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Study of Antioxidant Activity in Annona Leaves

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Abstract

This study aims to test the antioxidant activity of extracts of the leaves of the *Annona squamosal linn* plant, which is commonly used in folk medicine and is considered one of the most important anticancer agents at the present time. The DPPH reagent inhibition method was utilized to estimate the total antioxidants, (where (DPPH) is 2,2- diphenyl-1- picrylhydrazyl) revealing that the extract concentrations required for 50% inhibition of free radicals (IC_{50}) was 3766.14µg/ml. The antioxidant equivalent to ascorbic acid was 3823.22µg/ml, which was the highest efficacy for a concentration of 5000 mg/ml of the extract and the lowest at a concentration of 1000mg/ml. This study also assessed the contents of phenolics for their in vitro antioxidant activity. The total phenolic content of methanol extract of *Annona squamosal linn* measured by Folin-Ciocalteau reagent in terms of gallic acid ranged from 1189.75 to 3502.25 mg GAE/g of extract , (where(GAE) is gallic acid equivalent). The extract was tested against the following antibacterial types: *Staphylococcus aureus, Streptococcus spp, Escherichia coli, Klebsiella spp, Psuedomones spp,* and *Protues spp*. According to the findings, different extract concentrations effectively inhibited the growth of the chosen pathogenic microorganisms. The study revealed that the *Annona squamosal linn* leaves contain large amounts of phenolic compounds which were found to be the main contributor to its antioxidant activities, and the extract was found to exert low to moderate antibacterial activity. Hence, it can be concluded that the leaves of *Annona squamosal linn* can lead to the creation of compounds that can be used to develop new and more effective anticancer drugs.

Keywords: *Annona squamosa Linn*, Folin-Ciocalteau reagent, gallic acid, ascorbic acid, DHPP, antibacterial activity

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دراسة النشاط المضاد للأكسدة في أوراق نبات القشطة

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الملخص

تهدف هذه الدراسة إلى اختبار النشاط المضاد للأكسدة لمستخلصات أوراق نبات القشطة ، والتي تستخدم عادة في الطب الشعبي وتعتبر من أهم العوامل المضادة للسرطان في الوقت الحاضر. تم استخدام طريقة تثبيط كاشف DPPH لتقدير مضادات الأكسدة الكلية ، (حيث DPPH هو 2,2-diphenyl-1-picrylhydrazyl) ، وكان تركيز المستخلص الذي يثبط الجذور الحره بنسبة 50%(IC_{50}) 3766.14µg/ml. وكان (IC_{50}) لحمض الأسكوربيك 3823.22µg/ml، وكانت أعلى فاعلية لتركيز 5000 mg/ml من المستخلص وأقلها عند تركيز 1000mg/ml. كما قامت هذه الدراسة بتقييم محتويات الفينولات الكلية في مستخلص أوراق نبات القشطه ، حيث تراوح إجمالي محتوى الفينولات في المستخلص الميثانولي المقاس بواسطة كاشف Folin-Ciocalteau بإستخدام حمض الجاليك كمرجع بين 1189.75 - 3502.25 mg GAE/g ، (حيث (GAE) هو مكافيء حمض الجاليك) . تم اختبار المستخلص ضد ستة أنواع من البكتريا المسببة للأمراض وهي : *Staphylococcus aureus , Streptococcus spp , Escherichia coli , Klebsiella spp* and *Protues spp* , *Psuedomones spp* . وفقاً للنتائج ، فإن تركيزات المستخلص المختلفة تمنع بشكل فعال نمو البكتريا المسببة للإمراض . حيث كشفت الدراسة أن أوراق نبات القشطة تحتوي على كميات كبيره من المركبات الفينولية التي وجد أنها هي المساهم الرئيسي في أنشطتها المضادة للأكسدة ، ووجد أن المستخلص له نشاطاً مضاداً للبكتريا منخفضاً إلى متوسطاً، ومن تم يمكن الإستنتاج أن أوراق نبات القشطة يمكن أن تصنع منها أدوية جديدة تكون فعاله لمكافحة السرطان.

الكلمات المفتاحية : نبات القشطة ، كاشف فولن ، حمض الجاليك ، حمض الأسكوربيك ، DPPH ، النشاط المضاد للبكتريا.

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INTRODUCTION

The taxonomic designation, 'Annona', is derived from the Latin term 'anon', which signifies 'annual yield', alluding to the fruit-bearing characteristics of the various species encompassed within this genus. Annonaceae, commonly referred to as the custard apple family, constitutes a family of angiosperms comprising arboreal, shrubby, or infrequently lianaceous forms(syed *et al*., 2017). Comprising approximately 2300 to 2500 species across 130 recognized genera, the Annona genus is considered the prototypical genus of this diverse plant group. This botanical family predominantly inhabits tropical climates, with a limited number of species identified in temperate zones. Approximately 900 species belong to the Neo-tropical region, 450 to the Afro-tropical region, while the remaining species are classified as Indo-Malayan. Within the Annonaceae family, there exist 130 genera, of which several are extensively distributed. Notable genera include Annona, Anonidium, Rolliania, Uvaria, Melodorum, Asimina, and Stelechocarpus(Hasmila *et al*., 2019).

Annona squamosa Linn (Annonaceae), commonly referred to as custard apple, is widely recognized for its palatable fruits. The species have been traditionally employed in the therapeutic management of various conditions including epilepsy, dysentery, cardiovascular, disorders, syncope, helminthiasis, constipation, hemorrhagic episodes, pyrexia, and ulcers. They have also been utilized as an abortifacient(Gavamukulya *et al*., 2014).

The aqueous extract is derived from the leaves of Annona. Squamosa has been documented to mitigate hyperthyroidism, a condition frequently regarded as a contributing factor to diabetes mellitus(Qorina *et al*., 2019). An infusion derived from the leaves is regarded as effective in addressing prolapse in pediatric patients, while the pulverized leaves are inhaled to mitigate episodes of hysteria and syncope(Lee and cho., 2016).

The significance of the fruit is ascribed to its saccharine mesocarp, which possesses therapeutic properties. Furthermore, it acts as a substantial reservoir of carbohydrates (23.5%), proteins (1.6%), and minerals (0.9%)(de souza *et al*., 2015). In conjunction with these dietary components, the fruit encompasses substantial quantities of vitamins including folic acid and ascorbic acid, in addition to minerals such as calcium, phosphorus, and iron(souza *et al*., 2018). Furthermore, the custard apple serves as a significant source of naturally occurring antioxidant compounds, and all components of

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the plant are employed in traditional medicine practices worldwide(Fischer *et al*., 2017).

The antioxidant properties of fruit extracts, in addition to the significance of custard apple in mitigating oxidative injuries, have been documented in the literature(Leesombun *et al*., 2019;Ngnameko *et al*., 2019). The fruits of the custard apple are rich in polyphenolic compounds, which exhibit antiviral, antimicrobial, and anti-inflammatory effects, alongside their inherent antioxidant capabilities (Calzada *et al*., 2020;Huang *et al*., 2016).

In the realm of botany, there exist three primary categories of phytochemicals; namely alkaloids, terpenoids, and phenolic metabolites(Yu *et al*., 2020). Within these three classifications, phenolic compounds are of paramount importance in dietary contexts and have been the subject of extensive scholarly inquiry(Gajera *et al*., 2017). The identification of novel and non-toxic antioxidants derived from natural sources has garnered significant interest for their potential applications in functional food products(Ghawade *et al*., 2018). Antioxidants assume a pivotal function in the human physiological defense system against disorders induced by free radicals, serving as radical scavengers(Sokpe *et al*., 2020). Phenolics constitute a category of chemical entities that encompass both simple phenols and polyphenols. Polyphenols possess the capability to mitigate and avert damage to human tissues instigated by the promotion of free radicals(Kumar *et al*., 2018). Flavonoids have the capacity to engender mechanisms that may effectively impede invasion and induce apoptosis in neoplastic cells. Reactive oxygen species (ROS) are molecular species characterized as O_2 free radicals and exhibit a dualistic function, being both harmful and advantageous to biological systems(Sovia *et al*., 2017). In addition to their involvement in pathological conditions within the organism, ROS are recognized for their contribution to the degradation of food substances through the autoxidation of lipids and enzymatic oxidation processes occurring during the storage and processing of fats, oils, and lipid-containing edibles(Prasad *et al*., 2021). Antioxidants are compounds that exist in minimal concentrations compared to the oxidizable substrate, effectively inhibiting or delaying the oxidation process of the substrate(Al-Nemari *et al*., 2020). The human organism fails to produce an adequate quantity of antioxidants to counteract the deleterious effects of reactive oxygen species (ROS) (Ahmed ., 2020). Although synthetic antioxidants, including butylated hydroxytoluene, butylated hydroxy anisole, gallic acid esters, and tertiary butylated hydroquinone, are capable of attenuating free radical activity, they have faced scrutiny due to

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their potential toxicological implications, limited solubility, and moderate efficacy in antioxidant activity. Consequently, there exists a pressing necessity to identify novel natural sources of antioxidants(Kalidindi *et al*., 2015).

Thus, The present study was carried out to measure the total polyphenol and antioxidant activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) of the extract of Annona squamosal linn. The antibacterial Concentrations were tested on the six species of bacteria. extracts for their possible use as source of antioxidants and also as antibacterial agents that can be used to prevent food spoilage.

2. Materials and methods

2.1 Annona leaf Samples

Leaves of the plant were procured from the local market of Misurata city, and were originally dried and imported from Thailand. The dried leaves were finely ground using USA standard testing sieve (Fisher Company) to create a powder that is easily soluble in the organic solvent used in the extraction process. The powder is then stored in air-tight polyethylene bags in a desiccator.

2.2 Chemicals and equipment used

In this study, methanol solvent of analytical grade was used at a ratio of 95% from the company (PSPARK chemical). DPPH free radical scavenging assay: 2,2- diphenyl-1- picrylhydrazyl and Ascorbic acid from the company (BHD chemical). Total phenolic content: sodium carbonate, Folin-Ciocalteu reagent (Chem King). A spectrophotometer was used:
(Italy 40 40 Spectrophotometer) and (Eliza reader, Biotek, Germany).

2.3 Extraction method:

The Annona leaves were authenticated for their unambiguous identity by Prof. A.M. Amletan, Head of Botany Department, Misurata University, Libya. The cold extraction method (soaking) which involves placing the sample in a beaker with a limited amount of solvent sufficient to cover it, was followed(Ma QinGe *et al*., 2016). An amount of 5g of the extract was taken by using (Glossaries DHAUS) and soaked in 100ml of cold methanol , methanol was used because it is a good polar solvent for dissolving phenols found in plant leaves, Polar extracts were found to be better free radical scavengers compared with those less polar. The container was, then, placed into magnetic stirrer (Model: RT 15P; Serial: 2 930 700). After that, the samples were left to rotate for 24 hrs. Furthermore, a filtering process was

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applied to the clarified suspension by using Sartorius PTEF 0.45 µm filter and the beaker was wrapped with aluminum foil to protect the mixture from being spilled off and from exposure to light. The filtrate was left at room temperature until the solvent completely evaporated. The extraction yield was then calculated as a percent of the used powder. The precipitate was kept in the refrigerator until the analysis was conducted.

2.4 Antioxidant assays

2.4.1 Determination of Folin-Ciocalteu index for total phenolic contents (TPC)

For the quantification of Total Phenolic Content (TPC), the Folin-Ciocalteu Index (FCI) assay was employed with minor adjustments; the methodology utilized adheres to the protocol delineated by(Reshmae and Suganthi., 2024). 1mg of Annona leaf extract was taken and dissolved in 1ml of pure methanol. The samples were then diluted with methanol to obtain five concentrations (5000, 4000, 3000, 2000, 1000 µg/ml). Approximately, 1.5 ml of diluted Folin-Ciocalteu reagent (10%) was added to 0.1 ml(1mg/ml) of the sample extract for each concentration, which was then mixed individually and left to equilibrate for a duration of 5 minutes. Then, 1.2 ml (7.5%) of Na_2CO_3 (w/v) was added, and the mixture was left to rest for 30 minutes in a dark environment. The absorbance was measured at a wavelength of 765 nm utilizing a spectrophotometer UVD-3500 after a 2-hour duration. The results were, later, documented in terms of milligrams of gallic acid equivalent. A standard curve was plotted using different concentrations of Gallic acid. The absorbance obtained was converted to gallic acid equivalent (GAE) in mg per gm of dry material (mg GAE/g of extract) using gallic acid standard curve.

2.4.2 DPPH radical-scavenging activity

The capacity of the extract to scavenge the stable DPPH free radical was measured(Balderrama-Carmona *et al*., 2020). DPPH was used to determine the proton radical scavenging action of extracts of *Annona squamosal linn* , because it possesses a proton free radical and shows a characteristic absorbance at 517nm . The DPPH is a purple-black solid substance. This radical exhibit stability and its antioxidant capacity is determined by measuring the IC_{50} factor, which is defined as the concentration of the extract required to inhibit 50% of the DHPP radical.

A volume of 0.1 ml from each specified concentration was individually combined with 2.9 ml of a 0.1 mM DPPH solution prepared in advance. The control group consisted of 0.1 ml of the extract in conjunction with 2.9 ml of the DPPH solution. The negative control was established by amalgamating

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0.1 ml of methanol with 2.9 ml of the DPPH solution, which was subsequently allowed to incubate for 30 minutes in a dark environment. The absorbance measurements were acquired at a wavelength of 517 nm utilizing a spectrophotometer after a duration of 2 hours. The outcomes were documented in terms of milligrams of ascorbic acid equivalent. Methanol was used as a zero solution and the absorbance of solution DPPH was also measured without adding the extract, and the I% inhibition rate for the free radical DPPH was calculated according to the following relationship(Singh *et al*., 2024):

Radical scavenging activity (RAS%) = (Control absorbance – Sample absorbance / control absorbance) × 100

2.5. Determination of antibacterial activity of A. squamosa leaves extracts

Evaluation of Antibacterial Activity

With a few minor adjustments, the cup-plate agar diffusion method was used to measure the antibacterial activity. Agar that had been incubated was divided into two groups and 20 ml aliquots were placed into sterile Petri dishes. The agar was allowed to settle in each group's six cups, each of which had a diameter of 10 mm and was cut with a sterile corkscrew (No. 4). Each of the halves was designed for one of the test compounds. Separate Petri-dishes were created for the standard antibacterial chemotherapeutic agent. After removing the agar discs, 0.1 ml samples of each of the extracts and pure complexes were added to alternate cups using an adjustable volume micro titer pipette, and was allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours. This procedure was repeated for different concentrations of the complexes and the standard antibacterial chemotherapeutic. Following incubation, the growth inhibition zones that resulted were measured as well as their average diameter (Gul *et al*., 2016).

2.6 Statistical analysis

Statistical analyses were computed by using Microsoft Excel 2010 .

3. RESULTS AND DISCUSSION

3.1. Extraction yields

The extraction yield depends on solvents, time and temperature of extraction, as well as the chemical nature of the sample. Under the same time and temperature conditions, the solvent used and the chemical property of the sample are the two most important factors(Anaya- Esparza *et al*., 2020). The

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recommended effective extracting solvents are aqueous mixtures of methanol, ethanol and acetone(Punia *et al*., 2020).

In the present study, the extraction yield obtained for methanol solvent was determined by a percentage of (6.12%).

3.2. Total Phenolic Contents (TPC)

It is widely acknowledged that flora encompasses a myriad of phenolic compounds characterized by the presence of a hydroxyl group attached to an aromatic ring. These phenolic constituents impede the progression of chain oxidation reactions through the donation of a hydrogen atom or by chelating metal ions. Consequently, they function as reducing agents and antioxidants(Kumar *et al*., 2021). The assays were conducted utilizing the entire extract, as this approach may offer greater advantages compared to isolated constituents, due to the fact that a bioactive individual component can exhibit alterations in its properties when interacting with other compounds present within the extract(Kumar *et al*., 2020;Amalia *et al*., 2024).

The comprehensive quantification of phenolic compounds within the methanol extract derived from the leaves of Annona squamosa Linn was executed utilizing the Folin–Ciocalteu assay, revealing a total phenolic content of about 1189.75 - 3502.25 mg GAE/g of extract; as shown in (Table 1). Initially, a calibration curve was constructed employing various concentrations of gallic acid (figure 1). The findings strongly suggest that phenolic compounds are essential components of this botanical specimen, and certain pharmacological effects may be ascribed to the existence of this significant element. The review of the literature indicated that diverse extracts derived from the seed, leaf, root, and other anatomical parts of Annona squamosa contain flavonoids and phenolic compounds(Ruddaraju *et al*., 2019).

The results obtained in this study exhibit a high degree of consistency with numerous previously documented findings. This indicates that phenolic compounds typically exhibit greater solubility in polar organic solvents compared to aqueous solutions. Furthermore, the findings of this study are congruent with those of researchers indicating that among the solvents evaluated, a 50% methanol extract displayed the highest concentration of total phenolics derived from the leaves of Annona squamosa Linn(Chan *et al*., 2020). The findings reported by researchers indicate that ethanol and methanol exhibit greater efficacy than water in extracting total phenolics from Annona squamosa Linn leaves(Nguyen *et al*., 2020). Furthermore, in

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the assessment of total phenolic content utilizing the Folin assay, the various leaf extracts displayed a remarkably high phenolic content of approximately 13.0098 mg/g of extract, followed by the water and methanol extracts of the seed(Tabassum *et al*., 2024).Through our study,we noticed that the higher the concentration of the extract, the more phenols it contains.

Table (1) Total Phenolic Contents (TPC) and Extract Yield

Concentration((µg/ml)	Total phenolic Contents (TPC) mg GAE/g of extract	Extract yield (%)
5000	**3502.25**	**6.12%**
4000	**2392.25**	
3000	**1799.75**	
2000	**1467.25**	
1000	**1189.75**	

Table 1 illustrates the dose-dependent increase in Total phenolic Contents (TPC), with the highest value of Total phenolic Contents (TPC)observed at 5000 µg/ml and least value at 1000 µg/ml.

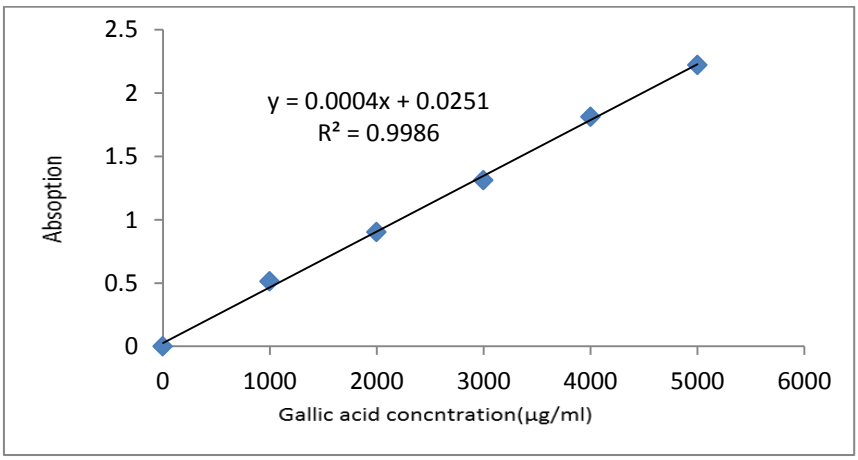

Figure 1. Standard gallic acid solution (µg/ml)

3.3 DPPH radical-scavenging activity

There exist various methodologies for the assessment of antioxidant activity pertaining to both synthetic and natural compounds. The DPPH assay represents a swift and cost-effective approach, commonly employed for the

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evaluation of the antioxidative capacity of diverse natural sources. The DPPH scavenging assay is extensively utilized to evaluate the free radical scavenging capabilities of plant extracts due to its sensitivity, simplicity, rapidity, and widespread availability in chemical laboratories. Antioxidants are capable of mitigating radical species through the mechanism of hydrogen donation, leading to a reduction in DPPH absorbance measured at 515 nm. The IC_{50} value represents the concentration of the sample at which the inhibition percentage attains 50%. Consequently, IC_{50} values exhibit an inverse relationship with antioxidant activity; a diminished IC_{50} value signifies an enhanced antioxidant efficacy of the evaluated sample. Table (2) illustrates the DPPH radical scavenging activity of methanolic extract of *Annona squamosal linn*. The IC_{50} value for the extract of *Annona squamosal linn* was determined to be 3766.14µg/ml (Figure 2), indicating significant antioxidant properties. This observed activity may be attributed to the presence of phenolic compounds. In comparison, the IC_{50} value for the standard reference ascorbic acid was recorded at3823.22µg/ml (Figure 3).
Previous studies on antioxidants of the Annona leaf extract using different solvents are aligned with the present study's results, including that the ethanol extract showed a high level of antioxidants, with an IC_{50} value of 132.96µg/ml, while the standard ascorbic acid had an IC_{50} of 2.64µg/ml (Safira *et al*., 2022). Through our study,we noticed that the higher the concentration of the extract, the more antioxidants it contains.

Table 2. DPPH radical scavenging activity of methanolic extract of *Annona squamosal linn* and ascorbic acid

Concentration µg/ml	DHPP	Control Abs	Sample Abs	RSA% Sample	RSA% Control
5000	0.747	0.281	0.263	64.79	62.38
4000	0.619	0.278	0.260	57.99	55.08
3000	0.425	0.260	0.257	39.52	38.82
2000	0.295	0.207	0.231	21.69	29.83
1000	0.125	0.107	0.109	12.80	14.40
IC_{50}(µg/ml)				3766.14	3823.22

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Table 2 illustrates the dose-dependent increase in antioxidants activity, with the highest percentage of antioxidants observed at 5000 µg/ml and least percentage at 1000 µg/ml.

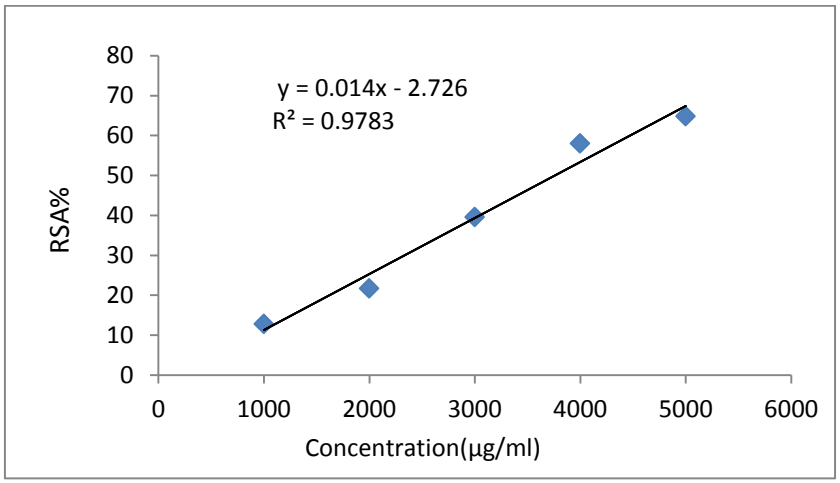

Figure 2. DPPH scavenging activity of methanolic extract of *Annona squamosal linn*

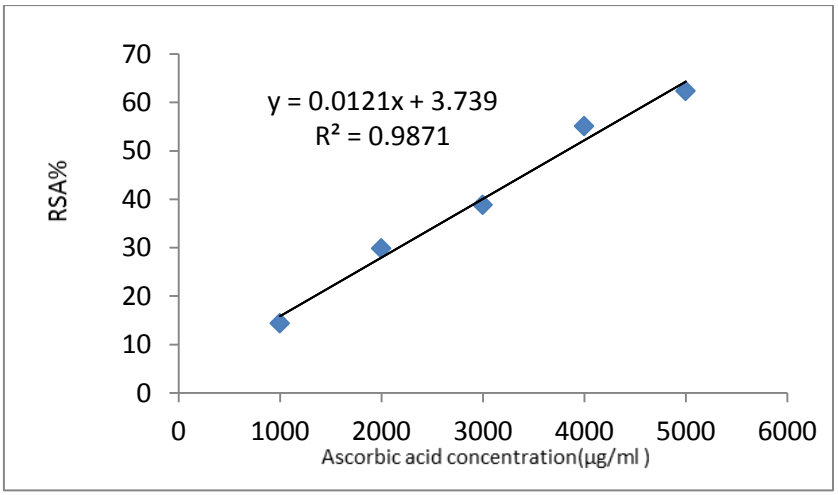

Figure 3. DPPH scavenging activity of ascorbic acid.

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3.4 Antibacterial Studies:

The antibacterial Concentrations were tested on the six species of bacteria including: (*Staphylococcus aureus, Streptococcus spp, Escherichia coli, Klebsiella spp, Psuedomones spp* and *Protues spp*) The extract was under a variable concentration (5000 , 4000 , 3000 , 2000 , 1000µg/ml). At the concentration of 1000 µg/ml, the extract showed a positive effect on the two types of bacteria and was (*Staphylococcus aureus, Streptococcus spp*). In a concentration of 2000 µg/ml, it showed a positive effect on all of the species of bacteria. In a concentration of 3000 µg/ml, the extract showed a highly positive effect on all types of bacteria. As for a concentration of 4000 and 5000 µg/ml, they showed the highest positive effect on all types of bacteria. The obtained results were consistent with the results obtained by researchers in the reference(Ling., 2020). Through our study,we noticed that the higher the concentration of the extract, the stronger its effect on bacteria.

Table (3) Antibacterial Activities of methanolic extract of *Annona squamosal linn*

Concentration µg/ml	*Staph.Aureus*		*Streptococcus* spp		*E. coli*		*Klebsiella* spp		*Pseudomonas* spp		*Protues* spp	
	A*	%	A*	%	A*	%	A*	%	A*	%	A*	%
5000	++	60	++	50	+	30	+	35	++	55	+	30
4000	++	55	++	45	+	25	+	30	++	50	+	25
3000	++	40	++	40	+	25	+	20	++	50	+	20
2000	+	30	+	35	+	20	+	20	+	30	+	20
1000	+	25	+	20	-	5	-	5	-	10	-	10

Percentage of Inhibition: Below 20% = (-) low active, 20% – 40% = (+) Active, 40% – 60% = (++) mildly active & 60% – 80% = (+++) moderately active, (80%, up) = (++++) highly active, *Activity

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Table 3 illustrates the dose-dependent increase in antibacterialactivity, with the highest inhibition observed at 5000 µg/ml and least inhibition at 1000 µg/ml .

Conclusion

Annona squamosa Linn (Custard apple) represents a valuable medicinal flora that has been utilized historically in oncological therapies. It exhibits pharmacological properties analogous to those of a significant succulent, aromatic perennial herb. Furthermore, the plant demonstrated notable antioxidant and antimicrobial properties. The current investigation emphasizes the potential application of A. squamosa L. leaf extracts as a source of antioxidants. It further aims to assess the total phenolic content within the leaves of Annona squamosa Linn and antibacterial activities. The phenolic concentration was identified to be within the range of 1189.75 - 3502.25 mg GAE/g of extract. The evaluation of antioxidant activity was conducted employing the DPPH assay. The IC_{50} value was determined to be 3766.14 µg/ml. In conclusion, the study revealed that the leaves of *Annona squamosa Linn*. contain a considerable quantity of phenolic compounds that were found to be the major contributor for their antioxidant and antibacterial activities.

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