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Comparative evaluation of the phenolic and antioxidant properties of the leaves, root, stem bark, and root bark of *Annona muricata* (Annonaceae)

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Abstract
Oxidative processes can compromise the immune system. The problem posed by oxidative processes is quite challenging in the field of internal medicine and oncology. This study was aimed at evaluating the phytochemical and *in vitro* antioxidant activity of different parts of *Annona muricata*. Methanol extracts of the leaves, root, root bark, and stem bark were analyzed for their total phenolic content, total flavonol content and antioxidant activities using Gallic acid equivalent (GAE), Quercetin equivalent (QE) and DPPH reduction as references. Phytochemical screening and the antioxidant assay were done using standard methods. Phytochemical screening revealed the presence of alkaloids, tannin, phenols, reducing sugar, anthraquinone, saponins, flavonoids, cardiac glycoside, and carbohydrates. The total phenolic content was found to be 74.9, 56.5, 47.6 and 51.5 (% mg/g GAE) for the leaves, root, stem bark and root bark respectively while the total flavonol content gave 365.6, 83.4, 264.8 and 195.6 (µg/mg QE) for the leaves, root, stem bark, and root bark respectively. The IC_{50} was observed to be 4.36, 14.37, 11.72 and 12.25 (µg/ml) for the leaves, root, stem bark and root bark respectively. These results suggest that different parts of *A. muricata* possess high levels of polyphenols and significant antioxidant capacities.

Keywords: *Annona muricata*, phenolic properties, antioxidant properties

1. Introduction
Plants owe their nutritional and therapeutic effectiveness to their metabolic products, this has led to the extensive exploration of various species of plants in order to establish an evidence based report on their phytochemical composition and its relationship to human health. Natural compounds from plants represent a major source of molecules with medicinal properties; amongst the important bioactive principles in plants are the antioxidants and polyphenols. The importance of these compounds is attached to their roles in protecting biological macromolecules from the damaging effects of reactive species. A medicinal plant is a plant that has constituents with similar properties as conventional synthetic drugs which have been used by humans throughout history to either cure or lessen symptoms from an illness. A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. [1]
Since prehistoric times, individuals of all continents have used hundreds to thousands of indigenous plants for treatment of diseases. The declining efficacy of synthetic drugs and the increasing adverse effects as well as contraindications of their use makes the usage of natural drugs topical again. The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine. Examples include the Western medicine with origins in Mesopotamia and Egypt, the Unani-tibb and Ayurvedic systems centred in western Asia and the Indian subcontinent, Chinese herbal medicine, the classical herbal medicine system based on Greek and Roman sources, traditional African medicine, and native American medicines. Ethno botany which is the study of traditional human uses of plants is recognized as an effective way to discover medicines [2]. Polyphenols are micronutrients with antioxidant properties, found abundantly in natural plant food sources such as fruits, vegetables, red wine, cocoa and dried spices. There are over 8000 identified polyphenols found in foods. Polyphenols play an important role in preventing and reducing the progression of diabetes, cancer, neurodegenerative diseases and cardiovascular diseases. They also play an important role as prebiotics, increasing the ratio of beneficial bacteria in the gut which is important for health, weight management and disease prevention. In humans, they help in fighting free radicals, and reduce the appearance of aging, they promote brain health and protect against dementia, and they support normal blood sugar levels, flavonoids and lignins are examples of polyphenols. *Annona muricata* has a long history of traditional use.

Common names include soursop, paw-paw, sirsak, graviola, or guanabana. The fruits are used extensively in preparing syrups, candies, beverages, ice creams and shakes. Different parts of the plant are responsible for a wide array of ethno medicinal activities and indigenous communities in Africa and South America extensively use this plant in their folk medicine. Numerous investigations have substantiated these activities, including anticancer, anticonvulsant and anti-arthrities.

Fig 1: Fruiting top of Annona muricata.

2. Materials and Methods

Sample Collection

The various parts of *A. muricata*, which include the leaves, stem bark and root bark, and root were harvested in the month of september from University of Port-Harcourt, Choba, Nigeria, and were identified and authenticated by Dr. Osuala of the department of pharmacognosy, Faculty of Pharmaceutical Sciences, University of Port-Harcourt, Choba, Rivers State, Nigeria. The voucher specimen was kept in the same institution.

Reagents and Chemicals

The reagents used in the course of the research work were analytical grade and they include; Methanol (Sigma Aldrich), Dragendorff's reagent, Mayer's reagent, Hager's reagent, Fehling's solution A and B, Sodium hydroxide, Hydrochloric acid, Molish reagent, Sulphuric acid, Ferric chloride, Gallic acid (Sigma Aldrich), Quercetin, Folin-Ciocalteu's reagent (Sigma Aldrich), 1,1 –diphenyl- 2-picrylhydrazyl (DPPH), Ascorbic acid (kermel).

Equipment

Apparatus used include analytical weighing balance, ultraviolet visible spectrophotometer (Pye-unicam, UK) water bath, and glass wares.

Extraction of Plant Sample

Fresh leaves, root, stem bark and root barks of the plant were air dried for 14 days after which it was pulverised using mechanical grinder. Three hundred gram (300 g) each of the pulverised plant parts was macerated (cold maceration) in 2.5 liter of methanol for 72 hours and the extracts obtained were dried and properly stored.

The percentage yield was calculated using the formula;

% yield = (weight of extract / weight of crude powder) ×100

Phytochemical Screening

The plant extracts (leaf, root, stem bark and root barks) were subjected to preliminary phytochemical analysis using the method described by Trease and Evans [3].

Free Radical Scavening Assay (Antioxidant Capacity)

Preparation of DPPH solution

5 mg of DPPH was weighed into a 100 ml volumetric flask which was wrapped in foil. 100 ml of methanol was added in aliquots to the reagent with intermittent shaking at intervals. The solution was kept in the dark at an ambient temperature.

DPPH radical scavenging capacity

The DPPH scavenging activity of the leaf, root, stem bark, and root bark extract was measured by colorimetric method [4]. In brief, an aliquot of 2 ml of different concentrations (4-20 µg/ml) of sample solution prepared with methanol was mixed with 2 ml of DPPH solution. The reaction mixture was incubated for 30 minutes in the dark at room temperature. The absorbance of the mixture was measured at 517 nm with a UV spectrophotometer. Methanol was used as blank and DPPH solution was used as control. Ascorbic acid was used as the standard, and all determinations were performed in triplicate. The free radical scavenging activities of the test samples, expressed as percentage of inhibition were calculated using the equation below

Percentage (%) inhibition of DPPH activity = [(AB – AA)/ AB] x 100

Where; AA and AB are the absorbance values of the test and of the control samples respectively [5] Plotting a graph of % inhibition against concentration, the IC_{5O} (which is the inhibitory concentration of test substance required to scavenge 50% of free radicals in a given sample) was determined using the regression equation. The values obtained were used as a measure of antioxidant activity.

Determination of Total Phenolic Content

Total phenolic content was quantified and expressed as Gallic acid equivalent according to the method proposed by Singleton *et al*,. 1965 [6]. The amount of the total phenolics in leaves, roots, stem bark and root bark extracts was determined with the Folin-Ciocalteu's reagent. 1 ml of standard solution of concentration 0.125, 0.25, 0.5 and 1.0 mg/ml of Gallic acid were prepared in methanol. A concentration of 0.1 mg/ml of each plant extract was also prepared in methanol and 0.5 ml of each sample was introduced into test tubes and mixed with 2.5 ml of a 5-fold dilute Folin-Ciocalteu's reagent and 4 ml of 10% sodium carbonate. The tubes were covered with aluminum foil and allowed to stand for 45 minutes at room temperature and the absorbance was read at a wavelength of 760 nm using a UV spectrophotometer. Gallic acid was used as a standard and the total phenolics were expressed as Gallic acid equivalents (GAE mg/g) using this formula;

TPC = GAE × V/M

Where;

TPC = Total content of phenolic compounds (% w/w)

GAE = Gallic acid equivalent (obtained from the standard curve using the regression equation)

V= volume of extract in ml;

M = weight of plant extract in g.

Determination of Total Flavonol Content Using Aluminium Chloride Colorimeter Method

Total flavonol content was quantified and expressed as Quercetin equivalent QE according to the method proposed by Kumaran *et al.* 2011 [7]. Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in methanol and then diluted to 6.25, 12.5, 25, 50, 80, and 100 µg/ ml. A calibration curve was made by measuring the absorbance of the dilutions at 440 nm (λ_{max} of quercetin)

using a UV/Vis spectrophotometer.
In brief, 2.0 ml of 2% $AlCl_3$ in ethanol was added to 2.0 ml of the various extracts. The mixture was incubated at room temperature for 1 hour, after which the absorbance was measured at 440 nm. Appearance of yellow colour indicated the presence of flavonols. Total flavonol contents were calculated as quercetin equivalent (µg/mg) using this formula;
TFC = QE × V/M
Where;
QE = Quercetin equivalence obtained from the standard curve
V = volume of extract in ml;
M = weight of plant extract in mg.

3. Results

Table 1: Percentage Yield

Plant part	Weight of extract (g)	Weight of pulverized powder (g)	Percentage yield (%)
laves	20.30	300	6.77
root	17.20	300	5.73
Root bark	19.25	300	6.41
Stem bark	15.73	300	5.24

Table 2: phytochemical screening results for various morphological parts of *A. muricata*

Phytochemicals	Leaf	Root	Root Bark	Stem Bark
Alkaloid	+	+	+	+
Saponnin	+	-	-	-
Flavonoid	+	+	+	+
Carbohydrate	+	+	+	+
Anthraquinone	+	-	+	-
Phenolics	+	+	+	+
Terpenoids	+	-	-	-
Reducing Sugar	+	+	-	-
Cardiac Glycosides	+	-	-	+

Key; + = Present - = Absent

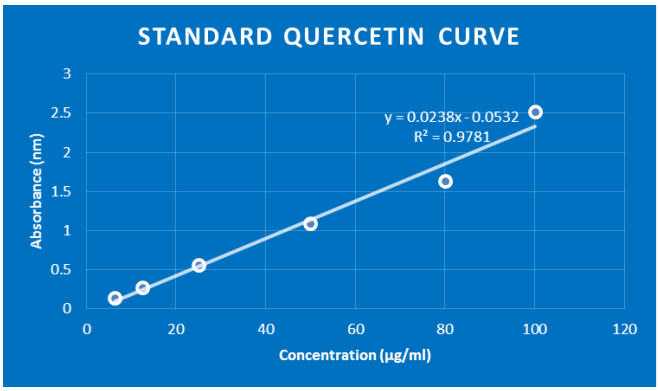

Fig 2: Graph of Absorbance (nm) against concentration (µg/ml) of Quercetin

Table 3: Total flavonoid content various parts of *A. muricata*

S/No	Plant Part	TFC (µg/mg)
1	Root	83.4
2	Root bark	195.12
3	leaves	365.6
4	Stem bark	264.8

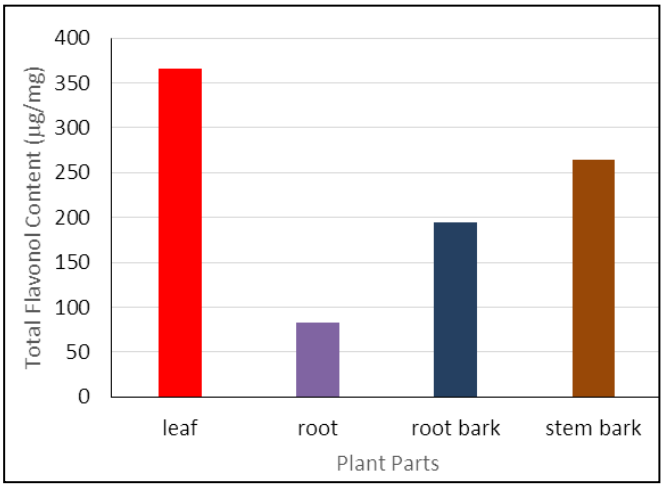

Fig 2: Bar chart representation of the Total flavonol content of various morphological parts of *A. muricata*

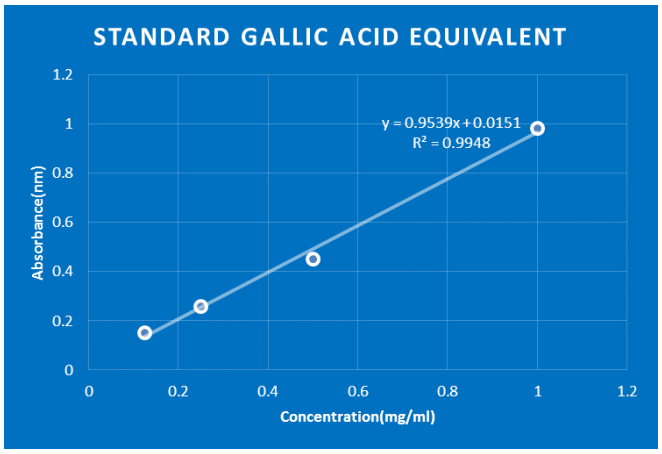

Fig 3: Graph of absorbance (nm) against concentration (mg/ml) of Gallic acid

Table 4: Total phenolic content of various parts of *A. muricata*

S/No	Plant Part	GAE (%mg/g)
1	Leaves	74.9
2	Root	56.5
3	Root bark	51.5
4	Stem bark	47.6

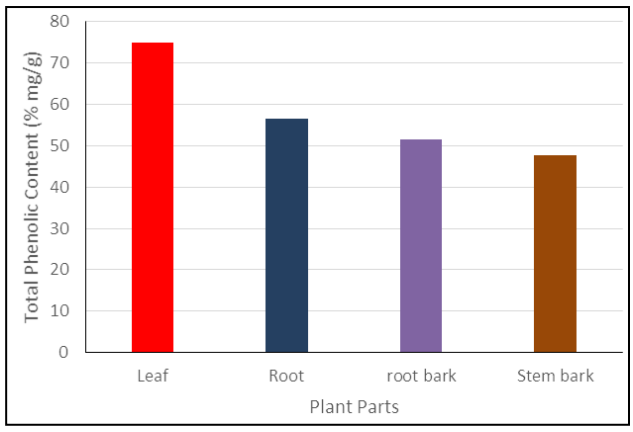

Fig 4: Bar chart representation of the Total phenolic contents of various morphological parts of *A. muricata.*

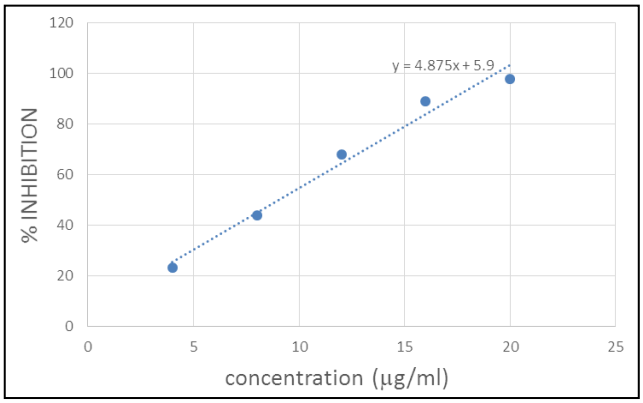

Fig 5: Graph of % inhibition against concentration (µg/ ml) of ascorbic acid

Table 5: DPPH radical scavenging activity of the various parts of *A. muricata*

	% Inhibition of methanol extract of different parts of *A. muricata.*			
Concentration (µg/ml)	**Leaves**	**Root**	**Root bark**	**Stem bark**
4	49.03	20.27	23.50	30.40
8	58.9	30.24	36.50	40.91
12	60.5	40.60	45.00	56.40
16	68.5	61.00	67.60	61.73
20	78.43	63.3	73.30	64.03

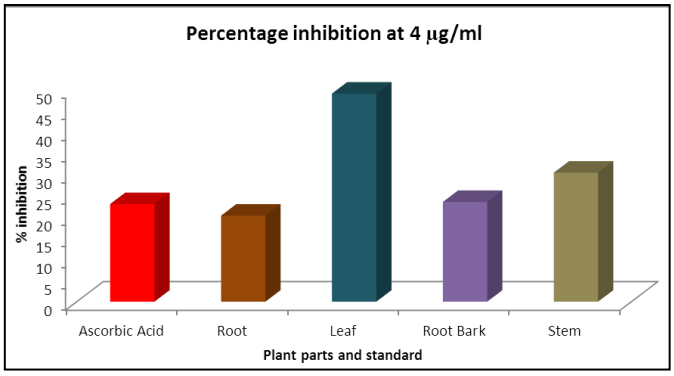

Fig 6: Bar chart representation of % inhibition of ascorbic acid and various parts of *A. muricata* at 4 µg/ml

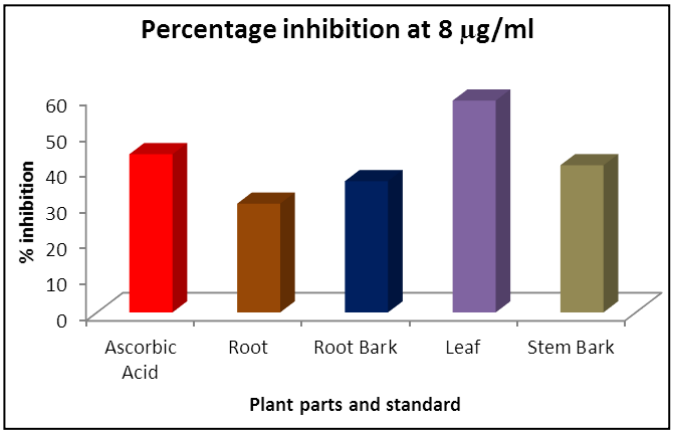

Fig 7: Bar chart representation of % inhibition of ascorbic acid and various parts of *A. muricata* at 8 µg/ml

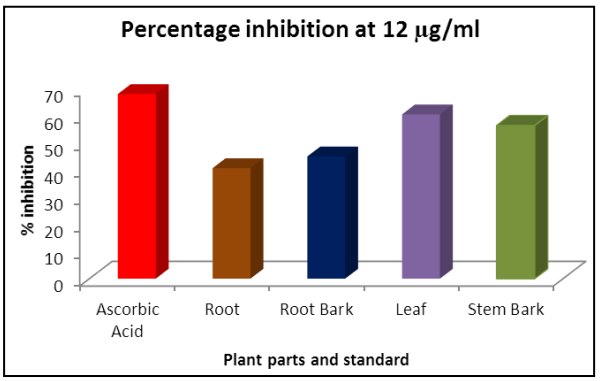

Fig 8: Bar chart representation % inhibition of ascorbic acid and various parts of *A. muricata* at 12 µg/ml

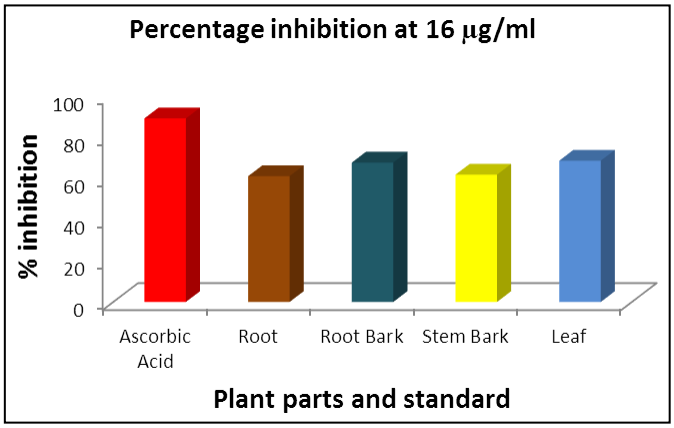

Fig 9: Bar chart representation of % inhibition of ascorbic acid and various parts of *A. muricata* at 16 µg/ml

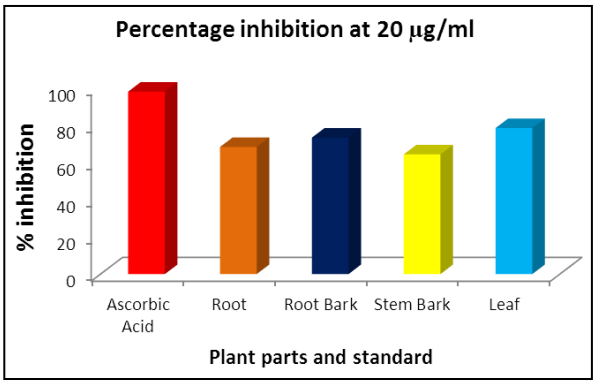

Fig 10: Bar chart representation of % inhibition of ascorbic acid and various parts of *A. muricata* at 20 µg/ml.

Table 6: IC_{50} of ascorbic acid and the various parts of *A. muricata*

S/No	Plant Part	IC_{50} (µg/ml)
1	Ascorbic acid	9.22
2	Leaves	4.36
3	Root	14.37
4	Root bark	12.25
5	Stem bark	11.72

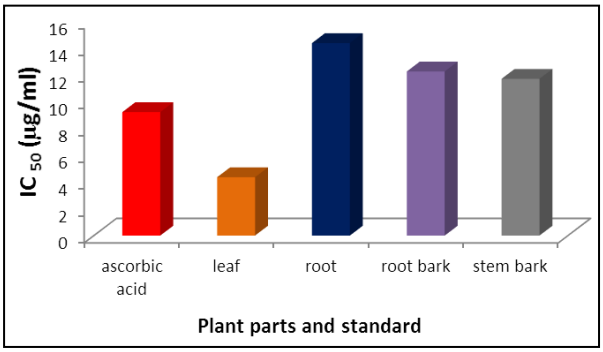

Fig 11: Bar chart representation of IC_{50} of ascorbic acid and various parts of *A. muricata*

4. Discussion

The extraction of 300 g of the different morphological parts of *A. muricata* with absolute methanol using maceration method for 72 hours gave a percentage yield of 6.77%, 5.73%, 6.41% and 5.24% for the leaf, root, root bark, and stem bark respectively (Table 1.1). Phytochemical screening revealed the presence of secondary metabolites such as alkaloids, flavonoids, tannins, phenol, and carbohydrate in all examined parts of the plant. Anthraquinone and reducing sugar were found in the leaf and root in contrast to the other parts whereas saponins, terpenoids and cardiac glycosides were found only in the leaf (Table 1.2). As such, the phytochemical constituents vary with different morphological parts. So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts [8]. The total flavonol content was found to be; 365.6, 264.8, 195.12, and 83.4 (µg/mg QE) (Table 2.0), for the leaves, stem bark, root bark and root respectively while the total phenolic content was observed to be 74.9, 56.5, 51.5, 47.6 (% mg/g GAE), (Table 3.0) for the leaves, root, root bark, and stem bark respectively. The result of total flavonol content TFC (µg/ml QE) and total phenolic content TPC (% mg/g GAE) of the different morphological parts as shown above reveal the leaves contain the highest amount of phenolics and flavonols while the root and stem bark were observed to contain the least amount of TFC and TPC respectively. Table 4.0 shows the concentration dependent antioxidant activities of the different parts of *A. muricata* based on % DPPH reduction. The various parts of the plant showed varying percentage inhibitions with the highest concentration (20ug/ml) showing the highest inhibition (%) for all the parts examined. The results obtained from IC_{50} determination (table 5.0) gave varying IC_{50} in the order 4.36, 11.72, 12.25, and 14.37 (µg/ml). The lower the IC_{50}, the higher the antioxidant activity; on this basis, it be can be said that the leaf possesses the highest free radical scavenging activity and is therefore the most potent of all the parts examined because it has the least IC_{50} value (4.36 µg/ml). The leaf was also observed to be more potent than ascorbic acid (IC_{50} of 9.22 µg/ml) which was used as standard.

5. Conclusion

In conclusion, it can be said that all the parts of the plant examined showed free radical scavenging activity. Also, the phytochemical screening results showed the presence of phenolic compounds as well as flavonoids which are responsible for the free radical scavenging activity they are also implicated in antioxidant activities of the plant morphological parts.

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