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

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Abstract

The aim of this research is to analyse the flavonoid, amino acid, and vitamin profiles of ethanol seed extract of *Annona squamosa* using high performance liquid chromatography (HPLC). The fruits of *Annona squamosa* were collected from Takum, Taraba State, the seeds were removed from the fruit washed with water, dried in the shade and then pulverized into fine powder. For the crude extraction, the method adopted by Yakubu *et al.* (2020) was adopted for this protocol. The IC50 of the seed was found to be 3.87. The total flavonoid content was significantly higher (182.76±0.61) compared with the total phenolic compound (70.34±3.04). A reliable detection and peak resolution were obtained from the HPLC analysis. The result showed the presence of nine amino acids, which include; Threonine (9.6%), Phenyl Alanine (17.0%), Aspartic Acid (26.0 %), Glutamic acid (16.0%), Leucine (5.7%), Iso-Leucine (5.3%), Glycine (5.0 %), Arginine (2.3), Glutamine (13.2%), three vitamins; vitamin K (0.4%), vitamin B1(63.0 %) and B3 (36.6%), and three flavonoid compounds Trolox (0.2%), Quercetin (2.1%) and Coumaric acid (97.7%) in plant sample. The phytochemical study confirms that the seed of *Annona squamosa* rich sources of amino acids such as and these herbal raw materials can exhibit a wide range of pharmacological activities.

Keywords: Phytochemicals, Antioxidants, Amino Acids, Vitamins

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INTRODUCTION

The sweetsop or custard apple tree (*Annona squamosa*) is a tiny shrub belonging to the Annonaceae family and genus Annona. Custard apple extracts and their active ingredients have been shown in numerous studies to have anti-inflammatory, analgesic, and antioxidant properties (26). Various parts of the *Annona squamosa* plant, including its bark, roots, leaf, stem, fruit, peel, and seeds, have been extracted and used in traditional pharmacological applications across various nations to treat a range of illnesses, including fever, tumors, dysentery, hemorrhage, and epilepsy (10). *Annona squamosa* seed powder is used to get rid of lice, leaf extract is used to cure ulcers and boils, and the fruit can be utilized to treat tumors and function as a sedative in heart disease situations (2). Custard apple has been found through phytochemical investigations to include a variety of phenol-based chemicals, including as proanthocyanidins, and 18 distinct phenolic compounds, most of which are flavonoids or alkaloids (15).

Annona squamosa is a semideciduous tree that can grow to a height of 3 to 8 meters. It is primarily resistant to drought. Fruits attract attention mostly because of their fragrant and sweet taste. Fruits have thick skins (rinds) with knobby, hexagonal portions. They are ovoid to conical in shape, 8–10 cm in diameter and length, and weigh 1-2 kg. Fresh fruit is consumed from the pulp of ripe, mature fruits. Astringent and inedible, unripe fruit (4). Different tribes in Nigeria have given this plant a variety different name. Thus, it is called Madodo or wiwi in Jukun, Gwanda daji or Fasa dabur in Hausa.

Because it grows and produces poorly in areas with frequent rainfall, *Annona squamosa* is perhaps the most drought-tolerant species in the Annonaceae family. More than 700 mm of annual rainfall is beneficial for its growth. For custard apples, a pH of 6.0 to 6.5 is optimal. It may grow in a variety of soil conditions, including clay, loams, and sandy soil (20). The pulp of ripe the fruit is consumed raw or added to ice cream and milk-based drinks as flavouring agent (33). Pseudomonas aeruginosa and Escherichia coli are actively inhibited by Annona squamosa. *Annona squamosa* seeds contain the antibacterial components cholesteryl glucopyranoside, squamocin, annotemoyin-1, and annotemoyin-2 (20).

Custard apple pulp has a sugar concentration of up to 28%, with sucrose (2.53%) making up the majority of the sugar content. Other sugars include dextrose (5.05%), laevulose (0.04%), and other rich, aromatic flavours. According to Patel et al. (18), it has notable

amounts of vitamin C, iron, calcium, thiamine, amino acid, potassium, carotene, riboflavin, niacin, and ascorbic acid, as well as magnesium and dietary fibre. Custard apples have a moderate glycemic load and a low glycemic index, despite their high sugar content. Among the specific compounds obtained are aliphatic ketones, such as palmitone. Purines and organic acids including octanoic and hexanoic acid (27).

Nowadays, phenolic compounds' natural anti-inflammatory, antibacterial, anticarcinogenic, and antioxidant properties are much sought-after in terms of application and research. The chemical structure of phenolic compounds is common and consists of an aromatic ring with one or more hydroxyl substituents. These compounds can be classified into many classes; flavonoids, phenolic acids, tannins, stilbenes, and lignans are the primary families of phenolic compounds (3). These phenolic compounds often have much to do with the plant's defensive mechanisms. Nevertheless, phenolic metabolites are crucial for other processes, such as adding pigments for defence against herbivores, incorporating attractive substances to speed up pollination, and having antibacterial and antifungal properties (1,12). Flavonoids, which are phenolic chemicals, have been identified in more than 8000 different vascular plant species. The majority of flavonoids that are consumed are broken down into different phenolic acids, some of which are still capable of scavenging free radicals (19). Fruits, herbs, stalks, cereals, nuts, vegetables, flowers, and seeds are the most common places to find them (9,25). As anticancer, antibacterial, antiviral, antiangiogenic, antimalarial, antioxidant, neuroprotective, antitumor, and anti-proliferative medicines, flavonoids have been widely utilised (32). The building blocks of protein synthesis are amino acids. They are components of the cell's structure and sources of energy required for healthy cell division, growth, and function. A variety of clinical problems, such as metabolic diseases, cardiovascular diseases, immunological diseases, and cancer, have been associated with abnormalities of amino acid metabolism (13). Amino acids play crucial roles in human nutrition as either a source of nutraceutical substances or as necessary dietary components. They also function as precursors for numerous primary and secondary metabolites (31).

MATERIALS AND METHODS

Plant Collection and Preparation

The fresh leaf and fruits of *Annona squamosa* were collected from Takum, Taraba State. The seeds were removed from the fruit. The leaf and the seeds were washed with water, dried in the shade and then pulverized into fine powder using traditional mortar and pestle, and store in a glass container.

Preparation of Ethanol Extract of *Annona squamosa*

The method adopted by Yakubu *et al.* (2020) was adopted for this protocol. 500g of pulverized sample each of leaf and seed was weighed into a plastic container and filled with 2000 mL ethanol (1:4 w/v) and was allowed to stand for 72 h with occasional shaking, thereafter, filtered with muslin cloth followed by Whatman No. 1 filter paper. The filtrate was concentrated using rotary evaporator under reduced pressure and concentrated with hot water bath at 45^{o}C to avoid denaturation of the active ingredients. The concentrate was transferred into air-tight container and preserved in the refrigerator prior to administration. Before the administration, it was re-dissolved in distilled water.

Determination of Total Phenolics Compounds (TP)

The Folin Ciocalteu (FC) method reported by Lachman *et al.* (2000), was used with slight modification as used by Yakubu *et al.*, (2014). 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.

Procedure

Exactly1ml of the sample was added to test tube containing 0.5ml Follin Ciocalteu reagent. After 5 minutes, 1.5ml 7.5% sodium carbonate was added. The total volume made up to 10ml using methanol water. The mixture was incubated at room temperature for 2 hours. The absorbance was taken at 750nm against blank. The results were expressed as GAE. The reactions were conducted in triplicates and absorbance of the sample was measured (11,36)

Estimation of total flavonoids content (TFC)

Flavonoids were determined using the aluminium chloride colorimetric method of Chang *et al.* (2002). 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 (5).

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 Singleton *et al.*, (2002) as reported by Yakubu *et al.* (2014). 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 (29,36).

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

HPLC analysis was carried out on the seed extracts using sykam HPLC machine (S3250 UV/visible detector).

RESULTS AND DISCUSSION

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

Concentration	% inhibition of the Seed
31.25	30.76
62.50	33.10
125.00	31.52
250.00	38.91
500.00	58.12
1000.00	85.28
	IC50=3.87

From the table above, the IC50 of the ethanol seed extract of *A. squamosa* was found to be 3.87 with different percentage of inhibition on different concentration.

Table 2. Result of total flavonoid and phenolic contents

PARAMETERS	TFC (mgEqQe)	TPC (mgEqAG)
Seed	182.76 ± 0.61^{a}	70.34 ± 3.04^{b}

* 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; mgEqQe: milligram equivalent of quercetin

From table 2, the result showed that the total flavonoid content was significantly ($p < 0.05$) higher compared with the total phenolic compounds. The polyphenolic chemicals found in significant amounts in Annona species are antioxidants that may aid in the prevention of oxidative stress-related disorders such as hepatoxicity (14). Due to their ability to scavenge free radicals, these compounds—which are highly significant plant constituents—have

attracted a lot of attention due to their potential antioxidant properties. The antioxidant properties may help prevent long-term health issues such cancer, heart disease, inflammation, and neurological illnesses (38).

Table 3. Flavonoid Profile of Ethanol Seed Extract *Annona Squamosa*

Fractions	Retention. Time [min]	Amount [ppm]	Amount% [%]	Compound Name
	1.775	4.419	2.1	Quercetin
	2.053	0.345	0.2	Trolox
	2.362	203.798	97.7	Coumaric Acid

Table 3 showed the amount of each amino acids present in ethanol seed extract of *Annona squamosa*. The table revealed the amount of flavonoid presence with their retention times (min) and absorbances. Coumaric acid was found to be the most prominent (203.798ppm) while Trolox was found to be the least (0.345ppm) flavonoid present in the seed. Fruits and vegetables are a great source of quercetin, a naturally occurring flavonoid compound. Numerous studies suggest quercetin may be used therapeutically to treat and prevent a number of illnesses. Equivalent to α-tocopherol, the most active form of vitamin E, Trolox is a synthetic water-soluble analogue that is used as a standard chemical to compare the antiradical activity of food in the form of Trolox equivalent antioxidant capacity (TEAC) (30).

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

Reten. Time [min]	Amount [uL]	Amount% [%]	Compound Name
1.795	145.006	9.6	Threonine
2.387	257.519	17.0	Phenyl Alanine
2.948	394.862	26.0	Aspartic Acid
3.233	243.015	16.0	GLutamic Acid
3.742	86.385	5.7	Leucine
4.060	79.914	5.3	Iso-Leucine
4.745	75.194	5.0	Glycine
5.027	34.972	2.3	Arginine
6.178	200.518	13.2	Glutamine

Table 4 showed the amount of each amino acids present in ethanol seed extract of *Annona squamosa*. The table revealed the amount of each amino acids presence with their retention times (min) and absorbances. Aspartic acid was found to be the most prominent (394.862uL) while arginine was found to be the least (34.972uL) amino acid present in the seed. One significant class of molecules with biological activity is free amino acids. In addition to serving as the building blocks of proteins and polypeptides, certain amino acids have also been shown to have specific roles in the central nervous system and to be antioxidants that are necessary for the maintenance of immunity, growth, metabolism, and reproduction (6,16). Arginine, cysteine, glutamine, leucine, proline, and tryptophan are all examples of essential amino acids (35,8). Essential amino acids valine, leucine, and isoleucine are powerful nutritional signalling agents that control the synthesis of mammalian protein (22).

Table 5. Vitamin Profile of Ethanol Seed Extracts of *A. Squamosa*

Retention. Time [min]	Amount [mg]	Amount% [%]	Compound Name
0.372	0.000	0.0	
1.805	2.958	0.4	Vitamin K
2.377	519.113	63.0	Vitamin B1
2.952	301.666	36.6	Vitamin B3

Table 5 showed the amount of each vitamins present in ethanol seed extract of *Annona squamosa*. The table revealed the amount of each vitamins presence with their retention times (min) and absorbances. Vitamin B1 (t_R = 2.38, peak 2) was found to be the most prominent (519.113mg) while Vitamin K was found to be the least (2.958mg) vitamin present in the seed. The vitamin contents of the *Annona squamosa* seed extract showed the presence of vitamin K (2.958 mg), B1 (519.113 mg) and B3 (301.666 mg). HPLC analysis of *Annona squamosa* seed revealed a valuable vitamin B1 concentration of 63.0%. The human body uses these vitamins for a variety of functions, including immune system function, bone formation, skin health maintenance, wound healing, and connective tissue strengthening. Vitamin B1 and B3 function as cofactors for different enzymes involved in oxidation-reduction reactions and carbohydrate metabolism (7).

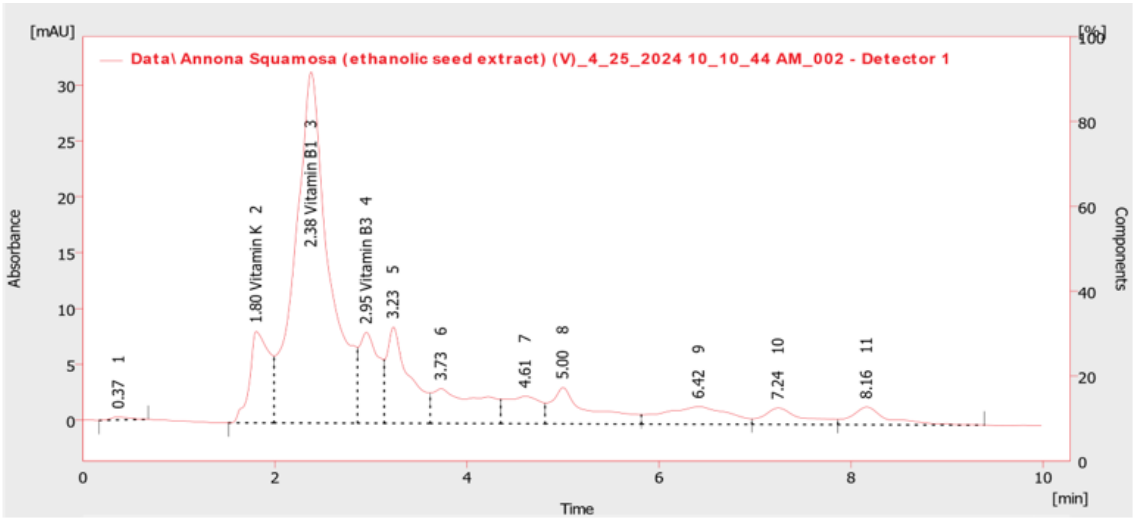

Figure 1. Chromatogram of the Vitamins profile for ethanol seed extract *Annona squamosa*

The chromatogram for ethanol seed extract *Annona squamosa*. The chromatogram showed three (3) different peaks with their retention times (min) and absorbances of each Vitamins. Vitamin B1 (t_R = 2.38, peak 2) was shown to be the most prominent Vitamin present in the seed.

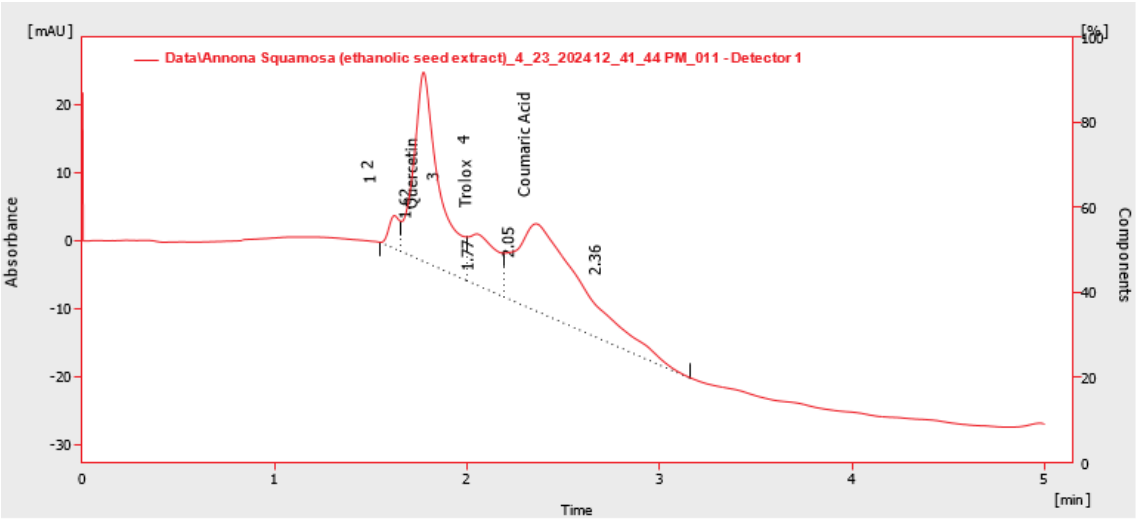

Figure 2. Chromatogram of the flavonoid profile of ethanol seed extract *Annona squamosa*

The chromatogram for ethanol seed extract *Annona squamosa*. The chromatogram showed three (3) different peaks with their retention times (min) and absorbances of each flavonoid. Coumaric acid (t_R = 2.38, peak 2) was shown to be the most prominent flavonoid present in the seed.

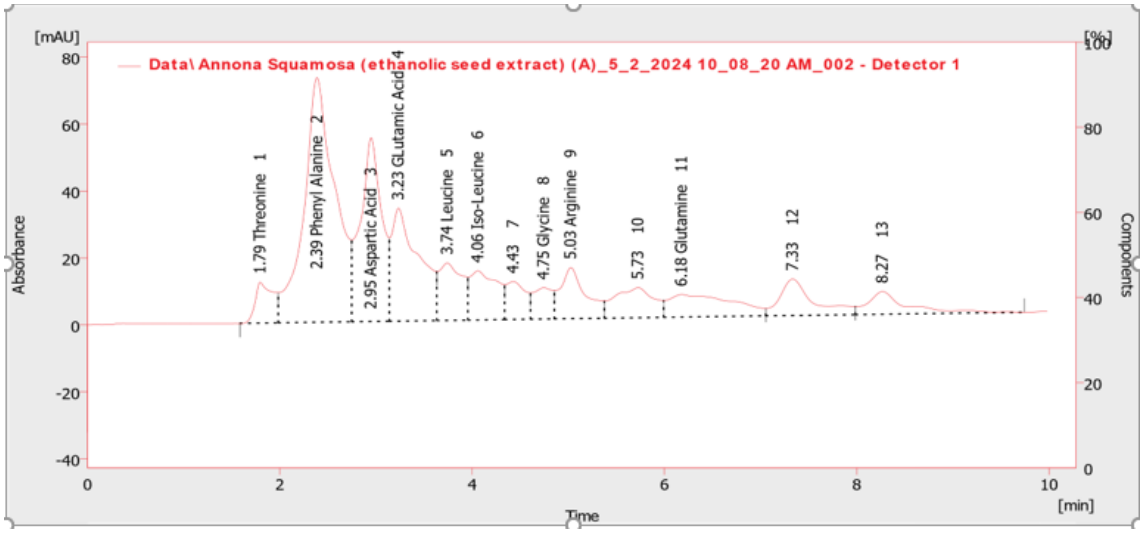

Figure 3. Chromatogram of the amino acid profile of ethanol seed extract *Annona squamosa*

The chromatogram for ethanol seed extract *Annona squamosa.* The chromatogram showed nine (9) different peaks with their retention times (min) and absorbances of each amino acid. Aspartic acid (t_R = 2.95, peak 3) was shown to be the most prominent amino acid present in the seed.

CONCLUSION

Extensive beneficial and pharmacological research has been conducted on *A. squamosa*, a tropical fruit tree. In the food sector, it plays a significant role. The bioactive constituents of the seed of *Annona squamosa* could be potential source of nutraceutical and flavouring agents. These results revealed that *Annona squamosa* seed may serve as viable food supplements to benefit human health due to the presence of these bioactive compounds. The seed also contains a valuable amount of phytochemicals and antioxidant which could be used in the treatment of diseases relating to oxidative stress.

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