World Journal of Advanced Research and Reviews, 2020, 07(03), 096–109

World Journal of Advanced Research and Reviews

e-ISSN: 2581-9615, Cross Ref DOI: 10.30574/wjarr

Journal homepage: https://www.wjarr.com

(RESEARCH ARTICLE)

Studies on standardization of a useful medicinal plant: *Annona muricata* Linn (Annonaceae)

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Publication history: Received on 01 June 2020; revised on 10 September 2020; accepted on 12 September 2020

Article DOI: https://doi.org/10.30574/wjarr.2020.7.3.0179

Abstract

Standardization of leaf, stem and root of *Annona muricata* (Annonaceae) were carried out to determine its pharmacognostic profile. The evaluation of the analytical standards as well as the preliminary phytochemical analyses of these parts was also carried out. The macromorphological and microscopical studies of the different parts revealed features that can be used for proper identification of the plant. The analytical standards determined were found to be: total ash 10.93, 10.23 and 10.87% for leaf, stem and root respectively; acid-insoluble ash 1.83, 1.98 and 1.70% for leaf, stem and root respectively; water-soluble ash 1.80, 1.85 and 3.75% for leaf, stem and root respectively; sulphated ash 7.3, 6.4 and 5.42 % for leaf, stem and root respectively. The alcohol-soluble extractive for the leaf, stem and root were 2.50, 7.50 and 5.00% respectively while the water soluble extractive for leaf, stem and root were 7.50, 2.50 and 10.00% respectively. The moisture content for the leaf, stem and root were 6.50, 5.50 and 4.50% respectively. The result of the phytochemical analysis revealed the presence of alkaloids, glycosides, tannins, terpenoids, reducing sugars, carbohydrates, saponins, flavonoids and steroids in all the parts studied. Resins are present only in the leaf and stem while oils are present only in leaf. These findings are of importance in the establishment of diagnostic indices for the identification and standardization of the plant.

Keywords: Pharmacognostic investigation; *Annona muricata*; Standardization; Macromorphological,

1. Introduction

Standardization is one of the essential means of evaluation of crude drugs, which eventually enters the market. It attempts to establish some standards for determining the overall quality and strength of a given crude drug and the contaminants [1]. According to WHO [2; 3; 4], standardization and quality control of herbals is the process involved in the physicochemical evaluation of crude drug covering aspects, such as selection and handling of crude material, safety, efficacy and stability assessment of finished products. It also involves the documentation of safety and risk based on experience and product information provision to consumer and product promotion. The pharmacopeia and other official compendia have defined standards for crude drug in terms of appearance, numerical constants, and active constituents' strength.

Annona muricata L. (Annonaceae) an upright small evergreen tree, 5-6 m high, has glossy dark green leaves and produces a large heart-shaped, edible fruit 15-23 cm in diameter, yellow-green in color, and has white flesh. *Annona muricata is an* indigenous tropical plant in the South and North America, including the Amazon. It is called Brazilian pawpaw, soursop, prickly custard apple, Soursapi. The fruit pulp is used for making drinks and sherbets and, though slightly sour-acid, can be eaten out of hand. Different parts, including the bark, leaves, roots, fruit, and fruit seeds of the graviola tree are used in natural medicine in the tropics having different activities. Numerous bioactive compounds and

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phytochemicals have been found in *Annona muricata*, as scientists have been studying its properties since the 1940s. Its many uses in natural medicine have been validated by scientific research. Studies by different researchers demonstrated that the bark and the leaves had hypotensive, antispasmodic, anticonvulsant, vasodilator, smooth-muscle relaxant, and cardio depressant activities in animals [5; 6; 7]. Several studies have demonstrated that leaf, bark, root, stem, and seed extracts of graviola are antibacterial in vitro against numerous pathogens [8; 9] and that the bark has antifungal properties. *Annona muricata* seeds demonstrated active antiparasitic properties [10], and a leaf extract showed to be active against malaria [11]. The leaves, roots, and seeds of *Annona muricata* demonstrated insecticidal properties, with the seeds demonstrating strong insecticidal activity [12]. In a clinical study, novel alkaloids found in graviola fruit exhibited anti-depressive effects in animals [13]. Annonaceous acetogenins, a novel set of phytochemicals found in *A .muricata* have demonstrated significant Antiparkisonian, antileishmanial, molluscicidal, antiviral, anti-depressive, antitumorous and anticancerous properties, and selective toxicity against various types of cancer cells without harming healthy cells [14; 15; 13;16; 17;18; 19]. Acetogenins have shown selective toxicity to tumor cells at very low dosages as little as 1 part per million [20; 21; 22; 23; 24; 25; 26; 27]. Due to the usefulness and medicinal uses of *A. muricata*, this work was undertaken to standardize its leaves, roots, and stems. The results from this study could be used to prepare a monograph for proper identification, standardization of the plant, and possible inclusion in the Pharmacopoeia.

2. Material and methods

2.1. Collection and identification of plant material

The leaves, roots and stems of *Annona muricata* were collected from Nsukka, Nsukka Local Government Area, Enugu State, Nigeria. The samples were collected and authenticated by Mr. Ozioko, a taxonomist at the International Center for Ethnomedicinal and Drug Development (inter CEDD).

2.2. Preparation of plant materials

The leaves, roots and stems were carefully separated, washed and excess water allowed to drain off. Representative samples of the leaves, roots and stems were kept for microscopic examination, while the stems and roots were allowed to dry under the sun and the leaves were shade dried. The samples were then separately pulverized and stored in separate sample containers from where they were collected and used for analysis. Transverse sections were cut from the representative using sledge microtome. The sections persevered in 70% ethanol until needed for studies.

2.3. Preliminary phytochemical tests

Phytochemical tests were performed on the stems, roots and leaves samples in order to detect the presence or absence of major secondary plant metabolites of pharmacognostic importance which may include: alkaloids, steroids, tannins, saponins, resins, flavonoids, glycosides, oils etc. The procedures adopted for these tests are those outlined by Harborne [28]; Trease and Evans [29].

2.4. Standardization

2.4.1. Macroscopic analysis

The leaves, roots and stem-barks are visually examined. The leaves' macroscopic characters, which include the type of margin, petiole, venation, base, shape, size, etc, were observed and noted. Macroscopic features such as size and shape, surface characters, fracture and texture of the roots and stem-barks were also observed. Finally, the organoleptic properties like colour, odour and taste of the plant materials were noted.

Microscopic examination of powdered materials

Little quantity of the powdered crude drug was placed on a slide and two drops of chloral hydrate was added to moisten the powdered drug. It was covered with cover slip and passed across the flame of Bunsen burner repeatedly until bubbles occur. Then it was allowed to cool. Two drops of glycerin were added for clarity of structures and the slide was viewed under microscope to reveal microscopical characters which were observed and noted [30].

Microscopic examination of transverse sections

The staining method as described by Odoh *et al*., [30] was used. Sledge microtome was for the sectioning of the specimen. The sections were transferred into staining jar and stained in Safranin for 5 min. The Safranin was drained off and sections were washed about times with distilled water. Then 97% of alcohol and absolute alcohol were used to

wash the sections for two times each. The sections were counter stained in 1% fast green for 5 min and washed with absolute alcohol for about three to four times. After that, sections were transferred into a staining jar containing 50/50 alcohol/Xylene and washed until they become clear. Finally, the pure Xylene was used to clear the sections and the Canada balsam mountant was used to mount the sections on the slide.

2.4.2. Determination of analytical standards

The analytical standard determination was carried out according to the method of Odoh *et al*., [31].

Total ash values

A tarred nickel crucible was ignited to a constant weight at a dull red heat, cooled and stored in a desiccator. A 2 g of the powdered material was weighed into the nickel crucible and heated gently until all the moisture had been driven off and the material had been completely charred. The heat was increased until most of the carbon had been completely vaporized after which the material was heated to about 450 0C to make the residue carbon free. The residue was cooled and weighed. The heating and cooling were continued until a constant weight was achieved.

Acid insoluble ash

The total ash gotten from (a) above was transferred to a beaker containing 5ml of dilute 30% hydrochloric acid, heated to boiling on a water bath for 5 min, filtered through ashless filter paper, the beaker and crucible were washed repeatedly with water and the washings were passed through the filter paper until it was free from acid. The filter paper was dried in the oven, folded into a narrow cone and heated at 150 °C until it was completely ashed. The residue was then heated more strongly and after cooling in desiccator, the crucible was re-weighed.

Water soluble ash

A nickel crucible was ignited to a constant weight at 450 °C and reweighed after 2 g had been put into it. The crucible with the drug was ignited at low heat, initially to burn off the carbon content. The heat was gradually increased until all the carbon was burnt off. The crucible was cooled in a desiccator and re-weighed and heating was continued until a constant weight was obtained. The content of crucible was transferred into a small beaker, 25 ml of distilled water was added and the beaker contents were boiled for 5mintues and filtered through an ashless filter paper. The paper together with the residue was dried in the oven and compressed into small or narrow cone. This was then transferred into the crucible as the heating was continued until the ashless filter was eliminated and the weight was noted.

Sulphated ash

A nickel crucible was ignited to a constant weight at a dull red heat in the oven. A 2 g of the powdered plant material was spread over the bottom of the crucible which was reweighed. The material was moistened with diluted sulphuric acid and ignited at low heat initially to burn off the carbon content. The crucible was cooled in a desiccator. More dilute sulphuric acid was added and heating continued to about 800 °C with occasional cooling and reweighing until a constant weight was obtained.

2.4.3. Determination of extractive yield

The determination of water soluble extractive and alcohol-soluble extractive is used as a means of evaluating drug constituent which are not readily estimated by other means. In some cases, the amount of a drug soluble in a given solvent is an index of its purity. The methods of Odoh *et al*., [31] used.

Alcohol soluble extractive

A 5g of the powdered plant material was weighed accurately and was placed in a 250 ml stopper conical flask; 100 ml of 90 % alcohol were added. The stopper was firmly replaced and the contents of the flask were shaken mechanically for 6 h and allowed to macerate for a further 18 h for a total of 24 h and filtered. 20 ml of the filtrate was evaporated to dryness in 25 ml beaker over a water bath. The residue was dried to a constant weight at 105 °C and then weighed.

Water soluble extractive

A 5 g of the powdered plant material was weighed accurately and was placed in a 250 ml stopper conical flask. 100 ml of distilled water was added. The stopper was firmly replaced and the contents of the flask were shaken mechanically for 6 h and were allowed to macerate for a further 18 h for a total of 24 h and then filtered. 20 ml of the filtrate was evaporated to dryness in a 25 ml beaker over water bath. The residue was dried to a constant weight at 105 °C.

2.4.4. Determination of moisture content

A tarred evaporating dish was heated to a constant weight and stored in a desiccators, 2 g of the powdered plant material was added to the dish and kept in an oven maintained at a temperature of 105 °C. It was allowed to dry until a constant weight was achieved. The difference in weight of the evaporating dish was noted.

2.4.5. Chemomicroscopic analysis

The method of Odoh *et al*., [30] was adopted.

Test for cellulose, lignin, starch and suberized wall

A little of powdered drug was placed on a slide and two drops of iodinated zinc chloride solution (20 g of zinc chloride in 8.5 ml of water + 1 g of potassium iodide and 0.5 g of iodine in 20 ml of water) was added to the slide. A cover slip was used to cover the slide and then viewed under a light microscope to observe the individual colour changes.

Test for secretory cells and ducts

A little quantity of powdered drug was placed on a slide and two drops of Sudan III solution (prepared with equal parts of glycerine and alcohol) added. The slide was cover with a cover slip, viewed under a light microscope and the colour noted.

Test for calcium oxalate crystals

A little quantity of powdered drugs was mounted with 80% H_2SO_4 and examined under a light microscope and the colour noted.

Test for tannins

A little quantity of powdered drugs was mounted with ferric chloride solution and examined under a light microscope and the colour noted.

Test for fiber

A little quantity of powdered drugs was mounted with saturated aqueous solution of picric acid and allowed to stand for 5 min. The slide was irrigated with water and examined under a light microscope and the colour noted.

2.5. Statistical analysis of results

Data obtained was analysed using students's t-test. Difference between means were accepted significant at $p<0.05$. Results were presented as Mean ± SEM.

3. Results and discussion

3.1. Results of phytochemical analysis

The phytochemical analysis of *Annona muricata* are as shown in Table 1.

Table 1 Results of the phytochemical analysis of *Annona muricata*

Constituent	**Inference**		
	Leaf	**Stem**	**Root**
Carbohydrates	++	++	++
Reducing sugars	++	+++	++
Alkaloids	+++	+++	+++
Glycosides	+	+	++
Saponins	+	+	+
Tannins	+++	+	+
Flavonoids	+	+	++
Resins	++	_	++
Proteins	++	+	+
Oils	+	_	_
Steroids	+	++	+++
Terpenoids	++	+++	+++
Acidic compounds	_	_	_

Key: + = slightly present, ++ = moderately present, +++ = highly present, - = absence of metabolite.

3.2. Result of the structural standards

3.2.1. Macroscopic analysis of leaf, stem and root of Annona muricata

Leaf

The leaves are dark green and glossy above, dull and paler below obovate to elliptic, smooth, alternate, have short petioles and are oblong-ovate to cylindrical, 14 to 16 cm in length and 5 to 7 cm in width. Simple, distichous, pinnatinerved, without stipules, petiolated, sub membranaceous, elliptic-oblong to obovate, rounded-acute at the base, with entire margins, rounded at the apex and shortly acuminate; glabrous, lustrous and dark green on the adaxial face, sericeous on the abaxial face when young but promptly glabrous and olive-colored, with 5 to 7 pairs of secondary nerves. Domatia are present on the abaxial face as a manner of pockets; they are at the angles formed by the main nerve and the lateral nerves. When crushed the leaves emit a stronger odour.

Root

The root system extensive and superficial, brittle, dark brown in colour, hard, spreading beyond the diameter of the crown although shallow rooted; juvenile plants have a taproot that is eventually lost.

Stem

The stems of *Annona muricata* are rounded, rough and not pubescent with a dark brown colour.

3.2.2. Microscopic analysis of powdered leaf, stem and root

Microscopy of the powdered leaf

The microscopy of the powdered leaf revealed the following features: epidermal cells, prisms of calcium oxalate crystals, fibers, xylem vessels, anisocytic stomata, bundles of fibres with calcium oxalate crystals, starch granules and unicellular triochomes (Figure 1).

Microcopy of the powdered stem

The microscopy of the powdered stem bark revealed the following features: cork cell in the surface view, bundles of fibers, single fibre, prism of calcium oxalate crystals, pitted vessel and scelereids (Figure 2).

Microscopy of the powdered root

The microscopy of the powdered root revealed the following features: bundles of fibers, cork cell in surface view, prisms of calcium oxalate crystal, bundles of fibres with calcium oxalate crystals and single pitted fibre with lumen (Figure 3).

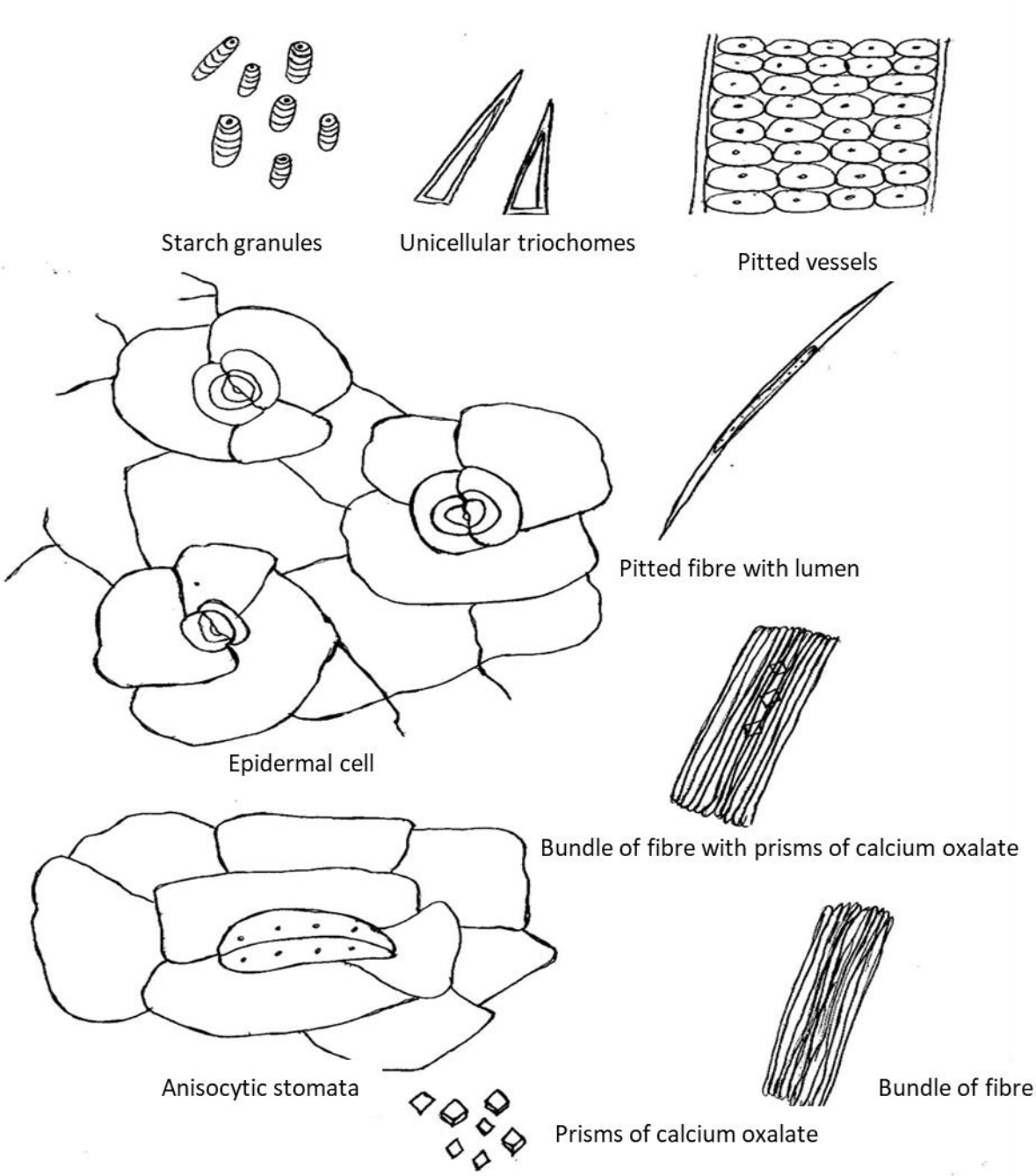

Figure 1 Microscopy of powdered leaf of *Annona muricata*

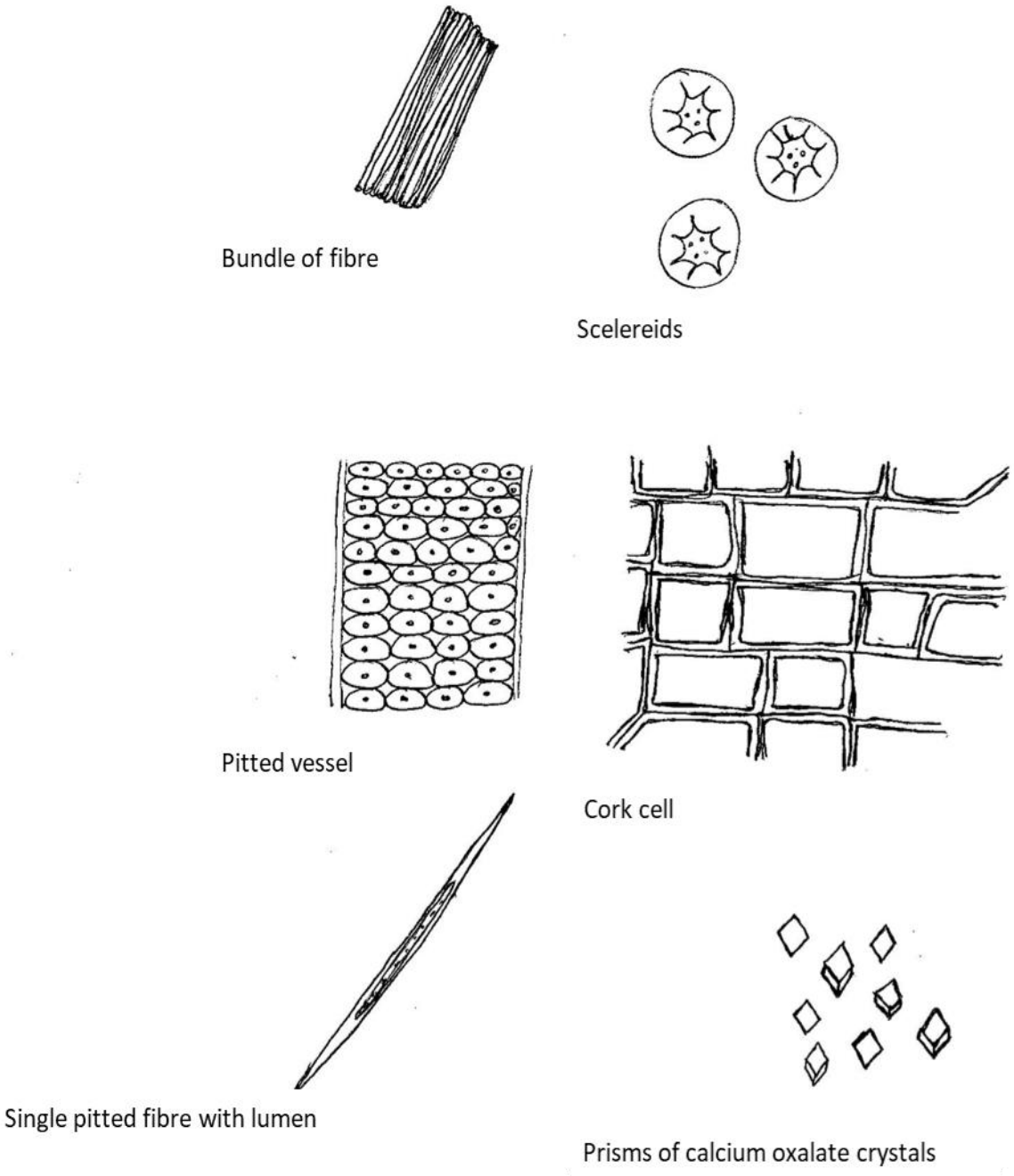

Figure 2 Microscopic of powdered stem of *Annona muricata*

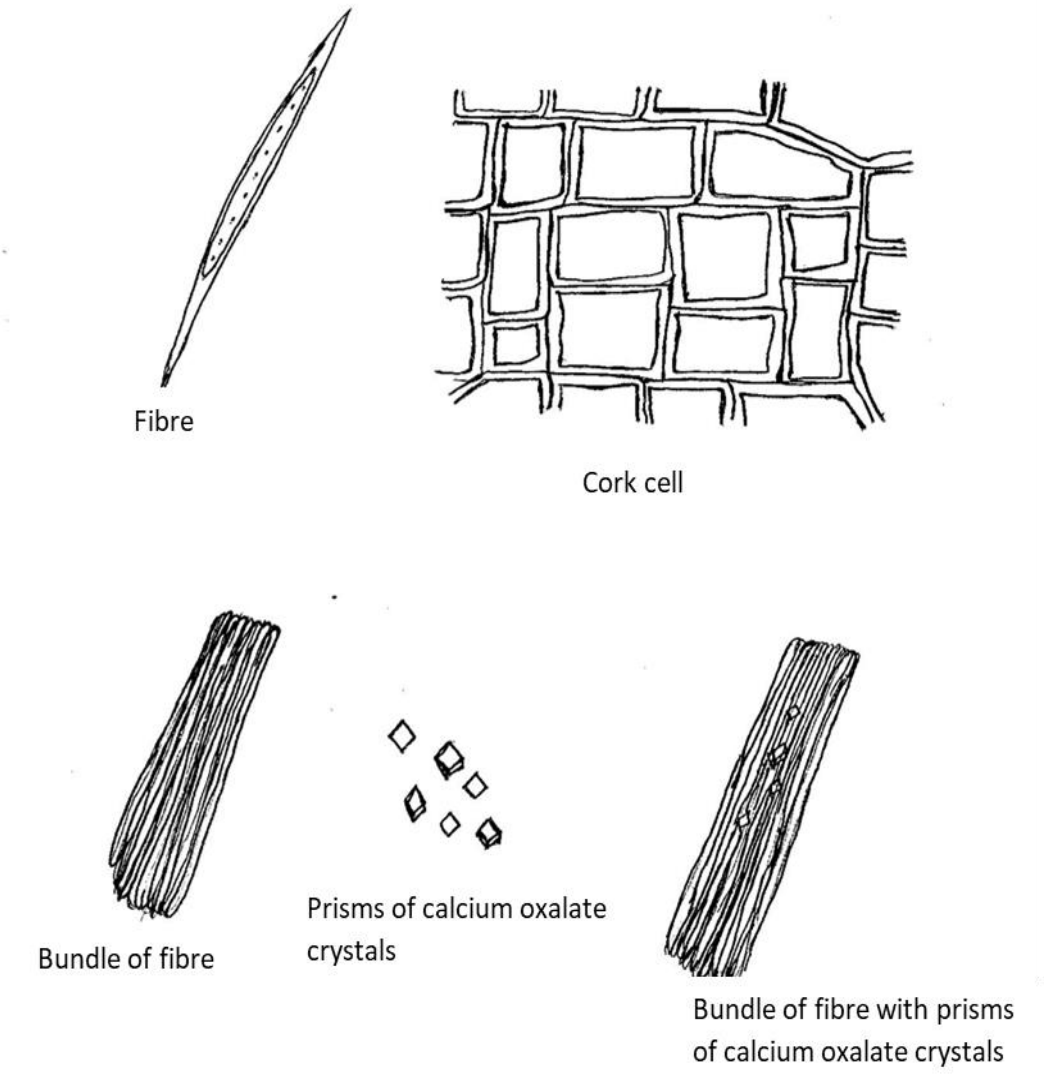

Figure 3 Microscopic of powdered root of *Annona muricata*

3.2.3. Microscopy of transverse section of leaf, stem and root

Transverse section of leaf of *Annona muricata*

The outer most cover is a thin waxy cuticle, followed by a layer of epidermal tissue. The palisade layer comes next followed by a wide zone of spongy mesophyll which has a lot of inter cellular air spaces. This was followed by crystal idioblast, on the outer part of the vascular bundle the phloem tissue is seen while the xylem vessels are at the inner part. The collenchyma, parenchyma and pith were also seen (Figure 4).

Transverse section of the stem of *Annona muricata*

The transverse section of the stem revealed the presence of epidermis. This was followed by the cork and the cork cambium. On the outer part of the vascular bundle the phloem tissue is seen while the xylem vessels are in the inner part. The vascular rays and pith were also seen (Figure 5).

Transverse section of the root of *Annona muricata*

The outer most cover is made up of thick multiple layered cork followed by the cortex and cork cambium (phellogen). Next to the cork cambium is a wide zone of parenchyma tissue. The phelloderm or secondary cortex is derived from the cork cambium. The cork, cork cambium and phelloderm make up the periderm which is the secondary protective tissue. To the inside of the periderm are the derivatives of the vascular cambium and to the inside of the vascular cambium lies

the xylem tissue which is made up of vessels, visible ray lines and a central scelerified parenchyma. Some patches of phloem fibres were seen in the secondary phloem area (Figure 6).

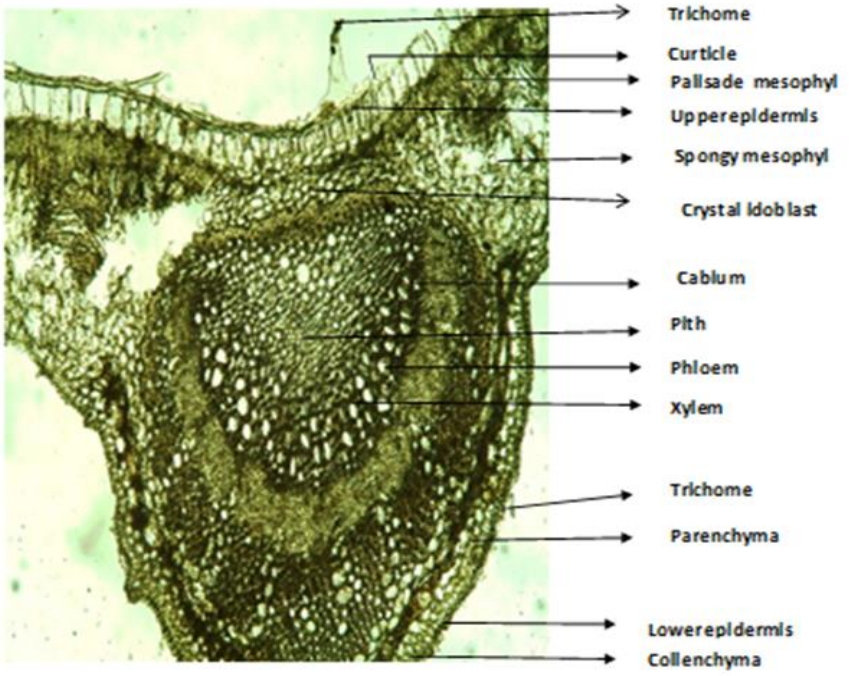

Figure 4 Transverse section of the leaf of *Annona muricata*

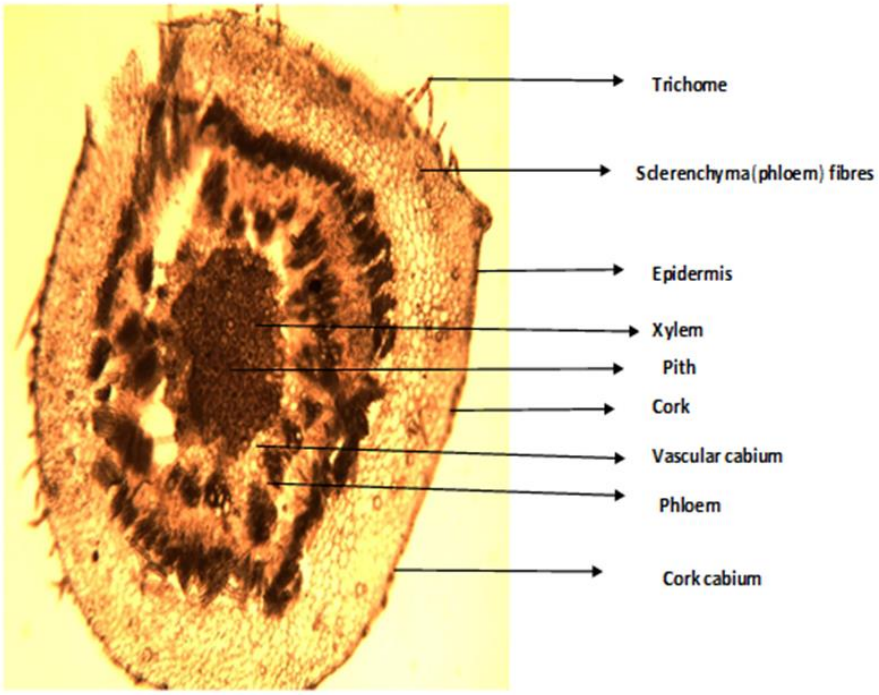

Figure 5 Transverse section of stem of *Annona muricata*

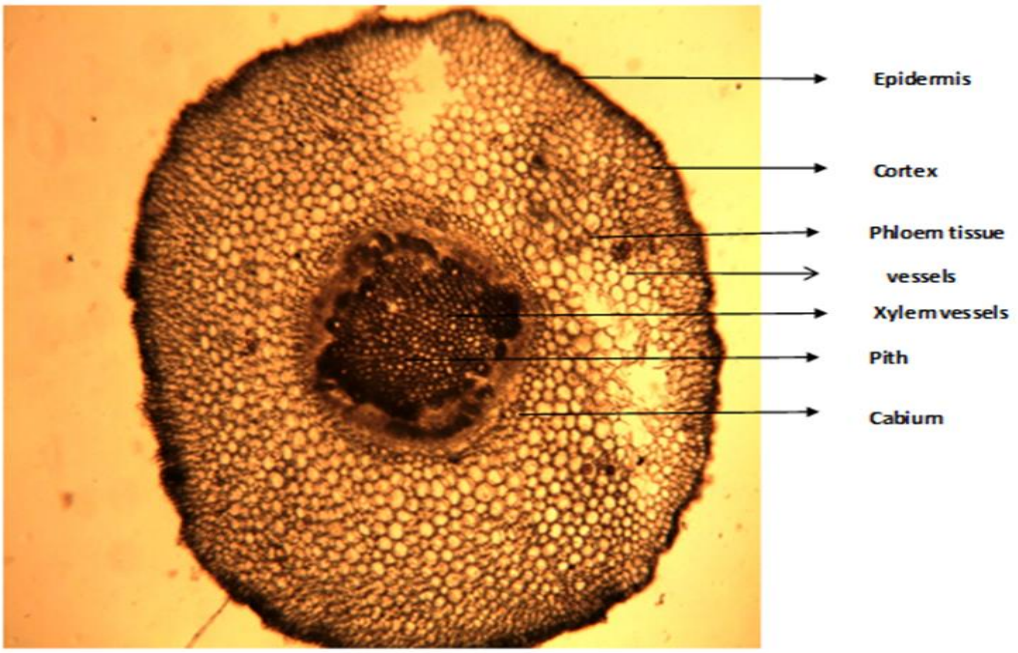

Figure 6 Transverse section of the root of *Annona muricata*

3.3. Result of analytical standards

The results of the analytical standard showed the percentage composition of the important parameters used as quality control tools in standardization of plant drugs (Table 3).

Table 3 Results of analytical standards of the leaf, stem and root of *Annona muricata*

Numerical Constant/ Standard	**Composition (%)w/w**		
	Leaf	**Stem**	**Root**
Total ash	10.93	10.23	10.87
Acid insoluble ash	1.83	1.98	1.70
Water soluble ash	1.80	1.85	3.75
Sulphated ash	7.30	6.40	5.42
Alcohol soluble extractive	2.50	7.50	5.0
Water soluble extractive	7.50	2.50	10.0
Moisture content	6.50	5.50	4.50

Values are ± SEM, n = 5

3.4. Results of chemomicroscopic analysis

The chemical microscopic analysis shows the presence of some features like lignin, starch, suberized walls, secretory cells, tannins, calcium oxalate crystals which are not all present in all the parts (Table 4).

Table 4 Result of the chemomicroscopic analysis of the leaf, stem and root of *Annona muricata*

Test reagent	Observation	Leaf	Stem	Root
Iodinated zinc chloride solution	Blue colour observed on epidermal cells	Cellulose -	Cellulose -	Cellulose -
Iodinated zinc chloride solution	Yellow colouration observed in the xylem vessels	Lignin -	Lignin +	Lignin -
Iodinated zinc chloride solution	Blue-black colouration observed on few grains in parenchyma cells	Starch +	Starch +	Starch+
Iodinated zinc chloride solution	Brown colouration observed	Suberized wall +	Suberized wall +	Suberized wall +
Sudan III solution	Pink-red colouration observed	Secretory cells +	Secretory cells +	Secretory cells +
Picric acid solution	Yellow colouration observed	Fibres +	Fibres +	Fibres +
80% H_2SO_4	Crystals of calcium oxalate dissolved	Calcium + oxalate +++=+=crystals +	Calcium + oxalate ++crystals +	Calcium + oxalate crystals +
Ferric chloride solution	No greenish colour in some parenchyma cells	Tannins +	Tannins -	Tannins +

Key: + = Present, - = Absent

4. Discussion

The identification, examination, and evaluation of the plant *Annona muricata* have been carried out, and the plant's characteristics were determined by the various analysis and tests performed.

Preliminary phytochemical test revealed the various chemical constituents of the leaf, stem and root of *A. muricata.* A systematic study of crude drug embraces through consideration of the secondary metabolites. The samples contain carbohydrates, alkaloids, flavonoids, glycosides, reducing sugars, resins, terpenoids, proteins, tannins, oils, saponins, and steroids for possessing therapeutic values. Flavonoids, one of the phytochemical compounds, are natural products of high pharmacological potency (antiallergic, antiinflammatory, antiviral and antioxidant activities), and are widely distributed in plants [32]. Glycosides, one of the phytochemical compounds detected, is known to increase the heart failure patients' force and contraction for most heart failure patients and thus found to be useful in treating congestive heart failure [33]. Glycosides have also been found useful in the treatment of cancer [34].

The plant's microscopical examination revealed the physical appearance of the morphological parts, which can be seen with the naked eyes. This gives an idea of the plant but cannot be relied on solely to identify the plant. The microscopical examination on the characteristic features found in the plants especially in its different morphological parts, are markers for proper identification. These characteristics features and their arrangement are usually not the same in all morphological parts but some are peculiar to all or few of them. For example, the results of the powdered leaf's microscopy showed prism of calcium oxalate crystals and bundles of fibres, which can be found in all the morphological parts. In contrast, the cork cells can be found in the root and stem, and anisocytic type of stomata occurred only in the leaf. Another necessary diagnostic is the presence of sclereids in the stem of *Annona muricata* which confers hardness and mechanical protection to the plant.

The total ash values can detect foreign organic matters and adulteration of the sand or earth [35]. They are also crucial in quality control within the British pharmacopeia (BP) specification [36]. The determination of ethanol-soluble extractive and water-soluble extractive is used to evaluate drug constituent, which is not readily estimated by other means [37]. In some cases, the amount of a drug soluble in a given solvent is an index of its purity. The crude drug's

moisture content is not too high (falls within the limit of the general requirement of 8 – 14%, indicating less probability of microbial degradation [37]. The results of analytical standards gave values, most of which are within BP limits.

Chemomicroscopy may be used to distinguish cellular structures. The result obtained for the plant shows lignin, tannins, calcium oxalates, fibres, and a Suberized wall, as shown in the table, which may be absent in other plants.

5. Conclusion

From this study that *Annona muricata* possesses phytoconstituents, which accounts for its usefulness as a medicinal plant. Also, the degree of their concentration varies according to the different morphological parts explaining variations in physiological and pharmacological activities. The study also showed that standardization is essential in determining the quality, purity, and strength of the crude drug and adulterants' nature. The analytical standards were determined, and the methods were used adequately within the limits of experimental error. Finally, the plant *Annona muricata* has been identified, evaluated, and standardized and may qualify to be included in official books.

Compliance with ethical standards

Acknowledgments

The authors acknowledged the taxonomist, Mr. A. O. Ozioko with International Centre for Ethnomedicine and Drug Development (InterCEDD) Nsukka, Nigeria for the identification and authentication of the plant material.

Disclosure of conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest.

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