Evaluation of antibacterial, antioxidant, and phytochemical properties of leaf, stem and root extracts of *Annona squamosa* in relation to pathogenic bacteria

Nirmala Patel, R. S. Chauhan

Department of Botany, PMCOE, Sanjay Gandhi Smriti Govt. Autonomous PG College, Sidhi M.P. 486661, India

Corresponding author

E-mail: ranupatel02@gmail.com

Abstract

In India, *Annona squamosa* is widely utilized as a traditional remedy for various ailments. The antibacterial properties of extracts derived from the leaves, stems and roots of *Annona squamosa* were examined against two strains of gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and two strains of gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The antibacterial compounds were extracted sequentially using solvents including hexane, chloroform, ethyl acetate, methanol and water. The antimicrobial efficacy of the extracts was assessed through the agar well diffusion method. It was observed that the root extracts of *A. squamosa* demonstrated greater antibacterial activity in comparison to the extracts from the leaves and stems. Notably, the chloroform extracts of *A. squamosa* roots were effective against *E. coli* and *B. subtilis*. Additionally, this study evaluated the antioxidant capacity of the leaf, stem, and root extracts of *A. squamosa* using the DPPH free radical scavenging method. The objective of this research is to present a detailed overview of the chemical constituents found in the leaf, stem, and root extracts of *A. squamosa*, along with the diverse range of phytochemicals identified in the study. Given the rising incidence of multi-drug resistant pathogens, these results bolster the therapeutic potential of this plant in traditional medicine, thereby enhancing its medicinal significance.

Keywords: Antibacterial, Annona squamosa, Agar well diffusion technique, Conventional medicine

Introduction

Annona squamosa, widely recognized as custard apple, is a member of the Annonaceae family and is classified as a small tree (Salman and Senthilkumar 2015). The Latin word "anon" translates to "yearly produce," which is the basis for the name "Annona," indicating the many fruit-bearing species within this genus, which includes over 2300 species (Leatemia and Isman 2004). The designation *A. squamosa* specifically pertains to this species, highlighting the knobby texture of its fruit (Vyas *et al*., 2012). The custard apple is an edible tree or large shrub that generally attains a height of three to five feet, although it can reach heights of six to eight meters under ideal conditions (Shashirekha *et al*., 2008). It is grown in gardens not only for its ornamental value but also as a significant source of fuel (Wayne *et al*., 2002). This tree is widely cultivated in India and other tropical regions primarily for its delectable heart-shaped fruits, which typically weigh around 150g. The mature fruit features a creamy and exceptionally sweet pulp, suitable for fresh consumption or as a flavoring for milk and ice cream (Vanitha *et al*, 2010). The pulp can

also be utilized to prepare a variety of delightful dishes, including jelly, squash and even wine (Al-Nemari *et al*., 2020 and Nagy *et al*., 1990).

Custard apples are known for their extensive therapeutic and non-therapeutic applications. In herbal medicine, different parts of the plant are employed to address various ailments, such as heart disease, diabetes, hyperthyroidism, and tumors (Shirwaikar *et al*., 2004). Historically, custard apple has been utilized to manage conditions including dysuria, illness, appetite loss, ulceration, ringworm, dysentery, nausea, hemorrhaging, painful urination, and as an agent for inducing abortion. The root of *A. squamosa* was thought to have potent purgative effects, and scrapings from the root bark were used to alleviate toothaches (Raj Sobiya *et al*., 2009) and (Yang *et al*., 2008). Internally, the roots were employed to treat spinal disorders and depression. The stem is recognized for its highly effective astringent qualities.

The leaves of *A. squamosa* are believed to be beneficial in treating prolapses in children. In instances of hysteria and fainting, crushed leaves are inhaled. A decoction made from the leaves is consumed to relieve dysentery (Gajalakshmi *et al*., 2011). Poultices created from the leaves are applied to boils and ulcers. The mature fruits of this plant are utilized in the treatment of cancerous tumors and assist in the process of suppuration. In Ayurveda, the fruits are regarded as a beneficial tonic, enhancing blood quality and increasing muscle strength, among other advantages. The powder obtained from dried unripe fruits is employed as a pesticide. However, it is important to note that the seeds of *A. squamosa* are toxic and acrid. Ground seeds are used to produce fish poison and insecticides. A paste made from the powdered seeds is applied to the scalp to eradicate lice. It is also utilized to kill worms in cattle wounds (Parvin *et al.*, 2003).

The seeds of *A. squamosa* can be utilized to extract premium oil, which is rich in fatty acids including oleic, linoleic, palmitic, and stearic acids (Mariod *et al*., 2010). These fatty acids have diverse applications in the manufacturing of alkyds, the production of soap, and the plasticizer sector (Ahmad *et al*., 2006). The residue from the seeds can be employed as a fertilizer (Khan *et al*., 1983), while the non-consumable seed oil acts as an insect repellent (Kamble and Soni 2010). Furthermore, it has been demonstrated that the alkaloidal extract from the plant effectively prevents the corrosion of C38 steel in a standard hydrochloric acid environment (Lebrini *et al*., 2010).

The growing interest in utilizing natural ingredients for a healthier lifestyle corresponds with the use of plants as sources of traditional medicine (Solikhah *et al*., 2021). Plants are abundantly found in our environment, rendering them accessible for medicinal applications (Safira *et al*., 2021). According to the World Health Organization, around 80% of the global population has employed herbal ingredients in their healthcare practices.

Through the investigation of the pharmacological properties of *A. squamosa*, researchers have revealed its potential as a medicinal plant (khairullah *et al*., 2021). This understanding can aid in the

formulation of natural remedies and therapeutic strategies (kalidindi *et al*., 2015). It is important to emphasize that additional research and clinical trials are essential to comprehensively assess the efficacy, safety, and specific uses of *A. squamosa* in various healthcare domains.

In summary, the growing interest in natural ingredients for promoting a healthier lifestyle, along with the pharmacological benefits of *A. squamosa*, underscores its potential as a significant medicinal plant. Ongoing research and investigation into its characteristics may pave the way for new treatment options and enhance the well-being of individuals globally.

The seeds of *A. squamosa* yield wax and latex, which serve as detergents, while their powdered form can be utilized as an effective hair shampoo when mixed with gram flour. In the realm of sustainable agriculture, employing pesticides derived from plants such as *A. squamosa* can significantly improve pest management strategies. These plant-based pesticides possess regenerative qualities and are comparatively less harmful to humans, non-target species, and natural predators. The aim of this study is to explore the antibacterial properties of crude extracts from the leaves, stems and roots of *A. squamosa*.

Materials and methods

A. Collection of plant material

The fresh leaves, stems and roots of mature *Annona squamosa* were gathered from Madhya Pradesh in the Rewa district.

B. Preparation of plant extracts

The leaves, stems and roots were meticulously cleaned and subsequently dried in the shade prior to being finely ground into a powder. Utilizing a Soxhlet apparatus, the dried and powdered plant materials were extracted using five distinct solvents: hexane, chloroform, ethyl acetate, methanol and an aqueous solution. Following the evaporation of the solvents, concentrated extracts were produced. These extracts were then preserved in tightly sealed containers and stored in a freezer at -40°C for future applications.

C. Antibacterial activity

Antibacterial activity was evaluated against two specific gram-positive bacteria, *B. subtilis* and *S. aureus*, along with two gram-negative bacteria, *E. coli* and *P. aeruginosa*. The bacterial strains utilized in this study were sourced from the Microbial Type Culture Collection (MTCC) at the Institute of Microbial Technology in Chandigarh. The Agar well diffusion technique was applied to ascertain the minimum inhibitory concentration of the plant parts (leaves, stems and roots) to assess their biological efficacy and potential applications. Each bacterial strain, including *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*, was inoculated into 20 millilitres of Muller Hinton Agar Medium (MHA) in petri dishes and permitted to incubate overnight. Wells approximately 6 mm in diameter were created using a cork borer and 20µl of each

sample extract (leaves, stems and roots) from a stock concentration of 1 mg/1 ml was introduced into the corresponding wells. The plates were subsequently incubated at 37°C for 24 hours. The antibacterial activity was assessed by measuring the diameter of the inhibition zones that developed around the wells in millimetres (Wayne *et al*., 2002). For the positive control, ciprofloxacin, a widely utilized antibacterial agent at a concentration of 5 µg/1 ml, was employed.

D. Antioxidant activity

DPPH free radical scavenging assay

A modified method based on the work of Kokate *et al*., (2003) was employed to evaluate the scavenging ability of the extracts through the DPPH free radical scavenging assay. A DPPH solution (0.1m/M) was created by dissolving 1.1829g of DPPH in methanol and adjusting the total volume to 30ml with additional methanol. This solution was then kept in the dark for 30 minutes to ensure the reaction was complete. Different concentrations of the extracts (0.2, 0.4, 0.6, 0.8, and 1mg/ml) were combined with 22µl of the DPPH solution and allowed to incubate at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The free radical scavenging activity was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Ac} - \text{At})}{\text{Ac}} \times 100$$

Where,

Ac = The absorbance of the test sample and

At = The absorbance of the control.

The testing of ascorbic acid was performed utilizing extract sample concentrations as a reference (Jamkhande *et al*., 2014). The antioxidant activity of the sample was quantified by the IC_{50} value, which is defined as the concentration of the sample that reduces the generation of DPPH radicals by 50% (Mulla *et al*, 2010) and (Chew *et al*., 2012).

E. Preliminary phytochemical Screening

A preliminary phytochemical screening was carried out to verify the existence of primary chemical constituents in the newly acquired extracts. The subsequent conventional tests were executed:

Alkaloid Test: A small quantity of the extract (mg) was heated in 2% sulfuric acid for 2 minutes in a separate test tube. Subsequently, a few drops of Dragendorff's reagent were introduced after filtration in another test tube. The appearance of orange or red precipitates signified the presence of alkaloids.

Phenol Test: Ferric chloride with 5% alcohol content was mixed with the substance in water. The presence of phenols was indicated by the emergence of a dark blue or green color.

Flavonoid Test: The substance in alcohol was treated with 10% NaOH or ammonia. The presence of flavonoids was confirmed by the development of a dark yellow color.

Saponin Test: A few milligrams of the extract were thoroughly combined with distilled water. The presence of saponins was verified when foam was produced.

Glycoside Test: The substance was treated with concentrated anthrone and sulfuric acid. Heating the mixture over a water bath revealed the presence of glycosides, indicated by the appearance of a green hue.

Terpenoid Test: Concentrated H2SO4 was added to a few milligrams of the extract in chloroform. The presence of terpenoids was indicated by the formation of a dark brown precipitate.

Steroid Test: In a dry test tube, 2 ml of chloroform were added to a few milligrams of the extract. After heating, a few drops of concentrated sulfuric acid and two drops of acetic anhydride were added, followed by a few drops of acetic acid. The presence of steroids was indicated by the emergence of a green coloration.

Protein and Amino Acid Test: A few drops of a diluted (1%) copper II sulfate solution were added to the sample solution in a test tube, followed by sodium hydroxide solution. Gentle stirring was performed to mix the substances. The presence of proteins was indicated by the purple color.

Reducing Sugar Test: 5ml of Fehling's solutions 1 and 2 (in a 1% ratio) were added to 2ml of the plant extract and heated for 5 minutes. The presence of reducing sugar was confirmed by the formation of red precipitates.

Result and discussion

A. Agar well diffusion method

The antibacterial properties of *Annona squamosa* leaf, stem and root extracts, which were dissolved in DMSO, were evaluated in vitro through the agar well diffusion method against various microbial strains, including *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*. The subsequent tables display the notable antibacterial activity recorded against gram-positive (*B. subtilis* and *S. aureus*) and gram-negative (*E. coli* and *P. aeruginosa*) bacterial strains utilizing the leaf, stem and root extracts of *A. squamosa* in conjunction with different solvents. Table 1 demonstrates the zone of inhibition produced by the leaf extracts of *A. squamosa* when combined with five distinct solvents against the bacterial strains on Muller Hinton Agar. Table 2 depicts the zone of inhibition established by the stem extracts of *A. squamosa* in combination with five different solvents against the bacterial strains on Muller Hinton Agar. Table 3 illustrates the zone of

inhibition generated by the root extracts of *A. squamosa* in conjunction with five different solvents against the bacterial strains on Muller Hinton Agar.

Table 1: Demonstrates the area of inhibition generated by the leaf extracts of *A. squamosa* when combined with five distinct solvents against the bacterial strains on Muller Hinton Agar

Test organisms	Zone of inhibition of leaf extracts					Positive control
	Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous solution	
B. subtilis	-	-	-	-	-	20
S. aureus	-	-	-	-	-	20
P. aeruginosa	-	-	10	-	-	20
E. coli	-	-	-	-	-	30

Table 2: Demonstrates the area of inhibition produced by the stem extracts of *A. squamosa* when combined with five distinct solvents against the bacterial strains on Muller Hinton Agar

Test organisms	Zone of inhibition of stem extracts					Positive control
	Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous solution	
B. subtilis	7	7	9	-	-	20
S. aureus	-	-	-	-	-	20
P. aeruginosa	-	-	-	-	-	20
E. coli	-	-	13	-	-	30

Table 3: Demonstrates the area of inhibition produced by the root extracts of *A. squamosa* when combined with five distinct solvents against the bacterial strains on Muller Hinton Agar

Test organisms	Zone of inhibition of stem extracts					Positive control
	Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous solution	
B. subtilis	7	14	-	-	-	20
S. aureus	-	-	-	-	-	20
P. aeruginosa	-	-	-	-	-	20
E. coli	6	12	-	-	-	30

Antibacterial activity of leaf extract

It is important to highlight that the ethyl acetate leaf extracts showed a zone of inhibition of 10 mm against *Pseudomonas aeruginosa*. Conversely, the leaf extracts mixed with five different solvents did not demonstrate any antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. A study published in the "International Journal of Pharma and Bio Sciences" explored the antimicrobial efficacy of *Annona squamosa* leaf extract against various pathogenic bacteria. The findings indicated that the leaf extract had notable inhibitory effects on several bacteria, including *Escherichia coli*, *Staphylococcus*

aureus, *Salmonella typhi* and *Pseudomonas aeruginosa*. This antimicrobial activity was linked to the presence of alkaloids and flavonoids within the leaf extract (Neethu *et al*., 2016).

Antibacterial activity of stem extract

The stem extracts, when combined with five distinct solvents, did not demonstrate any antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Nevertheless, the stem extracts in hexane, chloroform and ethyl acetate displayed inhibition zones of 7 mm, 7 mm, and 9 mm, respectively, against *Bacillus subtilis*. Additionally, the ethyl acetate stem extracts revealed an inhibition zone of 13 mm against *Pseudomonas aeruginosa*. Kachhawa and his associates performed a study that revealed a methanolic extract of the stem bark of *Annona squamosa* exhibited antimicrobial activity against both gram-positive and gram-negative strains of *Bacillus coagulans* and *Escherichia coli* in vitro (Kachhawa *et al*., 2012).

In a similar vein, Padhi and his colleagues documented favorable outcomes regarding the antibacterial characteristics of the plant when evaluated against *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Vibrio alginolyicus* (Padhi *et al*., 2011). In a separate investigation, Chavan and his research team (Chavan *et al*., 2010) successfully isolated a compound known as 18-acetoxy-ent-kaur-16-ene from a petroleum-derived extract of *A. squamosa* bark, which exhibited analgesic and anti-inflammatory effects.

Antibacterial activity of root extract

The root extracts, when combined with five different solvents, did not demonstrate any antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Nevertheless, the hexane and chloroform root extracts revealed zones of inhibition measuring 7 mm and 14 mm, respectively, against *Bacillus subtilis*. Furthermore, the chloroform root extract generated a 6 mm zone of inhibition against *Escherichia coli*, while the hexane root extract displayed a 12 mm zone of inhibition against the same strain. In his research, Vidyasagar indicated that by analyzing the crude extracts of *A. squamosa* root, researchers may have pinpointed specific phytochemicals that are responsible for the observed antimicrobial properties. These phytochemicals may encompass compounds such as alkaloids, flavonoids, tannins, phenols, or other secondary metabolites recognized for their antimicrobial activity (Vidyasagar *et al*., 2012).

They examined the bactericidal properties of five distinct solvents (hexane, chloroform, ethyl acetate, methanol and aqueous solution) at a concentration of 1 mg/1 ml. The chloroform root extracts exhibited the most significant antibacterial activity against *B. subtilis* and *E. coli* at the specified concentration. These results indicate the possibility of additional research in this field.

Antioxidant activity

The DPPH free radical scavenging assay was utilized to evaluate the antioxidant properties of the extracts. This assay is widely recognized for determining the capacity of compounds to neutralize free radicals and assess their antioxidant potential. The extracts were examined for their ability to scavenge DPPH radicals, which are both highly reactive and stable free radicals. The scavenging activity was quantified spectrophotometrically at 517 nm, and the percentage of inhibition was calculated based on the absorbance values of both the test sample and the control. Ascorbic acid served as a standard for comparison. The IC_{50} value, which indicates the concentration of the sample necessary to scavenge 50% of the DPPH radicals, was employed to assess the antioxidant activity of the extracts. This assay offers significant insights into the antioxidant potential of the evaluated extracts and their capacity to safeguard against oxidative stress.

DPPH free radical scavenging assay

The DPPH assay is a widely utilized technique for assessing the antioxidant activity of various samples. DPPH is a stable free radical that absorbs UV-Vis light at a wavelength of 517 nm. In the presence of an antioxidant within the sample, the DPPH radical is reduced, resulting in a reduction of its absorbance. The extent of inhibition is employed to evaluate the antioxidant capacity of the sample, with the concentration necessary to achieve 50% inhibition denoted as the IC50 value. Table 4 presents the percentage inhibition of ascorbic acid, which serves as a standard antioxidant, as determined by the DPPH assay method. Tables 5, 6 and 7 display the percentage inhibition of DPPH by methanolic extracts derived from the leaf, stem, and root of *A. squamosa*, respectively. These tables offer insights into the scavenging activity of the extracts and their efficacy in inhibiting DPPH radicals. The findings suggest that the methanolic extracts from the leaf, stem, and root exhibit proton-donating capacity and demonstrate significant inhibition of DPPH radicals. Figs. 1-4 illustrate the scavenging activity of the extracts in comparison to the standard ascorbic acid, visually showcasing their antioxidant potential.

Table 4: Demonstrates the percentage of inhibition of ascorbic acid, a recognized antioxidant, utilizing the DPPH assay method

S.N.	Conc. (mg/ml) of leaf extract	% inhibition	IC_{50}
1	0.2	48.40	**0.24 mg/ml**
2	0.4	59.64	
3	0.6	68.14	
4	0.8	80.75	
5	1	95.60	

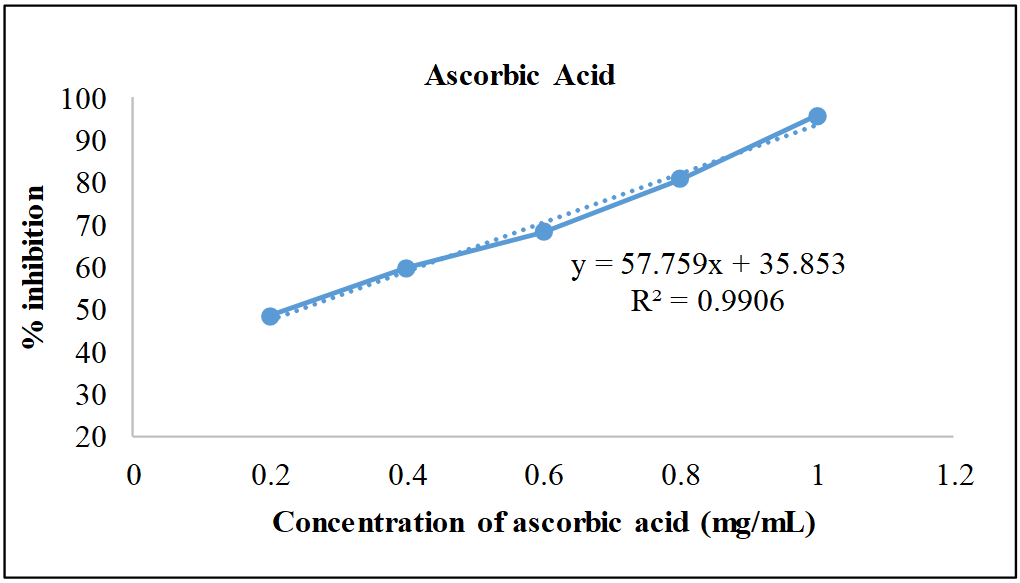

Fig. 1 Illustrate the scavenging action of standard ascorbic acid, visually showcasing its antioxidant capabilities

Table 5: Demonstrates the percentage of inhibition achieved by leaf extract, a recognized antioxidant, through the DPPH assay method

S.N.	Conc. (mg/ml) of leaf extract	% inhibition	IC_{50}
1	0.2	44.45	**0.27 mg/ml**
2	0.4	57.08	
3	0.6	68.06	
4	0.8	87.05	
5	1	89.57	

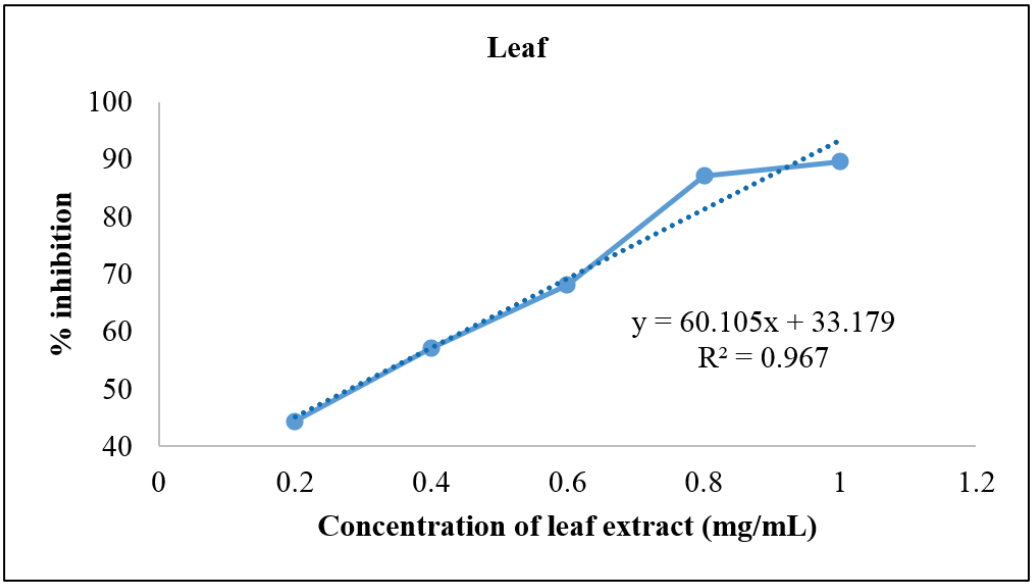

Fig. 2 Illustrate the scavenging activity of the leaf extracts in comparison to the standard ascorbic acid, visually demonstrating their antioxidant potential

Table 6: Demonstrates the percentage of inhibition achieved by the stem extract, a recognized antioxidant, through the DPPH assay method

S.N.	Conc. (mg/ml) of leaf extract	% inhibition	IC_{50}
1	0.2	34.36	**0.47 mg/ml**
2	0.4	44.08	
3	0.6	59.06	
4	0.8	70.91	
5	1	75.67	

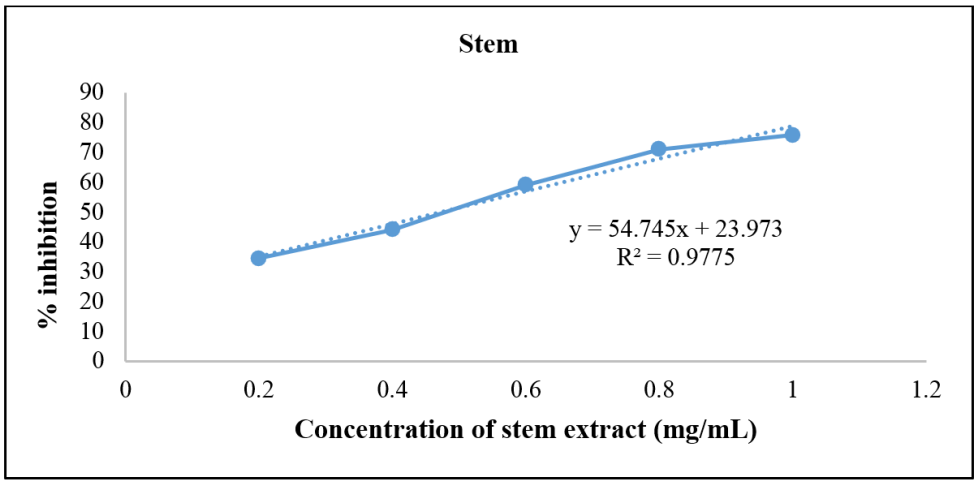

Fig. 3 Illustrate the scavenging activity of the stem extracts in comparison to the standard ascorbic acid, visually demonstrating their antioxidant potential

Table 7: Demonstrates the percentage of inhibition achieved by the root extract, a recognized antioxidant, through the DPPH assay method

S.N.	Conc. (mg/ml) of leaf extract	% inhibition	IC_{50}
1	0.2	31.09	**0.60 mg/ml**
2	0.4	36.21	
3	0.6	48.40	
4	0.8	57.98	
5	1	74.36	

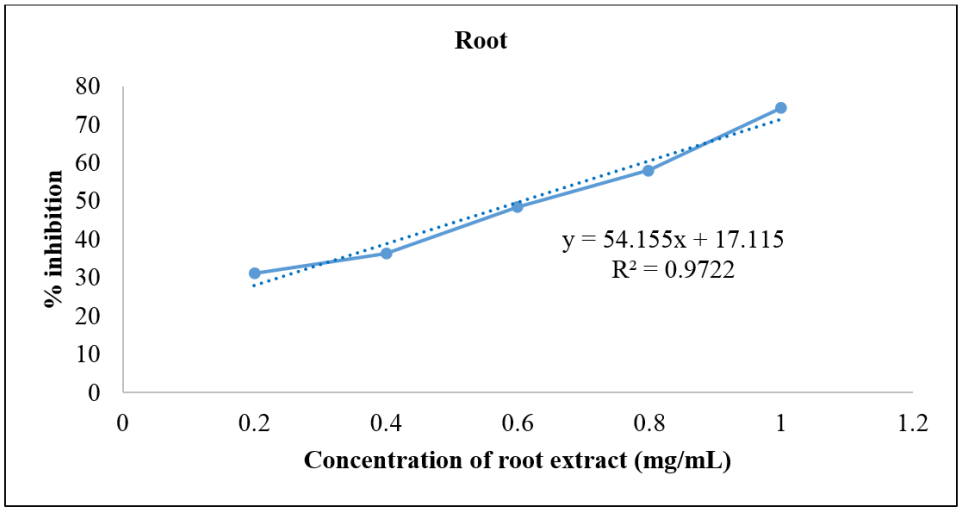

Fig. 4 Illustrate the scavenging activity of the root extracts in comparison to the standard ascorbic acid, visually demonstrating their antioxidant potential

Antioxidant activity of leaf extract

Although the average percentage of the leaf extract was lower than that of ascorbic acid, the established antioxidant, it displayed a comparable concentration-dependent free radical scavenging effect. The scavenging activity of the leaf extract was especially significant at concentrations of 0.8mg and 1mg. Through linear regression analysis, the IC_{50} values for ascorbic acid (0.24mg/ml) and leaf extract (0.27mg/ml) were calculated. Both ascorbic acid and leaf extract exhibited an IC50 value regarding their capacity to scavenge free radicals, as illustrated in Fig. 1 and 2, along with Table 4 and 5. The results from the study conducted by Neha and her colleague support the antioxidant activity of *A. squamosa* leaf extract (Neha and Dushyant 2011).

Antioxidant activity of stem extract

Although the average percentage of the stem extract was lower than that of ascorbic acid, it demonstrated a comparable concentration-dependent pattern of free radical scavenging activity. The scavenging effect of the stem extract was especially notable at concentrations of 0.8mg and 1mg. Through linear regression analysis, the IC_{50} values for ascorbic acid (0.24mg/ml) and stem extract (0.47mg/ml) were established. Figs. 1 and 3, along with Tables 4 and 6, depict the IC50 values and the free radical scavenging activity of both ascorbic acid and the stem extract. Neha and her colleague noted that the extract of *Annona squamosa* stem bark showed a significant scavenging effect on the DPPH free radical in their research (Neha and Dushyant 2011).

Antioxidant activity of root extract

While the average percentage of the root extract was less than that of ascorbic acid, a recognized antioxidant, it demonstrated a comparable concentration-dependent pattern of free radical scavenging

activity. The scavenging effect of the root extract was especially significant at concentrations of 0.8mg and 1mg. The IC_{50} values for ascorbic acid (0.24mg/ml) and root extract (0.60mg/ml) were calculated using the linear regression equation. Figs. 1 and 4, along with Tables 4 and 7, depict the IC_{50} values and the free radical scavenging activity for both ascorbic acid and the root extract. In their study, Abdalbasit and colleagues noted that the root of *Annona squamosa* exhibits greater antioxidant activity than the root of *Catunaregam nilotica* (Abdalbasit *et al*., 2012).

Preliminary phytochemical Screening

Phytochemical analysis of leaf extract

Qualitative assessments were performed on the leaf extract of *A. squamosa*, which was dissolved in five distinct solvents (hexane, chloroform, ethyl acetate, methanol, and an aqueous solution) to evaluate its phytochemical composition. The findings indicated the presence of alkaloids and glycosides across all tested solvents. Additionally, methanol and the aqueous solution exhibited the presence of phenols, whereas hexane and chloroform extracts were found to contain steroids. Reducing sugars were identified solely in the aqueous solution. Nevertheless, flavonoids, terpenoids, proteins and amino acids were absent in all leaf extracts, as shown in Table 8. In their research, Narasimharaju and his team identified the presence of glycosides, flavonoids, phenols, tannins, saponins, alkaloids, carbohydrates, and steroids in various extracts through a preliminary phytochemical analysis of *Annona squamosa* leaf extracts (Narasimharaju *et al*., 2015).

Table 8: Demonstrating the phytochemicals found in the leaf extracts of *A. squamosa*

S.N.	Phytochemicals	Solvents				
		Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous solution
1	Alkaloids	+	+	+	+	+
2	Phenols	-	-	-	+	+
3	Flavonoids	-	-	-	-	-
4	Saponins	-	-	-	-	+
5	Glycosides	+	+	+	+	+
6	Terpenoids	-	-	-	-	-
7	Steroids	+	+	-	-	-
8	Proteins	-	-	-	-	-
9	Amino acids	-	-	-	-	-
10	Reducing sugar	-	-	-	-	+

Phytochemical analysis of stem extract

According to the information provided, the *A. squamosa* stem extract was examined using five distinct solvents: hexane, chloroform, ethyl acetate, methanol and an aqueous solution. The analyses performed on the stem extracts from all five solvents indicated the presence of alkaloids. Below is a

summary of the chemical constituents identified in each solvent: the hexane extract contains terpenoids. There is no specific information available regarding the chemical composition of the chloroform extract. The ethyl acetate extract also contains terpenoids. The methanol extract is noted to contain phenol and terpenoids. The aqueous solution extract includes saponin, glycosides, and reducing sugar. According to Table 9, the *A. squamosa* stem extract was determined to be deficient in the following chemical constituents: flavonoids, steroids, proteins and amino acids. None of these components were detected in any of the solvent extracts that were tested. Neha and her colleague clarified that the phytochemical screening indicated that the primary constituents of the ethanolic extract of *Annona squamosa* stem bark were phenolic compounds, glycosides, alkaloids, among others. These constituents may contribute to the antioxidant activities (Neha and Dushyant 2011).

Table 9: Demonstrating the phytochemicals found in the stem extracts of *A. squamosa*

S.N.	Phytochemicals	Solvents				
		Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous solution
1	Alkaloids	+	+	+	+	+
2	Phenols	-	-	-	+	+
3	Flavonoids	-	-	-	+	+
4	Saponins	-	-	-	-	+
5	Glycosides	-	-	+	+	+
6	Terpenoids	-	-	-	-	-
7	Steroids	-	-	-	-	-
8	Proteins	-	-	-	-	-
9	Amino acids	-	-	-	-	-
10	Reducing sugar	-	-	-	-	+

Phytochemical analysis of root extract

The phytochemical constituents of *A. squamosa* root extract were examined utilizing five distinct solvents: hexane, chloroform, ethyl acetate, methanol and an aqueous solution. Qualitative analyses were performed to ascertain its chemical makeup. Alkaloids were identified in all five solvent extracts. The extracts from hexane and the aqueous solution revealed the presence of saponins, glycosides, and reducing sugars. The methanolic extract was found to contain phenolic compounds, whereas the extracts from hexane, chloroform, and methanol included terpenoids. Flavonoids, steroids, proteins and amino acids were not observed in the *A. squamosa* root extract, as shown in Table 10. No phytochemical analysis of *Annona squamosa* root extract has been performed prior to this study, signifying that this represents a novel research initiative.

Table 10: Demonstrating the phytochemicals found in the root extracts of *A. squamosa*

S.N.	Phytochemicals	Solvents				
		Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous solution
1	Alkaloids	+	+	+	+	+
2	Phenols	-	-	-	+	+
3	Flavonoids	-	-	-	+	+
4	Saponins	-	-	-	-	+
5	Glycosides	-	-	+	+	+
6	Terpenoids	-	-	-	-	-
7	Steroids	-	-	-	-	-
8	Proteins	-	-	-	-	+
9	Amino acids	-	-	-	-	+
10	Reducing sugar	-	-	-	-	-

Conclusion

Traditional plants have been acknowledged for their positive impacts on human health, which are linked to the presence of active phytochemical compounds. The current study suggests that *Annona squamosa* is abundant in antibacterial, antioxidant, and phytochemical properties. The research assessed the antimicrobial, antioxidant and phytochemical characteristics of extracts from the leaves, stems, and roots of various samples. The findings revealed that certain solvents activated activity in some extracts, while others did not. In particular, chloroform root extracts demonstrated notable antibacterial activity, whereas methanolic root extracts exhibited significant antioxidant properties. Furthermore, the majority of the phytochemicals tested were identified in the root extracts.

The main objective of this research was to investigate the potential application of *A. squamosa* extracts from leaves, stems, and roots as a source of antibacterial agents for treating digestive disorders. The study confirmed that *A. squamosa* roots have potent antibacterial and antioxidant properties, along with a variety of phytochemical constituents. This indicates that further exploration of *A. squamosa*, including its possible antidiabetic effects, could reveal additional advantageous properties and mechanisms.

Reference

- Ahmad, S., Naqvi, F., Sharmin, E., & Verma, K. L. (2006). Development of amine– acid cured *Annona squamosa* oil epoxy anticorrosive polymeric coatings. *Progress in organic coatings*, *55*(3), 268-275.

- Al-Nemari, R., Bacha, A. B., Al-Senaidy, A., Arafah, M., Al-Saran, H., Abutaha, N., & Semlali, A. (2020). Selective cytotoxic effects of *Annona Squamosa* Leaves against breast cancer cells via apoptotic signaling proteins. *Preprints*.

- Chavan, M. J., Wakte, P. S., & Shinde, D. B. (2010). Analgesic and anti-inflammatory activity of Caryophyllene oxide from *Annona squamosa* L. bark. *Phytomedicine*, *17*(2), 149-151.
- Chew, A. L., Jessica, J. J. A., & Sasidharan, S. (2012). Antioxidant and antibacterial activity of different parts of *Leucas aspera*. *Asian Pacific Journal of Tropical Biomedicine*, *2*(3), 176-180.
- Gajalakshmi, S., Divya, R., Deepika, V. D., Mythili, S., & Sathiavelu, A. (2011). Pharmacological activities of *Annona squamosa:* A review. *International Journal of Pharmaceutical Sciences Review and Research*, *10*(2), 24-29.
- Jamkhande, P. G., Wattamwar, A. S., Pekamwar, S. S., & Chandak, P. G. (2014). Antioxidant, antimicrobial activity and in silico PASS prediction of *Annona reticulata* Linn. root extract. Beni-Suef University *Journal of Basic and Applied Sciences*. *3*(2), 140-148.
- Kachhawa, J. B. S., Sharma, N., Tyagi, S., & Sharma, K. K. (2012). Screening of stem bark methanol extract of *Annona squamosa* for antibacterial activity. *Int J. Curr. Pharm. Res.*, *4*(1), 48-50.
- Kalidindi, N., Thimmaiah, N. V., Jagadeesh, N. V., Nandeep, R., Swetha, S., & Kalidindi, B. (2015). Antifungal and antioxidant activities of organic and aqueous extracts of *Annona squamosa* Linn. leaves. *Journal of food and drug analysis*, *23*(4), 795-802.
- Kamble, K. J., & Soni, S. B. (2010). Effect of steam blanching on quality of custard apple pulp. *Journal of Horticulture and Forestry*, *2*(5), 104-107.
- Khairullah, A. R., Solikhah, T. I., Ansori, A. N. M., Hidayatullah, A. R., Hartadi, E. B., Ram, S. C., & Fadholly, A. (2021). Review on the pharmacological and health aspects of apium graveolens or celery: An update. *Systematic Reviews in Pharmacy*, *12*(2), 595-601.
- Khan, M. W. Y., Ahmad, F., Ahmad, I., & Osman, S. M. (1983). Nonedible seed oils as insect repellent. *Journal of the American Oil Chemists' Society*, *60*, 949-950.
- Kokate, C. K., Purohit, A. P., & Gokhale, S. B. (2003). *Text book of Pharmacognosy*. *Pune: Nirali Prakashan*, *8*(66), 1-624.
- Leatemia, J. A., & Isman, M. B. (2004). Insecticidal activity of crude seed extracts of *Annona* sp., *Lansium domesticum* and *Sandoricum koetjape* against lepidopteran larvae. *Phytoparasitica*, *32*, 30-37.
- Lebrini, M., Robert, F. & Roos, C. (2010). Inhibition effect of alkaloids extract from *Annona squamosa* plant on the corrosion of C38 steel in normal hydrochloric acid medium. *International Journal of Electrochemical Science*, *5*(11), 1698-1712.
- Mariod, A. A., Abdelwahab, S. I., Elkheir, S., Ahmed, Y. M., Fauzi, P. N. M., & Chuen, C. S. (2012). Antioxidant activity of different parts from *Annona squamosa*, and *Catunaregam nilotica* methanolic extract. *Acta Scientiarum Polonorum Technologia Alimentaria*, *11*(3), 249-258.
- Mariod, A. A., Elkheir, S., Ahmed, Y. M., & Matthäus, B. (2010). *Annona squamosa* and *Catunaregam nilotica* seeds, the effect of the extraction method on the oil composition. *Journal of the American Oil Chemists' Society*, *87*(7), 763-769.
- Mulla, W. A., Kuchekar, S. B., Thorat, V. S., Chopade, A. R., & Kuchekar, B. S. (2010). Antioxidant, antinociceptive anti-inflammatory activities of ethanolic extract of leaves of *Alocasia indica* (Schott.). *Journal of Young Pharmacists*, *2*(2), 137-143.

- Nagy, S., Shaw, P. E., & Wardowski, W. F. (1990). Fruits of tropical and subtropical origin: composition, properties, and uses. *Florida Science Source*.

- Padhi, L. P., Panda, S. K., Satapathy, S. N., & Dutta, S. K. (2011). In vitro evaluation of antibacterial potential of *Annona squamosa* L. and *Annona reticulata* L. from Similipal Biosphere Reserve, Orissa, India. *Journal of agricultural Technology*, *7*(1), 133-142.

- Pandey, N., & Barve, D. (2011). Phsytochemical and pharmacological review on *Annona squamosa* Linn. *International Journal of research in pharmaceutical and biomedical sciences*, *2*(4), 1404-1412.

- Pandey, N., & Brave, D. (2011). Antioxidant activity of ethanolic extract of *Annona squamosa* Linn Bark. *Int J Biomed Pharmaceut Sci.*, *2*, 1692-1697.

- Parvin, S., Islam, E. M., Rahman, M., & Haque, E. (2003). Toxicological evaluation of annotemoyin-1isolated from *Annona squamosa* from long Evan's rat. *Pakistan Journal of Biological Science*, *6*(18), 1593-1596.

- Raj Sobiya D, Vennila Jannet J, Aiyavu C, Panneerselvam (2009). The heapatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in Swiss albino mice. *International Journal of Integrative Biology*, *5*(3), 182-18.

- Safira, A., Savitri, S. L., Putri, A. R. B., Hamonangan, J. M., Safinda, B., Solikhah, T. I., ... & Puspitarani, G. A. (2021). Review on the pharmacological and health aspects of Hylocereus or Pitaya: An update. *Journal of Drug Delivery and Therapeutics*, *11*(6), 297-303.

- Safira, A., Widayani, P., An-Najaaty, D., Rani, C. A. M., Septiani, M., Putra, Y. A. S., ... & Raharjo, H. M. (2022). A Review of an Important Plants: *Annona squamosa* Leaf. *Pharmacognosy Journal*, *14*(2)

- Salman, H. A., & Senthilkumar, R. J. A. J. P. C. R. (2015). Antibacterial activity of *Annona squamosa* L. and *Annona reticulata* L. against clinical isolates of mutans *Streptococci* the causative agents of dental caries. *Asian J Pharm Clin Res*., *8*(4), 152A155.

- Santhoshkumar, R., & Kumar, N. S. (2016). Phytochemical analysis and antimicrobial activities of *Annona squamosa* (L) leaf extracts. *Journal of Pharmacognosy and phytochemistry*, *5*(4), 128-131.

- Shashirekha, M. N., Baskaran, R., Rao, L. J., Vijayalakshmi, M. R., & Rajarathnam, S. (2008). Influence of processing conditions on flavour compounds of custard apple (*Annona squamosa* L.). *LWT-Food Science and Technology*, *41*(2), 236-243.

- Shirwaikar, A., Rajendran, K., & Kumar, C. D. (2004). In vitro antioxidant studies of *Annona squamosa* Linn. leaves. *Indian J Exp Biol*., *42*(8), 803-7.

- Solikhah, T. I., Solikhah, G. P., & Susilo, R. J. K. (2021). Aloe vera and Virgin Coconut Oil (VCO) accelerate healing process in domestic cat (*Felis domesticus*) suffering from scabies. *Iraqi J Vet Sci.*, *35*(4), 699-704.

- Vanitha, V., Umadevi, K. J., & Vijayalakshmi, K. (2010). *In vitro* assessment of alcoholic leaf extracts of *Annona squamosa* and *Aegle marmelos*. *Bioscan*, *5*(2), 225229.

- Vidyasagar, G. M. (2012). A comparative antimicrobial activity of methanolic root, leaf, seed cotyledon extracts of *Annona squamosa* L.

- Vyas, K., Manda, H., Sharma, R. K., & Singhal, G. (2012). An update review on *Annona squamosa*. *IJPT*. *3*(2), 107-118.
- Wayne, P.A. National committee for clinical laboratory standards. *Performance standards for antimicrobial disc susceptibility testing*. (2002); *12*, 01-53.
- Yang, Y. L., Hua, K. F., Chuang, P. H., Wu, S. H., Wu, K. Y., Chang, F. R., & Wu, Y. C. (2008). New cyclic peptides from the seeds of *Annona squamosa* L. and their anti-inflammatory activities. *Journal of agricultural and food chemistry*, *56*(2), 386-392.