Phytology

Phytochemical characterization, antioxidant and antimicrobial testing of ethanolic and ISO-propanolic extracts of *Annona squamosa* seeds

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Abstract

Annona squamosa, widely known as custard apple or sugar apple, is extensively consumed for its creamy white pulp having a sweet, tropical taste. The aim of this study is to identify the phytochemical constituents, antioxidant and antimicrobial testing of ethanolic and isopropanolic extracts of *Annona squamosa* seeds. The DPPH radical scavenging assay was used for antioxidant testing. It showed that the isopropanolic extract has high antioxidant potential (IC50 11.76mg/ml) while ethanolic has appreciable antioxidant potential with IC50 12.69 mg/ml. The IC50 value of control was 10.81 mg/ml. Qualitative analysis by phytochemical screening confirmed the presence of steroids, alkaloids, phenols and terpenoids. Antimicrobial testing of the seed extracts was done using the agar well diffusion assay, against the organisms *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. The isopropanolic and ethanolic extracts showed notable zones of inhibitions. The study concluded that *Annona squamosa* seeds are a potential antioxidant and antimicrobial agent.

Keywords: antioxidant activity-*Annona squamosa*, DPPH assay, phytochemical analysis, antimicrobial activity- *Annona squamosa*, agar well diffusion assay

Introduction

Nature, with an emphasis on the abundant plant plethora, is one of the most powerful tools for development, along with science. Nature has done its part by infusing some of the most biologically active compounds within plants. It is now our duty to explore and further study these compounds and develop varied solutions to our problems. Following the saying that "Nothing is wasted in nature", even the seeds have abundant potential, though much of it is still unexplored. Seeds, apart from having the capacity of growing an entire plant, have beneficial compounds like flavonoids, alkaloids, saponins, tannins, carotenoids, steroids, terpenoids, etc. These compounds possess several medicinal benefits like anticancer, anti-inflammatory, anti-diabetic, antioxidant properties, etc. which can help mankind.

India is an agrarian nation that grows a wide variety of tropical fruits, vegetables, and medicinal herbs. Amongst the many fruits cultivated in Maharashtra, Sitphal (*Annona squamosa*) is widely grown in Beed, Aurangabad, Parbhani and Solapur districts. It is a crucial component of many Indian dishes, mainly desserts, and has a distinctive sweet taste and creamy texture.

Annona squamosa is known as Sugar apple, Sweetsop, and Sitaphal in Marathi which belongs to the Annonaceae family. It is a small semi-deciduous tree, 3-8 meters tall with small alternate leaves 5-17 cm long and 2-6 cm wide. Round or heart-shaped, aggregate, soft fruits have a green bumpy surface with grainy pulp which matures in Sept-Oct month. Seeds are used for pest control as they have insecticidal properties (Tamang *et al*., 2021) [12]. The shape of the fruit which is given by the peel varies and is found to be irregular, lopsided, spherical, heart-shaped and round. The skin/peel of custard apple is tough and mostly green and black. The peel, pulp and seeds of *A. squamosa* were also observed to have antioxidant, antibacterial and anticancer properties (Shehata *et al*., 2021) [10] (Altaee *et al*., 2020) [7]. The pulp of *Annona squamosa* fruit with its tropical sweet taste is rich in calorie content. The fruit is a rich source of Vitamin A and Vitamin C, helping in the development of the fetus during pregnancy (Anary *et al*., 2016) [1]. Custard apple leaves have been widely studied for their activities including antimicrobial, antiobesity, and hepato-protective functions attributed to the diverse phytochemicals present in them (Kumar *et al*., 2021) [5].

It is a heavily seeded fruit. The seeds of *Annona squamosa* have a lot of beneficial properties like anti-microbial, anti-diabetic, anti-inflammatory and anti-tumoral etc. due to the flavonoids, alkaloids, phenols and Acetogenins present in them (Lopez-Romero *et al*., 2022) [6]. Acetogenins are chemical compounds possessing anti-tumoral, antiviral and antimicrobial properties. Acetogenins are unique to the *Annonaceae* family. These seeds have a lot of uses like the *Annona squamosa* seed oil is being used in the process of making biodiesel (Suthan *et al*., 2022) [11]. In spite of being a heavily seeded fruit with unique seed properties, the seeds are usually thrown away in every household, which generates seed waste.

Thus, *Annona squamosa* is a powerful plant with multiple uses for every part of the plant. In this study, ethanolic and isopropanolic extracts of *Annona squamosa* seeds were tested for their antioxidant and antimicrobial potential as well as the phytochemical screening was carried out for the above-mentioned extracts.

Materials and methods

1. Plant material

The fruits were bought from a local market in Mumbai, Maharashtra. The seeds were then procured, washed, and oven dried at 80°C. They were then crushed into powder and the powder was used to prepare organic extracts.

2. Preparation of extracts

The ethanolic and isopropanolic seed extracts were prepared using soxhlet extraction (Aryal D, 2022) [2] (Mali *et al*., 2018) [8]. Thimble was prepared containing 25 g of the seed powder. 300 ml of Ethanol and Isopropanol was used and the extraction was carried out for 4 hours at 70°C and 75°C respectively. The extract was then evaporated till a volume of 250ml of stock solution was obtained.

3. Phytochemical analysis

The isopropyl and ethanolic extract of the seeds were analysed for steroids, alkaloids, flavonoids, phenols, tannins, anthraquinone glycosides, saponins and terpenoids. Standard methods for preliminary phytochemical analysis were used with some minor modifications. A detailed description of the procedure is given below: (Shaikh & Patil, 2020) [9].

i) Test for steroids (salkowski's test)

Add 1 ml of chloroform in 1ml of sample extract and filter. The filtrate was then treated with concentrated H_2SO_4 from the sides of the test tubes. If there is formation of red colour, it indicates the presence of steroids.

ii) Test for alkaloids

The test samples were treated with Dragendroff's reagent. The formation of a red precipitate indicates the presence of alkaloids.

iii) Test for flavonoids (alkaline reagent test)

Add 1 ml of test samples in its respective test tubes. Treat the sample by adding 2% NaOH, which will give intense yellow colour and then become colorless on addition of dil. HCI. This colourless formation indicates presence of flavonoids in the test sample.

iv) Test for phenols (ferric chloride test)

Add 0.5ml of 1% FeCl3 in 2ml of test samples. Formation of dark green or brown color indicates presence of phenols.

v) Test for Tannins (ferric chloride test)

Add 1 ml of test samples in test tubes and dilute it with 4ml of D/w. 3 drops of 10% FeCl3 were then added in the test tubes. Formation of blue-green color indicates presence of tannins in samples.

vi) Test for Anthraquinone glycoside (hydroxyanthraquinone test)

10% KOH was added to 1 ml of sample. Formation of red color indicates presence of anthraquinone glycoside.

vii) Test for saponins (froth test)

1 ml of sample test is diluted in 2 ml of distilled water. The test tube was shaken for 15 min continuously. The formation of a layer of froth indicates the presence of saponins.

viii)Test for terpenoids

The sample extract is treated with 2 ml of chloroform and 1 ml of conc. H2SO4. The reddish-brown color formation indicates the presence of terpenoids.

4. Antioxidant assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test:

The antioxidant activity of the ethanolic and isopropanolic extracts of *A. squamosa* seeds was measured using the DPPH assay (Kothari & Seshadri, 2010) [4] (B. Vikas *et al*., 2017) [14], which is a stable free radical. The 0.1mM solution of DPPH was prepared by suspending the powder in methanol as the radicals are stable in it, thus making methanol the standard solvent used. The isopropanolic and ethanolic extracts were used in concentrations ranging from 05 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml. The volume of the stock solution was made to 01 ml by diluting it with methanol according to the concentrations. A 3ml DPPH solution was added and the test tubes were incubated in the dark for 30 mins. Also, control of the lemon peel powder was used in the same concentrations as the extracts. After incubation, the absorbance of isopropanolic and ethanolic extracts as well as the control was measured at 520nm using a colorimeter. The resultant values were presented in the form of IC50, which is the concentration of the antioxidant required to scavenge 50% of the free radicals.

5. Antimicrobial assay

5.1 Microorganisms

Ethanolic and isopropanolic extracts of *Annona squamosa* seeds were individually tested against a variety of Gram-positive organisms including *Staphylococcus aureus* and *Staphylococcus epidermidis* and gram-negative bacteria-*Klebsiella pneumoniae.* Pure bacterial cultures of *Klebsiella pneumoniae* and *Staphylococcus aureus* were obtained from the Department of Biotechnology, KET's V.G. Vaze College of Arts, Science and Commerce, Mumbai.

Staphylococcus epidermidis was isolated from human sweat samples of the face on Mannitol Salt Agar and incubated for 24 hours at 37°C. Four colonies namely S1, S2, S3, and S4 were selected from the obtained bacterial growth and plated separately on Mannitol Salt Agar plates. The purity and identity of the isolated colonies were confirmed by performing certain biochemical tests such as IMViC and gram staining (Tripathi N, 2022) [13]. From the results of the biochemical tests, S1 colony was identified as the *Staphylococcus epidermidis* isolate. Nutrient broth inoculum (incubated for 24 hrs) of all the three organisms- *Klebsiella pneumoniae, Staphylococcus aureus* and *Staphylococcus epidermidis* was used for the antimicrobial assay.

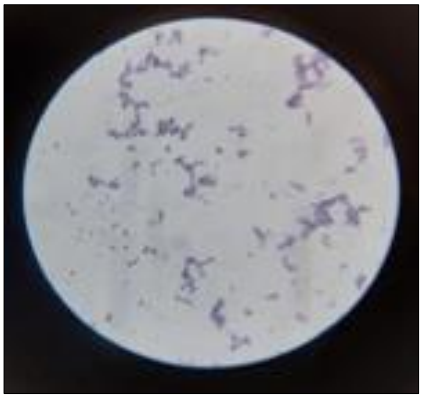

Fig 1: Gram staining of S1 colony

Fig 2: IMViC Test of S1 colony

5.2 Agar well diffusion assay

The antibacterial testing of *Annona squamosa* seed extracts against the above mentioned bacterias was done using the agar well diffusion assay (Balouri *et al*., 2015) [3]. The Mueller Hinton agar was bulk seeded with nutrient broth inoculations of *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, in individual flasks. The inoculated media was poured into petri plates and allowed to solidify. After solidification, bores or wells were made using a sterile borer under aseptic conditions. Different concentrations of organic extracts- isopropyl extract and ethanolic extract of *Annona squamosa* seeds were added to the wells. Concentrations ranging from 100mg/ml to 400 mg/ml were used for ethanolic extracts while testing of isopropyl extracts was done at concentrations - 75 mg/ml and 100 mg/ml. Ethanol and isopropyl alcohol were used as control, respectively. Cefuroxime and Neomycin solutions were used as antibiotic control against *Klebsiella pneumoniae* and *Staphylococcus* species, respectively. After additions of solutions in the respective wells, the plates were incubated at 37°C. Zones of inhibitions were measured after 24 hours of incubation.

Results and discussion

Phytochemical analysis

Phytochemicals such as steroids, alkaloids, phenols and terpenoids were determined in *Annona squamosa* seeds and are presented in Table 1.

Table 1: Phytochemical constituents of *A. squamosa* seeds in ethanol and isopropyl alcohol extracts

Phytochemicals	Ethanolic Extract	Isopropanolic extract
Steroids	+	+
Alkaloids	+	+
Phenols	+	+
Terpenoids	+	+

Antioxidant activity

Table 2: IC50 values obtained in DPPH assay for control and seed extracts of *Annona squamosa*

Extracts	IC50 value
Control	10.81 mg/ml
Isopropanolic extract	11.76 mg/ml
Ethanolic extract	12.69 mg/ml

The IC50 value of isopropanolic extract was found to be less than the ethanolic extract and hence has higher antioxidant activity. The IC50 value of isopropanolic extract (11.76 mg/ml) is close enough to the IC50 value of control (10.81 mg/ml). This proves that it has a notable antioxidant activity.

IC50 value of Isopropanolic extract (11.76 mg/ml) is the closest to that of control. Hence, amongst the tested extracts, isopropanolic extract is most comparable with the control in terms of antioxidant potential. Ethanolic, also shows remarkable antioxidant activity after isopropanolic extract. Aqueous extract shows the weakest antioxidant activity amongst these extracts as it has the highest IC50 value (lower the IC50 value, more the antioxidant capacity).

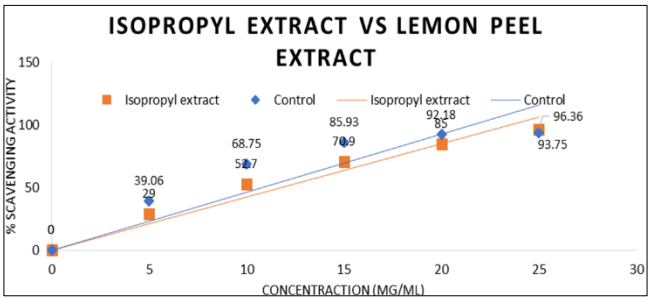

Fig 3: DPPH scavenging activity of *A. squamosa* seeds using isopropanol as solvent

The % scavenging activity of isopropyl extract ranges from 29% to 96.36%.

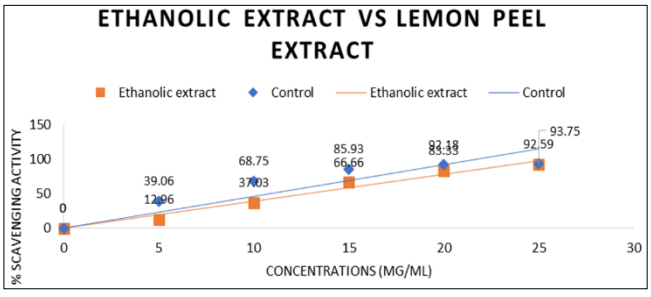

Fig 4: DPPH scavenging activity of *A. squamosa* seeds using ethanol as solvent

The % scavenging activity of the ethanolic extract ranges from 12.96% to 92.59%.

Antimicrobial activity

Klebsiella pneumoniae

Isopropanolic extract showed better results than ethanolic extract against *K. pneumoniae* with inhibition values 19mm (75 mg/ml) and 30 mm (100 mg/ml). The zone of inhibition for control (isopropyl alcohol) was 20.1 mm.

Concentrations ranging from 100 mg/ml to 400 mg/ml of ethanolic extract were used against *K. pneumoniae.* It showed appreciable results with inhibitions 16 mm (100 mg/ml), 17 mm (200 mg/ml), 19 mm (300 mg/ml) and 21 mm (400 mg/ml). Control (ethanol) showed an inhibition of 17.5 mm. Thus, isopropanolic extract and ethanolic extract both showed notable results against *K. pneumoniae.*

Isopropanolic extract did not show any activity against *Staphylococcus* species. Zone of inhibitions of Ethanolic extract against *Staphylococcus* species is depicted in Table 3.

Table 3: Zone of inhibitions of ethanolic extract against *Staphylococcus aureus* and *Staphylococcus epidermidis*

	100 mg/ml	200 mg/ml	300 mg/ml	400 mg/ml	Control	Antibiotic (neomycin)
Staphylococcus aureus	-	15 mm	16.5 mm	18.5 mm	-	16.25 mm
Staphylococcus epidermidis	12.5 mm	14.5 mm	18 mm	18.5 mm	12.75 mm	24 mm

Conclusion

It is appealing that the phytochemical screening of isopropanolic and aqueous extracts of *A. squamosa* seeds confirmed the presence of steroids, alkaloids, phenols and terpenoids. Further the results of DPPH radical scavenging assay like 96.36% scavenging activity (isopropanolic extract) and 92.59% scavenging activity (ethanolic extract) promised that *A. squamosa* seeds are a potential antioxidant. Although, isopropanolic extract showed no activity against *Staphylococcus* species, 18.5mm ZOI against *S. aureus* and *S. epidermidis* at 400 mg/ml concentration of ethanolic extract and 30 mm ZOI and 21 mm ZOI of isopropanolic (100 mg/ml) and ethanolic (400 mg/ml) extracts, respectively, reflect plant's medicinal impact. Therefore, in conclusion, *A. squamosa* seed extracts can be further tested for different properties using in-vitro and in-vivo models.

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