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In-Vitro Estimation of Total Phenolic, Flavonoid, Antioxidant Contents and Determination of Flavonoid, Amino Acid, and Vitamin Profiles of Ethanol Leaf Extract of *Annona squamosa* L.

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Abstract

Since the beginning of human civilisation, plants have been one of the main sources of medicines. The demand for pharmaceuticals, health products, food supplements, cosmetics, and medications made from plants is expanding. Since ancient times, traditional medicine has made use of the medicinal plant *Annona squamosa*. The aim of this research is to evaluate the total phenolic, flavonoid, antioxidant contents and the amino acid, and vitamin profiles of ethanol leaf extract of A*nnona squamosa* L. By using the DPPH free radical scavenging experiment, the antioxidant potential of the ethanol leaf extracts was calculated, and the absorbance was determined at 517 nm. The IC50 value of the leaf was estimated to be 4.24 µg/mL. The Folin Ciocalteu method was used to measure the total phenolic content (TPC) spectrophotometrically. Total flavonoid content (TFC) was measured by aluminium chloride colorimetric assay and Quercetin was used as standard, the absorbance was measured at 520nm. The results showed that *A. squamosa* leaf is a rich source

of phenolics and flavonoid (total Phenolic content: 26.01±1.45mg GAE/g and total flavonoid content: 71.20±35.74 mg QE/g). The high contents of phenolic compounds and flavonoid contents indicated that these compounds contribute to the antioxidant activity. *A. squamosa* can be regarded as promising plant species for natural plant sources of antioxidants with high potential value for drug preparation. The total amount of amino acids was found to be 331.887 uL Several detection and peak resolution were obtained from the HPLC analysis. The result showed the presence of eight amino acids including Asparagine (2.419uL), Threonine (50.828uL), Phenylalanine (37.533uL), Aspartic Acid (144.558uL), Leucine (8.650uL), Iso-Leucine (9.728uL), Glycine (54.060uL) and four vitamins Vitamin K (2.289mg), Vitamin B1 (958.597mg), Vitamin A (13.568mg) and Vitamin E (62.103mg).

Keywords: *Annona squamosa*, Custard Apple, Sugar Apple, Phytochemicals, Antioxidants, Vitamins, Amino Acid

INTRODUCTION

Many communities have long utilised medicinal plants as a means of treating ailments. The potential for little to non-existent negative effects when taken as prescribed makes herbal medications appealing (36). According to WHO survey, traditional medicine serves as the primary source of healthcare for 80% of the population residing in underdeveloped nations. Investigating the chemical components of plants and conducting pharmacological screenings could give us the basis for creating leads for developing of novel agents (28). Many medicinal plants contain natural antioxidants that prevent the negative consequences of oxidative stress. These plants have flavonoids and polyphenols that lower oxidative stress, scavenge free radicals, and may be used as an alternative treatment for a number of severe human diseases (29). Free radicals are extremely reactive substances that can react with proteins, lipids, carbohydrates, and DNA. They can originate from either nitrogen or oxygen. Reactive oxygen species and antioxidants may be out of balance, which can result in oxidative stress and subsequent cellular damage and a host of human diseases, including cancer, ageing, diabetes, ischemia heart disease, arthritis, gastritis, immunosuppression, and neurodegenerative diseases (12)

Worldwide, tropical and sub-tropical climates are home to the cultivation of *A. squamosa*, referred to as custard apple, sweet sop, and sugar apple. Because of its analgesic and

anticancer properties, it has been utilised in traditional medicine (20). Annona squamosa is a member of the Annonaceae family, which has about 2,300 species and roughly 135 genera (32). With 166 species, Annona is the most significant genera in terms of species count. The plant is an evergreen tree with a height range of 3 to 8 metres. The fruits of this plant are 5–10 cm in diameter, with numerous rounded protuberances that might be heart-shaped, conical, ovate, or round. The leaves are lanceolate, measuring 6–17 cm in length and 3–5 cm in breadth. The plant produces smooth, glossy, blackish or dark brown seeds that are 1.3–1.6 cm long (8).

Several biological activities attributed to *A. squamosa* have been linked to compounds like terpenoids, phenols, acetogenins, alkaloids, and steroids that have been identified from various preparations of the plant. The crude extract and compounds extracted from *A. squamosa* are reported to have anticancer, hepatoprotective, antioxidant, and antidiabetic properties (21). the different chemical components that were separated from the plant's stems, leaves, and roots; these included aporphine, coryeline, isocorydine, norcorydine, glaucine, and anonaine. Leaf extract contains 4-(2-nitro-ethyl 1)-1-6-((6-o-β-Dxylopyranosy1-β-D-glucopyranosyl)-oxy)benzene, Benzyltetrahydroisoquinoline, Borneol, and Anonaine). *Annona squamosa's* volatile ingredients. from the essential oil produced by steam distillation, bark was identified and examined using GC/MS. An alkaloid called annonaine, which is contained in the bark, is reported to have several characteristics. Essential oils found in the root are β caryophyllene, α pinene, α-humulene, and α gurjunene. Annotemoyin is one of the active ingredients found in the plant *A. squamosa* Linn's chloroform extract. *Annona squamosa* Linn. aqueous extract contains flavonoids that have been shown to have antibacterial activity. One such substance that demonstrated antitumoral and pesticidal action in vitro is bullatacin. According to reports, the ethanolic extract of leaves and stem has anticancerproperties **(**31**)**. It is thought to be helpful for cancer, diabetes, hyperthyroidism, and heart problems. The root is thought to have strong purgative properties (8). According to (1), the fruit of *A. squamosa* contains hematinic, sedative, stimulant, and expectorant qualities. It is also beneficial in treating anaemia and burning sensations. The seeds can be used to cure hair lice infections (19).

Chemical components found in the bark have cytotoxic effects. It has been demonstrated that the bark's annosquamosins A, B, and C can stop the growth of A2780 ovarian cancer cells and 95-D lung cancer cells (37). Additionally, leaf extract of *Annona squamosa* is

effective against Enterococcus faecalis, Vibrio alginolyticus, Klebsiella pneumoniae, Streptococcus pneumoniae, and Proteus species (27,42).

Figure 1. *Annona squamosa* Linn. (Fruits on a branch)

Source: (26)

Plant Collection and Preparation

From Takum in Taraba State, the fresh leaves and fruits of *Annona squamosa* were collected. The fruit's seeds were taken out. Following a water wash, the leaves were allowed to dry in the shade before being ground into a fine powder using a mortar and pestle and stored in a glass jar.

Preparation of Ethanol Extract of *Annona squamosa*

For this procedure, the method adopted by (41) was used. 500g of ground leaf and seed samples each were weighed into a plastic container, which was then filled with 2000 mL of ethanol (1:4 w/v). The mixture was let to rest for 72 hours, shaking occasionally, before being filtered through muslin cloth and Whatman No. 1 filter paper. In order to prevent the active components from becoming denaturated, the filtrate was concentrated using a hot water bath and a rotary evaporator operating at decreased pressure and 45 degrees Celsius. Before being administered, the concentrate was moved into an airtight container and kept in the refrigerator. In distilled water, it was redissolved prior to delivery.

Determination of Total Antioxidant

The scavenging action of the plant extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined spectrophotometrically at 517nm using Trolox as standard according to the method described by (35) as reported by (41). Initially, the sample serial dilution was

performed to obtain final concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µl/mL solutions from 1.0 mg/mL stock sample. The absorbance was measured for each concentration. Total antioxidant activity capacity (TAC) was calculated as mg/ml of Trolox equivalent (TE) using the regression equation from the calibration curve.

Procedure

Exactly 39.4mg of DPPH was dissolved 1L 80% Methanol to make a 0.1Mm2ml of DPPH solution was pipette into a cuvette followed by 100µL of sample. This was repeated for each concentration It was then mixed thoroughly and incubated at 37 for 20minutes and the absorbance of each was read at 517nm against the reagent blank.

Determination of Total Phenolics Compound

The Folin Ciocalteu (FC) method reported by (18), was used with slight modification as used by (40). During the experiments, the reagents and sample solutions were prepared as follows: The FC reagent was diluted to 1:10 with distilled water just before the experiment. Sodium carbonate (7.5% w/v) was prepared in distilled water.

A calibration curve was obtained by using gallic acid as standard. The TP was calculated from the standard curve prepared by the addition of two milligrams of gallic acid with 10 ml of methanol. Concentrations of 100, 50, 25 and 12.5 µg/ml were prepared from the stock solution. Both 0.5 mL of standards and extract were taken and mixed with 2.5 mL of Folin – Ciocalteu 50% and 2.5 mL of distilled water, after incubated for 5 min at 40 °C. Finally, 2 mL of Na2CO3 solution (7.5%, w/v) was added. The final mixture was shaken and then incubated for 15 min at 40 °C. The absorbance of all standards and samples were measured at 765 nm using UV – Vis spectrophotometer. The results were expressed as mg of gallic acid equivalents (GAE)/g

Estimation of total flavonoids content (TFC)

Flavonoids were determined using the aluminium chloride colorimetric method of (7). Quercetin was used for derivation of the calibration curve. Total flavonoids were expressed as mg/ml quercetin equivalent (QE).

Procedure:

About 10% Aluminium chloride was prepared by dissolving 10g of aluminium chloride in 100ml of distilled water. 1M potassium acetate was prepared by dissolving 98.15g in 1L methanol. 1.5ml methanol was added to a test tube followed by 0.1ml of 10% aluminium

chloride ($AlCl_3$) solution. 0.1ml of 1M potassium acetate (CH_3COOK) was then added, about 0.5ml (500µl) of the diluted sample was added into test tube. It was incubated at room temperature for 30 minutes and the absorbance of the reaction mixture was read at 520nm. The amount of 10% Aluminium chloride was substitute for water by the same amount of water in blank. The concentration of flavonoids in the sample is estimated from the calibration curve.

Analysis of Flavonoid, Amino Acid and Vitamin Profiles of the Ethanol Leaf Extract of *A. Squamosa* Using High Performance Liquid Chromatography (HPLC)

The bioactive compounds were evaluated using a sykam HPLC system connected to a S3250 UV/visible detector.

Statistical analysis

All experimental data analyses were performed using Microsoft Excel 2010. The reported results were presented as means ± standard deviation (SD). Statistical analyses were conducted using SPSS (Statistical Package of Social Sciences followed by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Table 1 Result of percentage of inhibition of leaf extract of *A. squamosa*

Concentration	% inhibition of the Leaf
31.25	37.02
62.50	42.61
125.00	39.61
250.00	41.09
500.00	50.19
1000.00	67.31
	IC50=4.24 µg/mL

From the table above, the IC50 of the ethanol leaf extract of *A. squamosa* was found to be 4.24 with different percentage of inhibition on different concentration.

The EC_{50} value which is the concentration of the antioxidant required to lower the initial DPPH concentration by 50%, is frequently used to describe the antioxidant activity (6,38,25).

Table 2. Result of Total Flavonoid Content and Total Phenolic Compound

PARAMETERS	TFC (mg QE/g)	TPC (mg GAE/g)
Leaf	71.20±35.74[a]	26.01±1.45[a]

Results represent mean ± standard deviation of group results obtained. Values in the same row with different superscript are statistically significant ($p < 0.05$).

mgEqAG: milligram equivalent of gallic acid; mg QE/g: milligram equivalent of quercetin

From table 1.2, the result showed that the total flavonoid content was significantly ($p < 0.05$) higher compared with the total phenolic compounds. When it comes to plant-based antioxidants, phenolic chemicals are thought to be quite significant. Increased antioxidant activity is frequently linked to a higher phenolic content, which is good for human health. The biological properties of phenolic compounds, such as their antibacterial, anti-inflammatory, and anticancer properties, have been extensively researched. The antioxidant capacity of flavonoids and polyphenolic substances is attributed to their well-known ability to scavenge free radicals. These substances function as hydrogen donors, reducing agents, and singlet oxygen quenchers, all of which contribute to their enhanced antioxidant qualities (2). The total phenolic content of medicinal plants and its antioxidant capacity are frequently assessed using the Folin–Ciocalteu (F–C) redox method and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. These techniques are quick, easy, and yield accurate results (15). The presence of phenolic compounds as antioxidant agents may lessen lipid peroxidation by regulating the generation of reactive oxygen species (4). Phenolic substances stabilise cell membrane networks and prevent the production and expression of inflammatory cytokines such as TNF-α, TGF-β, and several interleukins (IL-6, IL-2, and IL-8) (34).

Due to their antioxidant properties, flavonoids are especially beneficial in preventing cardiovascular disease, some types of cancer, and age-related cellular component deterioration. As a result of their polyphenolic composition, they can scavenge harmful free radicals such hydroxyl and super oxide (10). In experimental animal models, a range of dietary plant flavonoids prevent the growth of tumours (17).

Table 3 Vitamin Profile of Ethanol Leaf Extracts of *A. Squamosa*

Retention. Time [min]	Amount [mg]	Amount% [%]	Compound Name
1.815	2.289	0.2	Vitamin K
2.478	958.597	92.5	Vitamin B1
2.728	13.568	1.3	Vitamin A
5.687	62.103	6.0	Vitamin E
Total	1036.557	100.0	

Table 3 revealed the amount of each vitamins present in ethanol leaf extract of *Annona squamosa*. The table showed the amount of each vitamins presence with their retention times (min) and absorbances. Vitamin B1 (t_R = 958.597mg) was found to be the most prominent (519.113mg) while Vitamin K was found to be the least (2. 289mg) vitamin present in the leaf.

Vitamins are wide a group of organic compounds that are required for normal body function. Today there is increased interest in nutritionally rich foods that are either natural or minimally processed. (3)

Vitamin K is traditionally connected with blood coagulation, since it is needed for the posttranslational modification of 7 proteins involved in this cascade. However, it is also involved in the maturation of another 11 or 12 proteins that play different roles, encompassing in particular the modulation of the calcification of connective tissues (24) Vitamin K acts as a cofactor in the conversion of glutamate into Gla. Gla-containing proteins (MGP and osteocalcin) regulate many anticalcification and bone-forming processes in the body, which are dependent on vitamin K in order to be produced. Low levels of vitamin K impair activation of osteocalcin and decrease the activity of osteoblasts (cells important for building bone) (5). Low vitamin K status (indicated by undercarboxylated MGP) is associated with increased vascular calcifications, and these levels can be improved by effective vitamin K supplementation (39,11)

Scientific evidence suggests that Vitamin K also has anti-inflammatory activity, a vital component against various chronic aging diseases (33). Vitamin K inhibits the activation of the nuclear factor kappa B (NF-κB) and thus decreases the production of proinflammatory cytokines. Vitamin K is significantly and inversely related to individual inflammatory

biomarkers and inflammatory processes due to its anti-inflammatory effects (13). Vitamin A is essential for human existence and well-being. It serves as vital component for several physiological functions, such as reproduction, immune system modulation, target tissue growth and differentiation, and retinal function. A lack of vitamin A increases the risk of serious infections, a wide range of illnesses, and pathological abnormalities, such as problems with the immune system, bones, eyes, and epithelium (23). Alpha-tocopherol, also known as vitamin E, is a fat-soluble antioxidant and necessary micronutrient that could potentially shield tissues against uncontrolled lipid peroxidation (14). Vitamin E prevents cardiomyocyte apoptosis, which promotes cardiovascular health. Furthermore, under conditions of food preparation and storage, vitamin E provides considerable protection against lipid peroxidation. In order to prevent off-flavours and colour changes in food contents, vitamin E stabilises the lipid components of oils, fats, and active packaging (9).

Table 4 Result of Amino Acid Profile of Ethanol Leaf Extracts of *A. Squamosa*

Retention. Time [min]	Amount [uL]	Amount% [%]	Compound Name
0.390	2.419	0.7	Asparagine
1.768	50.828	15.3	Threonine
2.417	37.533	11.3	Phenyl Alanine
2.945	144.558	43.6	Aspartic Acid
3.755	8.650	2.6	Leucine
4.080	9.728	2.9	Iso-Leucine
4.755	54.060	16.3	Glycine
6.190	24.110	7.3	Glutamine
Total	331.887	100.0	

The table above revealed the amount of each amino acids presence with their retention times (min) and absorbances. Aspartic acid was found to be the most prominent (144.558uL) while asparagine was found to be the least (2.419uL) amino acid present in the seed. Amino acids are a class of important biomolecules that contain both amino groups ($-NH3+$) and carboxylate groups ($–COO^-$) (22). Amino acids are the building blocks of peptides and proteins. Amino acids are essential component needed for human health. They are typically present as free or bound into protein backbones, the

determination of amino acids is important to evaluate the nutritional value of protein-containing foods (30).

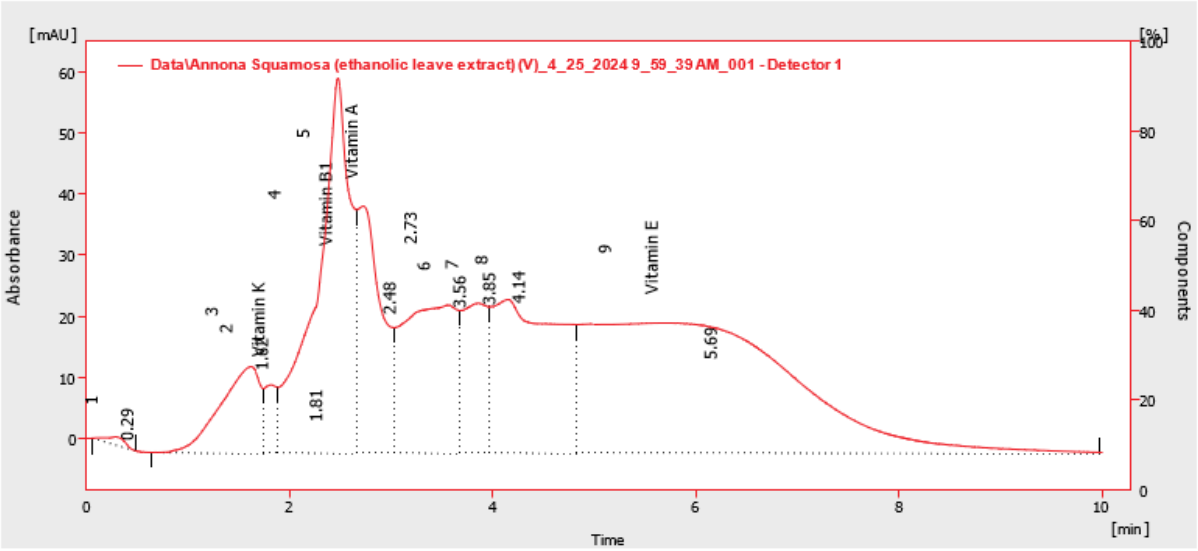

Figure 2. Chromatogram of the Vitamins profile for ethanol Leaf extract *Annona squamosa*

The chromatogram for ethanol leaf extract *Annona squamosa.* The chromatogram showed all the amino acids present in the leaf with their retention times (min) and absorbances

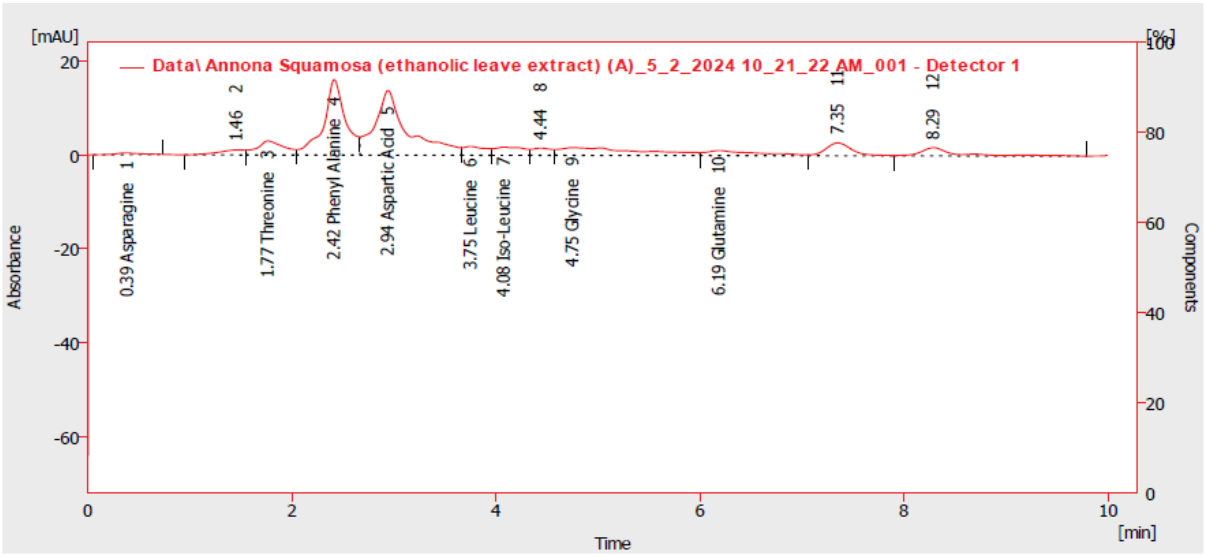

Figure 3. Chromatogram of the Amino Acids profile of ethanol leaf extract *Annona squamosa*

The chromatogram for ethanol leaf extract *Annona squamosa*. The chromatogram showed all the amino acids present in the leaf with their retention times (min) and absorbances

CONCLUSION

This study revealed the phenolic and flavonoid spectrum of *Annona squamosa* leaf. The high contents of phenolic compounds and flavonoid contents indicated that this plant contribute to the antioxidant activity. *A. squamosa* can be regarded as promising plant species for natural plant sources of antioxidants with high potential value for drug preparation. *A. squamosa* leaf may likely be used as an ingredient in the nutraceutical and food/nutrition industry due to the presence of these vitamins and amino acids benefiting human health.

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