc. H_2SO_4 was added. The yellow colour shows the presence of flavonoids.
9. **Terpenoids:** To 1 ml extract, 2 ml chloroform was added and mixed well. To this, conc. H_2SO_4 was added. The reddish-brown layer indicates the presence of terpenoids.
10. **Steroids:** To 1 ml extract, 2 ml chloroform, and 1 ml conc. H_2SO_4 was added. A reddish-brown ring indicates the presence of steroids.
11. **Anthraquinones:** To 3 ml extract, 3 ml benzene, and 5 ml 10% ammonia were added. The red colour shows the presence of anthraquinones.
12. **Tannins:** To 2 ml extract, 2 ml distilled water and 2-3 drops ferric chloride was added. The green colour indicates the presence of tannins.
13. **Quinones:** To 2 ml extract, sodium hydroxide was added. Formation of blue, green, or red colour confirms the presence of quinones.

Antibacterial activity:

3.8 g Mueller-Hinton agar was dissolved in 100 ml distilled water and autoclaved at 121ºC for 20 min at 15 lbs pressure. The bacteriostatic property of the compounds was tested by the agar well diffusion method.[25] The extracts were dissolved in sterile 1% dimethyl sulphoxide. Streptomycin in 1% DMSO was used as positive drug control. The extracts were serially diluted to obtain concentrations of 10 mg, 5 mg, 2.5 mg, 1.25 mg, and 0.625 mg per 50 µl for testing antibacterial activity. After incubating, the diameter of the zones of inhibition were measured (in mm) using a ruler. The following bacterial cultures were used for screening of antibacterial activity: *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Enterobacter cloacae*.

Table 1: Phytochemicals of *A. squamosa* seed extracts

Compound	Hexane	Petroleum ether	Chloroform	Ethanol
Alkaloids	+	++	+	+
Carbohydrates	+	+	-	+
Glycosides	-	-	-	-
Saponins	+	+	+	+
Proteins	-	-	-	-
Amino acids	-	-	-	-
Phenols	-	-	-	-
Flavonoids	-	+	-	-
Terpenoids	++	++	-	+
Steroids	+	++	+	+
Anthraquinones	-	-	-	+
Tannins	+	+	-	+
Quinones	-	-	-	-

(+ : mild, ++ : moderate, +++ : intense, - : absence)

Results and Discussion

The results of qualitative screening for primary and secondary metabolites in *A. squamosa* seed extracts are tabulated below (Table 1). Hexane extract of *A. squamosa* seeds contains alkaloids, carbohydrates, saponins, terpenoids, steroids, and tannins accompanied with a characteristic colour change. Petroleum ether extract has alkaloids, carbohydrates, saponins, flavonoids, terpenoids, steroids, and tannins. Chloroform extract has only alkaloids, saponins, and steroids. And in the ethanol extract, alkaloids, carbohydrates, saponins, terpenoids, steroids, anthraquinones, and tannins were found.

The antibacterial activity of *A. squamosa* seed hexane extract against the tested pathogenic microorganisms is represented (Table 2). A concentration-dependent increase in inhibition was noted against all the strains tested.

The antibacterial activity of *A. squamosa* seed petroleum ether extract against different pathogenic microorganisms (Table 3) infers that the extract was ineffective against *P. aeruginosa. E. cloacae* culture showed zones from 1.25 mg concentration of the extract. *S. aureus* inhibition was independent up to 5 mg concentration. The other cultures were highly susceptible along an increasing concentration gradient.

The antibacterial activity of *A. squamosa* seed chloroform extract against different pathogenic microorganisms is tabulated (Table 4). *E. cloacae* were the most vulnerable among the five tested strains, portraying high zones of inhibition at the lowest concentration. Notable susceptibility can be observed in *E. coli* cultures as well. The other cultures exhibited a moderate decline in bacterial population around the disc.

The antibacterial activity of *A. squamosa* seed ethanol extract against different pathogenic microorganisms is presented (Table 5). This extract is found to be the most potent among all the tested extracts as witnessed by the large inhibition circles formed around the well.

In all the experiments, the negative control with 1% DMSO did not form any zones.

Commercially available antibiotics are in danger of losing their efficacy because of the increase in developing microbial resistance. In recent years, its impact is considerable with the failure of treatment. This indeed is a heightened global concern to public health.[26] For this reason, the search for new antibiotics is an important objective. Since ancient times, natural products have been still one of the major sources of obtaining new drug molecules today. No doubt, plant products occupy the major part of the antimicrobial compounds discovered until now.[27]

Custard apple (*Annona squamosa* L.), a popular fruit with high medicinal and nutritional properties, is widely cultivated in tropical South Asia and America.[28] The seeds have been selected for the study. The review suggests that *A. squamosa* has extensively been used in traditional and folkloric medicine with many biological activities.[29] It is an edible fruit and its seeds have been used to treat malignant sores, as an insecticide[30] and an excellent vermifuge.[31]

Extraction was done with four solvents of different polarities (hexane, petroleum ether, chloroform, and ethanol) to identify the effective solvent against the bacterial strains. The final quality of the herbal drug depends on the solvents that separate medicinally active portions of a plant. In the maceration process followed by us, the coarsely powdered crude drug is agitated with the solvent until the soluble matter has dissolved. The mixture then is strained, the marc is pressed, and the combined liquids are clarified by filtration or decantation after standing.[24]

Qualitative biochemical estimations were conducted to detect the presence of phytochemicals in the

Table 2: Zone of inhibition formed by *A. squamosa* seed hexane extract.

Bacteria	Zones of Inhibition (mm)					
	0.625 mg	1.25 mg	2.5 mg	5 mg	10 mg	Standard
P. aeruginosa	9	10	12	13	13	45
E. coli	10	11	11	13	14	30
E. cloacae	9	11	13	14	15	31
K. pneumoniae	8	10	11	11	12	32
S. aureus	9	10	10	12	13	30

Table 3: Zone of inhibition formed by *A. squamosa* seed petroleum ether extract.

Bacteria	Zones of Inhibition (mm)					
	0.625 mg	1.25 mg	2.5 mg	5 mg	10 mg	Standard
P. aeruginosa	0	0	0	0	0	29
E. coli	8	9	10	12	13	33
E. cloacae	0	7	8	9	10	32
K. pneumoniae	7	8	10	11	12	32
S. aureus	7	7	7	7	8	38

Table 4: Zone of inhibition formed by *A. squamosa* seed chloroform extract.

Bacteria	Zones of Inhibition (mm)					
	0.625 mg	1.25 mg	2.5 mg	5 mg	10 mg	Standard
P. aeruginosa	7	8	8	9	11	24
E. coli	10	11	13	14	14	35
E. cloacae	12	13	15	16	18	34
K. pneumoniae	8	9	10	10	11	33
S. aureus	6	7	8	8	10	33

Table 5: Zone of inhibition formed by *A. squamosa* seed ethanol extract.

Bacteria	Zones of Inhibition (mm)					
	0.625 mg	1.25 mg	2.5 mg	5 mg	10 mg	Standard
P. aeruginosa	9	10	11	12	13	35
E. coli	11	13	14	16	18	36
E. cloacae	14	15	15	16	17	34
K. pneumoniae	8	9	10	11	13	36
S. aureus	13	13	15	15	18	37

A. squamosa seed extracts. The more polar and the less polar solutes dissolve completely in the more polar and less polar solvents respectively. However, for lipophilic compounds, lipophilic solvents can be used. Seeds being oil-rich, lipophilic solvents were selected for extraction. Our results highlight that all the extracts contain saponins, flavonoids, alkaloids, steroids, terpenoids, or tannins. However, phenols, flavonoids, terpenoids, and tannins were absent in the chloroform extract. It is due to the poor solubility

of these phytochemicals in chloroform. Plants typically produce several phytochemicals that act as a protective mechanism against environmental stressors; the more environmental stressors, the more phytochemicals a plant produces and hence also varies with growing conditions.[32]

Antibacterial screening of *A. squamosa* seeds was done by agar well diffusion assay. This assay is not only simple but easy to reproduce, cheap, makes reading and interpretation easier, and better correlates to the reference National Committee for Clinical Laboratory Standards (NCCLS) microdilution test. Therefore it represents a better methodology for antimicrobial drug susceptibility testing.[25]

A. squamosa seed extracts were tested against *Escherichia coli, Klebsiella pneumoniae, P. aeruginosa, Staphylococcus aureus,* and *Enterobacter cloacae*. A large number of microorganisms are responsible for hospital infections and any microbe may have the capacity to cause opportunistic infections in hospitalized patients.

Hexane, petroleum ether, chloroform, and ethanol extracts of *A. squamosa* seeds showed varying zones of inhibition. In a previous study, ethanolic, acetone, and aqueous fruit extract of *A. squamosa* was reported effective against six bacteria and one fungus.[33] leaves and bark of *A. squamosa* against two causative agents of dental caries also have proved efficient.[34] Susceptibility with chloroform extract is consensus with an earlier antibacterial report of the chloroform extract of *A. squamosa* seeds by broth dilution technique.[35] We have not tested against fungi since previous investigations with fungal strains showed no[36] or comparatively less anti-fungal activity.[37] However, a study on seed cotyledons extracts of *A. squamosa* against four fungi showed promising activity.[38] Nevertheless, our study matches with exploration often extracts of *A. squamosa* seed and leaf, water, methanol, and hexane extracts against six enteric bacterial strains.[39]

The inhibitory effect of all the extracts can be rightly attributed to the synergistic effect of the phytochemicals in the extract that has prevented the growth of bacteria. Phenolic compounds possessing a C_3 side chain are often cited as antimicrobials eliciting toxicity due to vital enzyme inhibition[40]. Quinones form complex irreversibly with nucleophilic amino acids in proteins[41] often leading to inactivation of the protein, the probable targets in the microbial cell being surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. These often make substrates unavailable to the microorganism. Flavones, flavonoids, and flavonols form a complex with extracellular soluble proteins and with bacterial cell walls. More lipophilic flavonoids disrupt microbial membranes.[42] Anti-infective action of tannins is by complexing with proteins through hydrogen bonding, hydrophobic effects, and covalent bond formation[43] leading to inactivation of microbial adhesins, enzymes, cell envelope transport proteins, etc. They also complex with polysaccharides.[44] Terpenes involve in membrane disruption.[45] Steroids are highly bactericidal and effectively permeabilize the outer membranes of Gram-negative bacteria sensitizing these organisms to hydrophobic antibiotics.[46] The mechanism of action of highly aromatic alkaloids is attributed to their ability to intercalate with DNA.[47]

Thus, the phytochemicals in the extracts interfere chemically with the synthesis and function of vital components of micro-organisms and kill microorganisms outright or simply prevents their growth by inhibiting cell wall synthesis, protein synthesis, nucleic acid synthesis, enzymatic activity, and folate metabolism or damage the cytoplasmic membrane which is the fundamental property of any good anti-bacterial.[48]

Conclusion

A comparative study with four different extracts of *A. squamosa* seed demonstrates that hexane, petroleum ether, chloroform, and ethanol extracts are effective in preventing bacterial growth. This is the first report on seeds that directly compares four extraction solvents. It is noteworthy that the *A. squamosa* seeds are potent against Gram-positive and Gram-negative bacteria. Among the four extracts, ethanol extract was the best in inhibiting the growth of all the tested bacterial strains, followed by chloroform and hexane extracts. Petroleum ether extract exhibited the least activity. The study can be extended to isolate chemical constituents from the extract and to explore its activity against multi-drug resistant strains.

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