Journal of Chemical Health Risks

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JCHR (2024) 14(5), 2004-2013 | ISSN:2251-6727

Antidiabetic & Antioxidant Activity of *Annona Squamosa* bark using Successive Solvent Extraction Method

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(Received: 11 June 2024 Revised: 16 July 2024 Accepted: 10 August 2024)

KEYWORDS

Diabetes mellitus, Annona squamosa, Pharmacognostic, Antidiabetic, Phytochemical, Antioxidant

ABSTRACT:

Diabetes mellitus, a metabolic disorder characterized by insulin dysregulation, poses significant health risks globally. This study investigates the pharmacognostic properties and antidiabetic potential of Annona squamosa bark extract. Pharmacognostic parameters including macroscopic and microscopic features, physicochemical properties (such as ash values and extractive values), and phytochemical analysis were evaluated. Extracts were prepared sequentially using n-hexane, ethyl acetate, and methanol, with highest yields observed in methanol (21.39%). Phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, steroids, and carbohydrates in the ethyl acetate extract. Acute toxicity studies demonstrated the safety of the chloroform extract up to 5000 mg/kg in rats. Antidiabetic activity was assessed using streptozotocin-induced hyperglycemia in Wistar rats. Treatment with ethyl acetate extract significantly reduced blood glucose levels in a dose-dependent manner compared to diabetic controls over a 14-day period. Additionally, the extract prevented weight loss associated with diabetes. The DPPH radical scavenging assay indicated antioxidant potential. These findings suggest that Annona squamosa bark possesses promising antidiabetic properties, possibly mediated through its phytoconstituents and antioxidant activity. Further research is warranted to elucidate its mechanisms of action and potential therapeutic applications.

Introduction

Diabetes mellitus is an insulin-regulated disruption of the metabolic balance that results in anomalies related to the metabolism of fat and carbohydrates(Cooke et al. 2008). It has been connected to reduced insulin secretion and peripheral sensitivity, as well as eventual b-cell dysfunction. Diabetes mellitus has long been a source of concern because of its high rates of occurrence and mortality, as well as the high costs of its administration and treatment. (Ohiagu, Chikezie, and Chikezie 2021). Type 1 diabetes, which is immune-mediated or idiopathic, accounts for the majority of cases of diabetes mellitus. Type 2 diabetes, also known as non-insulin-dependent diabetes, is the most common kind of disease and is marked by elevated blood sugar, resistance to insulin, and a relative lack of insulin(Baynest 2015). Maintaining glucose homeostasis requires both the secretion and action of insulin. Insulin secretion goes through a biphasic phase if the glucose challenge lasts for ten minutes or less. The duration of this phase of insulin secretion is prolonged to the point where elevated blood sugar levels act as a trigger. Three interconnected mechanisms regulate glucose homeostasis: inhibition of endogenous glucose synthesis, elevation of hepatic glucose uptake, and elevation of peripheral glucose uptake. These processes increase the concentration of insulin in plasma and take place following the activation

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of insulin release(Raptis and Dimitriadis 2001). The significance of oxidative stress has received a lot of attention lately, and it has been suggested that oxidative stress may be a major and frequent factor in the pathophysiology of secondary diabetes problems(Ceriello 2000). Antioxidants have been explored as potential treatments for oxidative stress, which has been connected to numerous problems of diabetes mellitus, such as retinopathy and atherosclerotic vascular disease, the primary cause of death in diabetes(Cunningham, n.d.). Custard apple, sharifa (Hindi), sitappalam (Tamil), sitaphala (Kannada), and Sita phalamu (Telugu) are some of the common names for Annona squamosa Linn. (Family: Annonaceae). India is another country where Annona squamosa L is grown. Plant components have antispasmodic and insecticidal properties; they are also used to treat rheumatism and spleen discomfort. Antidiabetic, Antioxidant, analgesic, antipyretic, anti-inflammatory, antiulcer, antiseptic, and abortifacient properties are all attributed to the plant(Pratap Singh, Kumar Singh, and Malik 2014).

Materials and Methods:

Collection & Authentication of Plant Material

The bark of the plant *Annona squamosa* was collected in February 2024 from Garrison Engineer Park in Prayagraj, Uttar Pradesh, India. The plant was identified and Authenticated by Botanical Survey of India, Prayagraj, India. The specimen of *Annona squamosa* bark was certified by Mr. Vinay Ranjan, Scientist-E and Head of office, Botanical Survey of India Central Regional Centre, Chatham Lines, Prayagraj 212002. A specimen with voucher number ……. Has been deposited at the Botanical Survey of India, Prayagraj, India.

Plant Profile

Originally from the West Indies, Annona squamosa L. (Annonaceae), also referred to as the custard apple, is grown all over India, mostly for its delicious fruit. The plant is said to have anti-tumour, antifertility, antioxidant, analgesic, antipyretic, anti-inflammatory, antiulcer, and antiseptic effects on rats and mice. The young leaves of Annona squamosa are widely used by tribal men in and around the villages of Uttar Pradesh's Aligarh district and the people of Bihar's Chotanagpur district for their antidiabetic properties(Shirwaikar et al. 2004).

Pharmacognostic study of plant

The macroscopic, microscopic and physiochemical parameters of Annona Squamosa bark were examined in the Pharmacognostic investigation.

Determination of Physicochemical property

Loss on drying

The weight loss in % w/w. It calculates the volume of any volatile material, including water, that can be driven off in the given conditions (desiccator or hot air oven). If the sample is in the shape of big crystals, crush it fast to a powder in order to reduce the size(Liu 2019).

$$Loss\ of\ Drying = \frac{Initial\ weight\ of\ sample - weight\ of\ sample\ after\ drying}{Initial\ weight\ of\ sample} x\ 100$$

Swelling index

One easy standard test for assessing the tendency toward swelling is the swelling index test. In order to conduct these experiments, water was added to a 2 g sample of

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dry powder in a 100-ml cylinder. After that, the sample was covered to keep out contaminants for 16 to 24 hours. The increase in the volume of the mixture was assessed as a measure of swelling after full hydration(Ahmed and Hasan 2015).

$$Swelling\ Index\ \% = \frac{Vol.of\ swollen\ material(ml) - Original\ Vol.of\ liquid(ml)}{Weight\ of\ dry\ material(gm)} x\ 100$$

Ash value:

Ash is the substance left over when the crude medicine is burned. The residue that is formed typically consists of the inorganic salts that are present in the medication naturally and stick to it. It might also contain inorganic material that has been added with the intention of adulterating

Total ash

A portion of the entire ash that becomes insoluble in diluted hydrochloric acid is known as the acid insoluble ash. Five grams of powdered medication that had been air-dried and accurately weighed were placed in a tared silica crucible and burned in the furnace by progressively raising the temperature to a dull red hot (4000C) until carbon was removed. After cooling and weighing, repeat for a consistent result. Next, the air-dried medication was used to calculate the percentage of total ash(Mandal et al. 2017).

$$\%\ Total\ Ash\ Value = \frac{Weight\ of\ Ash}{Weight\ of\ Drug} \times 100$$

Acid insoluble ash

After following the instructions under complete ash, 25 ml of 2N HCL was heated with the ash for five minutes. After gathering the insoluble material on ash-free filter paper, it was cleaned with hot water, dried, lit, and weighed. Next, using the medicine that had been air-dried as a reference, determine the percentage of acid insoluble ash(Kadam 2012).

$$\%\ Acid - Insoluble\ Ash\ Value = \frac{Acid\ insoluble\ Ash\ Weight}{Weight\ of\ Drug} \times 100$$

Water soluble ash

Five minutes were spent boiling the entire amount of ash with 25 milliliters of water. After gathering the insoluble material on ash-free filter paper, it was heated to a maximum temperature of 450 C for 15 minutes and then cleaned with hot water. The weight of the total ash was deducted from the total mass of the insoluble substance. The water-soluble ash is represented by the mass differential(Tamboli, Tambare, and More, n.d.).

$$\%\ Water - Soluble\ Ash\ Value = \frac{Total\ Ash\ Wt. - Wt.of\ water\ insoluble\ ash}{Wt\ of\ Drug} \times 100$$

IDENTIFICATION OF EXTRACTIVE VALUE:

Alcohol soluble extractive value

Five grams of precisely weighed powdered drug were placed in a sealed conical flask, to which 100 ml of 90% alcohol was added. The mixture was shaken continuously for six hours in an electrical shaker, and the mixture was left overnight to macerate. The extractive was then carefully filtered, the filter was evaporated to the point of dryness, and the weight was recorded. The percentage was then computed using the drug that had been air-dried(Rubiyanti Poltekkes Kemenkes Tasikmalaya et al. 2017).

$$\%\ Alcohal\ soluble\ extractive\ value = \frac{Weight\ of\ extract}{Weight\ of\ drug} \times 100$$

Water soluble extractive value

A sealed conical flask containing 5 grams of precisely weighed powdered drug was filled with 100 ml of chloroform water. The flask was then shaken continuously for 6 hours in an electrical shaker to allow for maceration. The extractive was then carefully

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filtered, evaporated to dryness, and its weight was determined. The percentage was then computed using the drug that had been air-dried(pal singh and Gupta 2018).

$$\%\ Water\ soluble\ extractive\ value = \frac{Weight\ of\ extract}{Weight\ of\ drug} \times 100$$

PREPARATION OF PLANT EXTRACT

Using an apparatus called the Soxhlet and organic solvents, a serial extraction was performed in order to prepare the extracts. The solvents that were employed were n-hexane, Ethyl acetate, and methanol in that sequence. To prepare the extracts, 100 grams of powder & 400 mL of the primary solvent—n-hexane—were added to a Soxhlet apparatus. The extraction process took place for six hours at 45–50 °C. The leftover solid material was again undergone for extraction with the next solvent system, that is Ethyl acetate, at 45–50 °C3333 for six hours, after undergoing a second extraction process, again we have used Methanol as a third solvent system for the Successive Extraction of remaining solid material at the same temperature for six hours. The solvent was extracted under low pressure using a rotary vacuum evaporator to produce the residues. Distilled water was utilized as the solvent in the experiment to create aqueous extracts. The extracted form was stored in a desiccator till further investigation was conducted(Murugan et al.).

Phytochemical Tests:

Using established test procedures, the extracts of Annona squamosa have been subjected to qualitative analysis for the identification of several active ingredients, including mucilage, tannin, glycosides, steroids, alkaloids, flavonoids, and carbohydrates(Nguyen et al. 2020).

Animals

Wistar rats of the both gender and approximate age, weighing between 170 and 200 g, were used for the antidiabetic activity. These rats were provided by M/S CHAKRABORTY ENTERPRISE, Kolkata (Registration number: 1443/PO/Bt/s/11/CPCSEA). They were kept in the animal house for 14 days at controlled temp. in a 12-hour light/dark cycle. They had free access to water and pellet diet. The Institutional Animal Ethics Committee accepted the study, and all experimental procedures were conducted with full adherence to ethical guidelines.

Acute toxicity studies

Acute toxicity tests were performed on albino Wistar rats of both sexes using the fixed dosage method in compliance with OECD guidelines. The rats were administered extract at varying dosages (5, 50, 300, 2000, and 5000 mg/kg). After two hours of continuous observation, the animal was observed sporadically for a further four hours. The animal's survival and death were recorded over the course of the night, and the LD50 was discovered(Saleh et al. 2021).

STZ Induced Hyper-glycemia in Rats

To experimentally develop diabetes in rats, a single intraperitoneal injection of a freshly prepared STZ solution at a concentration of 60 mg/kg of body weight in a 0.1 M cold citrate buffer solution with a pH of 4.5 was employed. After a duration of 72 hours, blood was drawn from the tail veins of the rats while they were under chloroform sedation. The blood glucose levels were assessed using glucosidase peroxidase reactive strips and a glucometer. An animal was considered diabetic if its level of blood glucose was greater than 250 mg/dl; only these animals were included in the study. The control rats received a single injection of citrate buffer(Asif et al. 2006).

Experimental design for antidiabetic activity

The rats were separated into five groups, with six individuals in each group, as follows(Mujeeb 2009).
Group I: Normal control rats given only buffer.
Group II: Diabetic controls (STZ, 60 mg/kg body weight).
Group III: Diabetic rats treated with Glimepiride (100 mg/kg body weight)
Group IV: Diabetic rats treated with ethyl acetate extract of *Annona squamosa* (200 mg/kg body weight)
Group V: Diabetic rats treated with ethyl acetate extract of *Annona squamosa* (400 mg/kg body weight)

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Glucoxidase peroxidase reactive strips and a glucometer (one touch basic plus) were used to monitor the blood glucose levels of the experimental animals at 0, 4, 7, 10, and 14 days after they were administered the plant extract.

DPPH Radical Scavenging Activity:

Free radical scavenging activity of the Ethyl acetate extract of *Annona squamosa*, based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of given reference. Different volume of extracts/standard (20 – 100µg/ml) was taken from stock solution in a set of test tubes and methanol was added to make the volume to 1 ml. To this, 2 ml of 0.1mM DPPH reagent was added and mixed thoroughly. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated by using the equation:

$$\%\ Scavenging\ activity = \frac{A0 - A1}{A0} \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. Lower the absorbance, the higher is the free radical scavenging activity. The curves were prepared and the IC50 value was calculated using linear regression analysis(Ali et al. 2013).

FTIR (Fourier Transform Infrared Spectrum):

Models were created using Fourier transform infrared spectra. The 400–4000 cm1 range was used to scan the spectra. The potassium bromide pellet technique was used to acquire the spectra. The 1 mg sample was combined with 100 mg of potassium bromide (AR grade) that had been vacuum-dried for 48 hours at 100 degrees Celsius. FTIR analysis was performed on the combination. Plotting the acquired spectra as intensity against wave number was done(Chandran et al. 2016).

HPTLC (High Performance Thin Layer Chromatography):

The sample was applied to plates that had already been coated. A known amount of of extract was dissolved in the appropriate solvent, and samples were applied using an applicator on a precoated plate. Using the CAMAG TLC sacnner 3 equipment, solvent systems optimised for TLC research were selected for HPTLC analysis(Agrawal, Itankar, and Vyas, n.d.).

Statistical analysis

Values are reported as mean S.E.M. for the six values. The one-way analysis of variance (ANOVA) was used to establish statistical significance. The statistical significance was established when $P<0.01$ and $P<0.05$ were compared with the diabetes control group.

RESULTS

1. Physiochemical Parameter

Table 1.1: Physiochemical Parameter Analysis of Ethyl acetate's extract

S.N.	Parameters	% w/w
1	Total ash value (% w/w)	**11**
2	Acid insoluble ash value	**0.70**
3	Water soluble ash value	**6.4**
4	Loss of drying at 105	**16.48**
5	Water soluble extractive value	**0.6**
6	Alcohol soluble extractive value	**5.3**
7	Swelling index	**0.5**

2. Percentage Yield

Table 2.1: Percentage Yields in different solvents

Solvents	% Yield
n-hexane	06.54
Ethyl acetate	13.18
Methanol	21.39

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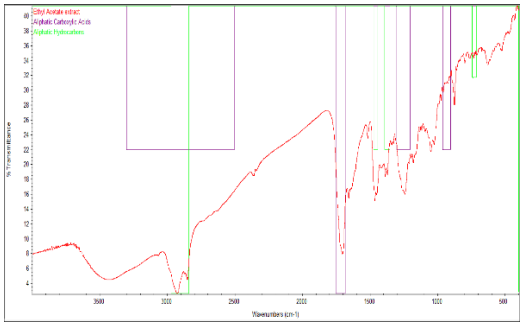
3. Preliminary Phytochemical screening

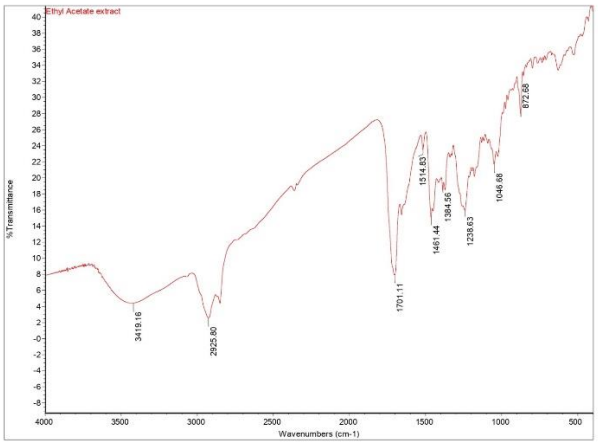
Table 3.1: Preliminary phytochemical screening of Ethyl acetate extract of the bark of Annona squamosa

S.N.	Test Performed	N-hexane extract	Ethyl acetate extract	Methanol extract
1	**Alkaloids**			
	Dragnodroff reagent	(+)	(+)	(+)
	Mayer test	(-)	(+)	(-)
2	**Flavonoids**			
	Lead acetate	(-)	(++)	(+)
	Shinoda test	(-)	(+)	(-)
3	**Tannin**			
	Fecl3	(+)	(++)	(+)
4	**Carbohydrate**			
	Molish	(+)	(+)	(-)
5	**Glycosides**			
	Killer- killani	(-)	(-)	(-)
6	**Steroid**			
	Salkowski	(+)	(+)	(+)
	Libermann-Burchard	(-)	(++)	(+)

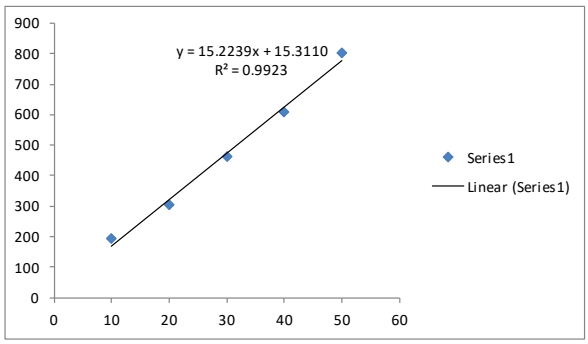
3. FTIR

There are numerous peaks in the FITR spectra for the Annona squamosa fraction. These peaks at 3419 cm-1, 2925 cm-1, 1701 cm-1, 1514 and cm-1, 1461 cm-1, and 1238 cm-1 show that aliphatic hydrocarbons and carboxylic acids are present.





5. HPTLC:



4. Acute Toxicity Study

The current study revealed that, when given orally to rats at a maximum dosage of 5,000 mg/kg body weight, the chloroform extract of Annona squamosa bark indicated non-toxic behaviour. The oral median lethal dosage (LD50) of the extract in rats was determined to be higher than 5,000 mg/kg body weight using the OECD guidelines for oral acute toxicity studies.

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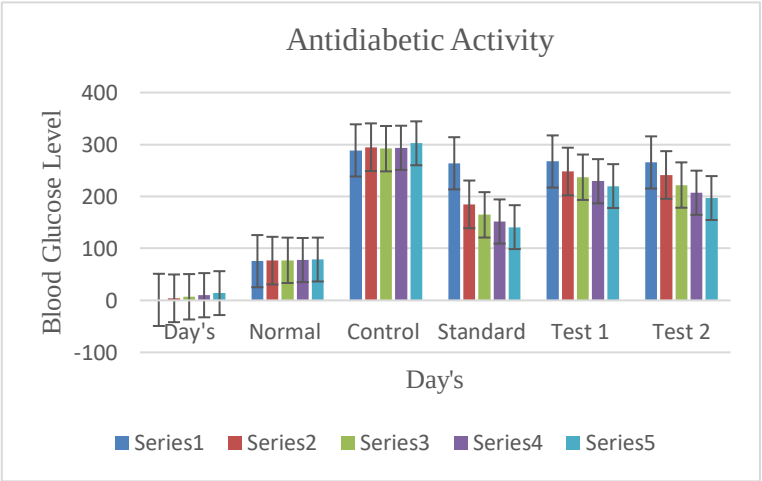
JCHR (2024) 14(5), 2004-2013 | ISSN:2251-6727

5. Blood Glucose Level Changes

Table 5.1: Effect of *Annona squamosa* Ethyl acetate's bark extract on changes in blood glucose level

Groups	1 Day	4 days	7 days	10 days	14 days
Control	75.5 ± 0.42	76.5 ± 0.76	77.1 ± 0.60	77.6 ± 0.84	78.6 ± 0.71
Diabetic control	288.6 ± 2.36	294.8 ± 2.84	292 ± 2.69	293.6 ± 1.60	302.3 ± 1.80
Metformin	263.8 ± 3.65	184.8 ± 2.40	164.6 ± 2.57	151.8 ± 1.88	141 ± 0.96
AS extract 200mg	267.3 ± 3.71	248 ± 3.65	237 ± 1.71	229.3 ± 1.25	220 ± 1.23
AS extract 400mg	265.5 ± 3.96	241.3 ± 2.40	222 ± 1.43	207.1 ± 1.24	197 ± 1.63



Graph 1 Graph represents the Blood Glucose Level *Vs* Day's, of Ethyl acetate extract of *Annona squamosa* extract

6. Body Weight Changes

Table 6.1: Effect of Ethyl acetate's extract of *Annona squamosa* bark on changes in body weight (grams)

Group	1 day	7 days	14 days
Control	172.3 ± 2.57	175.3 ± 1.49	174.5 ± 1.25
Diabetic control	174.8 ± 2.07	167.3 ± 1.60	148.5 ± 2.75
Metformin	175.6 ± 2.77	169 ± 2.30	168.6 ± 2.20
AS extract 200mg	172.8 ± 2.18	166.3 ± 2.34	166.8 ± 1.93
AS extract 400mg	174.3 ± 2.82	169.5 ± 2.44	168.5 ± 1.54

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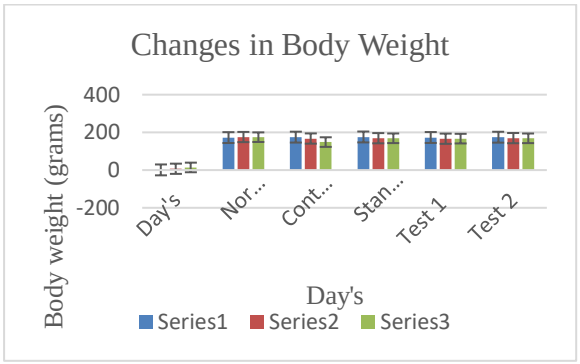


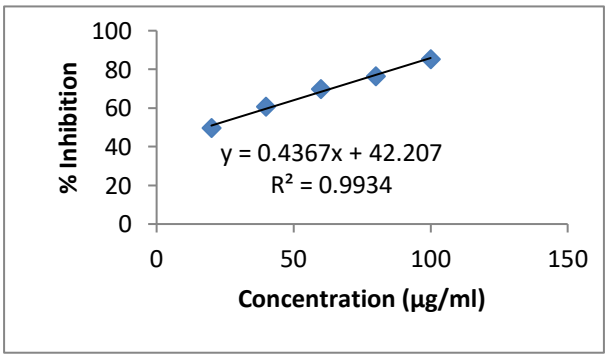
Graph 2 Graph represents the Changes in body weight *Vs* Day's, of Ethyl acetate extract of *Annona squamosa* extract

7. DPPH Activity

7.1 In-vitro Anti-oxidant Activity of Ethyl Acetate extract of Annona squamosa V/S Ascorbic acid

7.1.1 Table 1 DPPH radical scavenging activity of Ascorbic acid

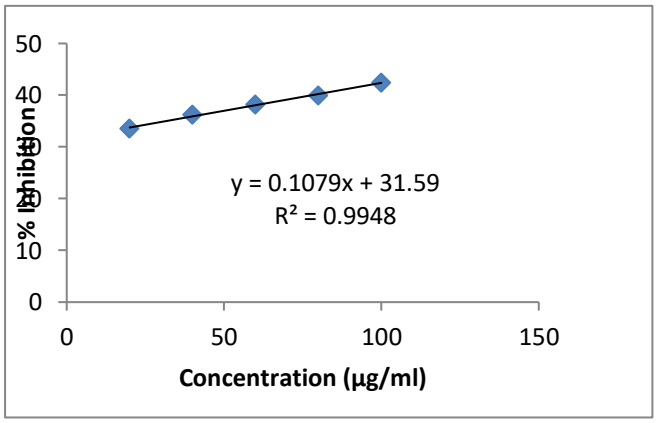
Concentration	Absorbance	% Inhibition
20	0.481	49.686
40	0.373	60.669
60	0.289	69.769
80	0.225	76.464
100	0.139	85.460
Control	0.956	
IC50	**17.89**	



Graph 3 Graph represents the Percentage Inhibition *Vs* Concentration of Ascorbic acid

Table 2 DPPH radical scavenging activity of Ethyl Acetate extract of *Annona squamosa*

Concentration	Absorbance	% Inhibition
20	0.619	33.512
40	0.594	36.197
60	0.575	38.238
80	0.559	39.957
100	0.536	42.427
Control	0.931	
IC50	**172.056**	



Graph 4 Graph represents the Percentage Inhibition *Vs* Concentration of Ethyl Acetate extract of *Annona squamosa* extract

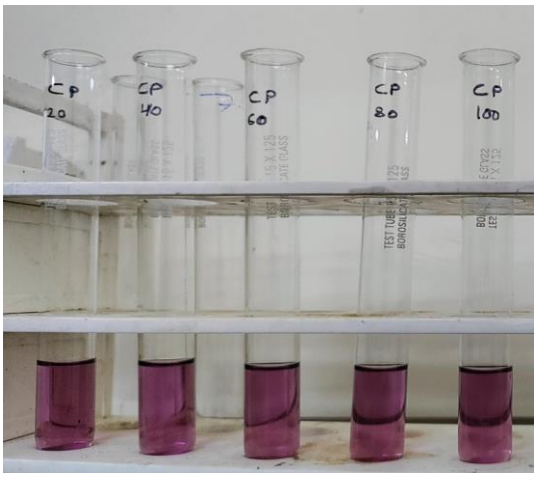


Figure 1 DPPH activity of Ethyl acetate extract of *Annona squamosa* extract

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DISCUSSION:

The results of this study add to the increasing amount of data demonstrating the Pharmacognostic and antidiabetic qualities of Annona squamosa bark extract, which is being used to treat diabetes. The study showed the potential therapeutic uses of this traditional medicinal plant by methodically evaluating a number of criteria, such as phytochemical composition, antioxidant activity, and antidiabetic effects. Significant physicochemical characteristics were found in the bark of Annona squamosa during the Pharmacognostic examination. For example, a total ash value of 11% suggested the presence of inorganic components and potential adulteration. The comparatively low extractive values (both water-soluble and alcohol-soluble) indicated that the bioactive chemicals' solubility was restricted. The low swelling index can be a reflection of the bark's structural properties. All things considered; the evidence suggests that Annona squamosa has a variety of phytochemicals that may enhance its medicinal effectiveness.

Conclusion

The study offers strong support for the antidiabetic and antioxidant qualities of bark extract from Annona squamosa, indicating that it may be used as a natural treatment for diabetes mellitus. More investigation is necessary to fully investigate this plant's medicinal potential in clinical settings and to clarify the precise mechanisms of action of its bioactive components. Long-term impacts and chronic toxicity should be taken into account in future research to provide thorough safety profiles prior to clinical use.

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