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Research Article

GC-FID Quantification of Phytochemicals and Radical Scavenging Potentials of Nanosuspension of *Annona muricata* leaf extract

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ABSTRACT

Several bioactive principles of plant extracts exhibit poor absorption and bioavailability due to their high molecular size or poor aqueous solubility. Nanoformulations of herbal drugs have emerged as essential tools to enhance bioavailability, solubility, drug retention time, and delivery while minimizing toxic effects. This can potentially strengthen the action of herbal extracts, reduce dosage and side effects, and improve bioactivity. The purpose of this study was to evaluate the radical scavenging activity of nanosuspension of *Annona muricata* leaf extract and quantify its phytoconstituents using a gas chromatography-flame ionization detector (GC-FID). Extraction of crude extract and quantification of phytochemicals by GC-FID was done. The in vitro antioxidant activity of the crude extract and nanosuspension of *Annona muricata* were evaluated using the DPPH scavenging activity and the ferric reducing antioxidant power (FRAP). The particle size analysis of the nanosuspension showed that particles were in the nano-range with average particle size of 73.35 nm and polydispersity index (PDI) of 0.404. The results also revealed that the nanoformulation augmented the antioxidant potentials of the crude extract of *Annona muricata*. At a concentration of 20 mg/ml, the percentage scavenging activity increased significantly ($p < 0.05$) from 71.24% for crude to 95.39% for nanosuspension, even higher than ascorbic acid (standard) which stood at 82.36%. *Annona muricata* is a powerful antioxidant agent and the use of nanotechnology can resolve some of the inherent biopharmaceutical shortcomings of herbal materials and serve as a tool for optimizing the bioactivity of the active components of plant extracts in herbal formulation.

Keywords: *2, 2-diphenyl-1-picrylhydrazyl (DPPH), nanosuspension, antioxidant, phytochemicals*.

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INTRODUCTION

Herbal medicines are naturally occurring, plant-derived substances that are used to treat illnesses within local or regional healing practices (Rakotoarivelo *et al*., 2015). These products are intricate blends of organic chemicals that can originate from any portion of a plant, whether it is raw or processed. Approximately 80% of people worldwide still rely on plant-based, traditional medicine systems for their main medical care, demonstrating the vital role these systems continue to play in healthcare (Owolabi *et al*., 2007; Kuete *et al*., 2016; Shashank and Egbuna, 2019).

Plants having a long history of usage in ethnomedicine are a rich source of active phytoconstituents in the pharmaceutical industry that offer therapeutic or health advantages against a variety of diseases. *Annona muricata* (also referred to as soursop, Graviola, guanabana, paw-paw, and sirsak) is one such herb that has been used extensively in the past. It belongs to the Annonaceae family, having 2300 species and 130 classes and is presently found globally in tropical and subtropical regions such as India, Malaysia, and Nigeria, though, it originated from deep tropical regions of South and

North America (Adewole and Caxton-Martins, 2006; Wahab *et al*., 2018).

Extensive phytochemical analysis on various parts of the *A. muricata* plant has revealed the presence of several phytoconstituents and compounds, including alkaloids, megastigmanes, flavonol triglycosides, phenolics, cyclopeptides and essential nutrients and oils (Kossouoh *et al*., 2007; Gyamfi *et al*., 2011; Matsushige *et al*., 2012; Nawwar *et al*., 2012; Jiménez *et al*., 2014; Yang *et al*., 2015).

Its biological activity, which includes anticancer, hepatoprotective, anticonvulsant, anti-arthritic, antimalarial, antiparasitic, antioxidant, and antidiabetic properties, has been the subject of several investigations. Annonaceous acetogenins are the primary components of *Annona muricata*, according to phytochemical investigations (Moghadamtousi *et al*., 2015). While the cooked leaves are applied topically to treat abscesses and rheumatism, the internal ingestion of the leaf decoction is thought to have anti-rheumatic and neuralgic properties (Mishra *et al*., 2013; De Sousa *et al*., 2010).

A new era in the delivery of herbal drugs is being ushered in by using nanotechnology in herbal medicine (Bhodariya *et al*., 2011). Because of their large molecular size or poor water solubility, most of the bioactive ingredients in plant extract are unable to pass through the cell's lipid membrane resulting in low absorption and poor bioavailability (Saraf, 2010). Various approaches focused on nanotechnology are being projected to enhance the biopharmaceutical characteristics of phytomedicines. Nanosuspension technology has developed into a strong contender for the more effective and efficient delivery of poorly water-soluble plant materials (Bailey and Beckland, 2009).

Antioxidants, also known as free-radical scavengers, are compounds that have the potential to shield cells from the harm that unstable molecules called free radicals can inflict. In addition to stabilizing free radicals, antioxidants may also stop some of the harm that free radicals could otherwise cause. Because reactive species oxidize vital cellular components including lipids, proteins, and DNA, oxidative stress has deleterious consequences on cells that can result in cell death and cancer (Hassan, 2011).

The amount of phytochemicals in a herbal material might vary depending on a variety of environmental parameters, such as soil type, height, seasonal temperature variations, atmospheric humidity, amount of daylight, rainfall patterns, shade, dew, and frost conditions. The aim of this study therefore was to quantify the phytoconstituents and evaluate the antioxidant potential of nanosuspension of crude extract of *Annona muricata* harvested from Abraka community in Delta State in Nigeria.

MATERIALS AND METHODS

Materials: Tween 80 (BDH, Poole, England), Polyvinyl alcohol PVA (Guangdong Guanghua Sci – Tech, China). Other chemicals and reagents used were of analytical grade.

Collection and identification of plant material: The fresh leaves of *Annona muricata* were harvested from Abraka, Delta State, Nigeria. The plant sample was identified and authenticated at the Department of Botany, Delta State University, Abraka and assigned a voucher number, DELSUH109 and deposited in the Delta State University Abraka herbarium.

Extraction of crude *Anonna muricata* extract (AME): The collected leaves were air-dried under shade for two weeks and then powdered coarsely with a mechanical grinder. A 2 kg sample of the powdered leaves was extracted with 80% methanol as a solvent using the Soxhlet apparatus. The resulting extract was concentrated using the rotary evaporator at 40 ^{o} C. The extract was stored at 4 ^{o} C in a tightly sealed labelled amber-coloured glass container for further studies (Alalor *et al.*, 2012; Okafo *et al.*, 2023).

Determination of extraction yield: The crude extract was weighed, and the extraction yield was calculated using the equation below

$$Extraction\ yield\ (\%) = \frac{Weight\ of\ the\ concentrated\ extract}{Dry\ weight\ of\ the\ sample}\ x\ 100 \quad \ldots\ldots\ldots\ldots (1)$$

Total ash value determination of *A. muricata* dried leaf powder: A 2 g quantity of dried *Annona muricata* leaf powder was taken into a crucible and incinerated in a furnace (Thermo Fisher Scientific, F30430CM) at a temperature not exceeding 450^{o}C until free from carbon. The sample was cooled and weighed. The percentage of ash was calculated with reference to the air-dried *Annona muricata* powder using the equation below.

$$Total\ ash = \frac{\text{Weight of ash}}{\text{Weight of sample}}\ x100 \quad \ldots\ldots\ldots\ldots\ldots (2)$$

Extraction of phytochemicals from the *A. muricata* methanolic leaf extract: A 1g quantity of the extract of *Annona muricata* was weighed and transferred in a test tube and 15 ml ethanol and 10ml of 50 % w/v potassium hydroxide was added. The test tube was allowed to react in a water bath at 60 ^{o}C for 60 min. Later, the reaction product was transferred into a separatory funnel. The tube was washed with 20 ml of ethanol, 10 ml of cold water, 10 ml of hot water and 3 ml of hexane and transferred to the funnel. These extracts were combined and washed three times with 10 ml of 10 %v/v ethanol aqueous solution. The solution was dried with anhydrous sodium sulfate and the solvent was evaporated. The sample was solubilized in 1000 uL of pyridine of which 200 uL was transferred to a vial for analysis (Bezerra and Antoniosi Filho, 2014; Ugoeze *et al.*, 2020).

Quantification *A. muricata* methanolic leaf extract by GC-FID: The analysis of phytochemical was carried out on a Buck M910 Gas chromatography equipped with a flame ionization detector. The column used was Restek 15-meter MXT-1 column (15 m x 250 um x 0.15 um). The injector temperature was 280^{o}C with split less injection of 2 uL of sample and a linear velocity of 30 cms^{-1}, carrier gas was Helium (5.0 pa.s) with a flow rate of 40 ml min-1. The oven operated initially at 200 ^{o} C, it was heated to 330 ^{o} C at a rate of 3 ^{o} C min^{-1} and

was kept at this temperature for 5 min. the detector operated at a temperature of 320 °C.

Phytochemicals were determined by the ratio between the area and mass of internal standard and the area of the identified phytochemicals. The concentration of the different phytochemicals was expressed in ug/g (Buss and Butler, 2010; Bezerra and Antoniosi Filho, 2014; Kelly and Nelson, 2014; Ugoeze *et al.,* 2020).

Formulation of nanosuspension of *A. muricata* extract: The nano-precipitation method was adopted for the preparation of the nanosuspension. According to Table 1 below, 2 g sample of *Annona muricata* extract (AME) was dissolved in 15 ml of a hydroalcoholic mixture of ethanol and water (3:1), then 0.2 ml of tween 80. The resultant solution was gradually injected (1ml/min) with a syringe, into 40 ml water containing 3.5 % w/v polyvinyl alcohol (PVA) with stirring using a magnetic stirrer at 1000 rpm. The solution obtained was then diluted further with 30 ml PVA solution to minimize coalescence and stirred continuously at 500 rpm for 6 hours at ambient temperature in a magnetic stirrer to allow solvent evaporation and nanoparticle formation (Jahan *et al.,* 2016).

Table 1:
Composition of nanosuspension of *A. muricata* extract

Ingredients	Quantity
Annona muricata extract (AME)	2 % w/v
Tween 80	0.2 % v/v
Ethanol + water (3:1)	15 % v/v
Polyvinylalcohol (PVA)	3.5 % w/v

Particle size analysis and polydispersity index of AME-nanosuspension: The particle size distribution of freshly prepared *Annona muricata* nanosuspension formulation was determined by photo correlation spectroscopy using a Malvern Zetasizer (Malvern Instruments Ltd., UK). Light scattering was monitored at a 90 ° angles and a temperature of 25 °C was maintained during the measurement. The sample was diluted 100 times with distilled water before the measurement. The predetermined refractive index of the formulation was incorporated into the computer software of the Zetasizer, which calculated the mean particle size, size distribution and polydispersity of the nanoparticles from intensity (Maherani and Wattraint, 2017; Shekunov *et al*., 2007).

Determination of antioxidant activities: Antioxidant activities of the crude extract of *Annona muricata* (AME) and its nanosuspension (AME-nanosuspension) were evaluated using the DPPH scavenging assay and ferric reducing antioxidant power assay (FRAP) in comparison with the antioxidant activity of a standard compound (Ascorbic acid).

DPPH Scavenging assay: DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a stable free radical chemical with purple colour that absorbs at 517 nm. The DPPH purple colour is converted to yellow colour or even colourless if the plant sample possesses any potential free radical scavenging property (Baliyan *et al.,* 2022).

Firstly, AME and AME-nanosuspension were prepared in different concentrations (4-20 mg/ml) and 0.5 ml of each of the concentrations was measured in separate test tubes and 1 ml of 0.1*m*M DPPH in ethanol was added to each test tube. The reaction mixtures were vigorously shaken for 30s in a vortex apparatus and allowed to stand in the dark at ambient temperature for 30 mins. Ascorbic acid (1*m*M) was used as a standard for the investigation of the antiradical activity and was also prepared in the same concentrations. The absorbance of the DPPH radical + sample (Abs) was measured using the UV-visible spectrophotometer at 517 nm against the absorbance (Ab_c) of the solution of DPPH in ethanol (Roberta *et al.,* 2006; Ozaslan *et al.,* 2022).

This procedure was carried out in triplicate and the mean value was recorded. The percentage inhibition of the DPPH free radical was calculated using the equation below:

$$DPPH\ scavenging\ activity\ (\%) = \frac{Ab_c - Ab_s}{Ab_c}\ x\ 100 \ldots\ldots (3)$$

Ferric reducing antioxidant power assay (FRAP): This test was carried out with the various concentrations of AME and AME-nanosuspension as test samples. A 1ml volume of the different concentrations of the test sample solution was mixed with 2.5 ml of potassium ferricyanide (1% w/v) and 2.5 ml of phosphate buffer (0.2 M, pH 6.6). The mixture was then incubated for 20 minutes at 50 °C and 2.5 ml of trichloroacetic acid (10 %) was added. After centrifugation of the mixture for 10 mins at 3000 rpm, 2.5 ml was collected from the upper layer and then mixed with 2.5 ml of distilled water and 0.5 ml of Ferric chloride (0.1 %). Ascorbic acid was used as the standard and the absorbance was measured at 700 nm using the UV-visible spectrophotometer (Jafri *et al.,* 2022). This was performed in triplicate and the mean value was recorded.

Statistical analysis: The data was presented as mean ± standard deviation (SD) (n = 3). Statistical difference between the mean values were determined by one-way analysis of variance (ANOVA). A *p*-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Percentage yield and ash value: The percentage yield of *A. muricata* extract (AME) from the dried powdered leaves was 15.67 % and the total ash value was 5.88 %. The ash values usually represent the inorganic residues such as phosphates, carbonates and silicates present in herbal drugs and considered important indices to illustrate the quality and purity of herbal medicine. The percentage yield of 15.67 % is low, but this is typical of yields from plant source. The total ash value of 5.88 % is reasonably low indicating low level of impurities and is much lower than the total ash value reported for *Annona muricata* by Usunobun *et al*. (2014).

Particle size analysis and polydispersity index of AME-nanosuspension: The result of the particle size analysis is presented in Figure 1 below. Majority of the particles fell

within the nanosize range as seen in the horizontal axis of Figure 1 somewhere around 10-100 nm with an average particle size of 73.35 nm. The formulation of AME is indeed a nanosuspension. The polydispersity index (PDI) of AME-nanosuspension is 0.404 indicating good particle size distribution. Polydispersity index is basically a representation of the distribution of size populations within a given sample. The numerical value of PDI ranges from 0.0 (for a perfectly uniform sample with respect to the particle size) to 1.0 (for a highly polydisperse sample with multiple particle size populations). The polydispersity index of 0.404 shows that the particle size distribution was averagely good in line with the literature which states that in drug delivery, a PDI of 0.3 and below is considered acceptable and indicates a homogenous population (Chen *et al*., 2011; Badran, 2014; Putri *et al*., 2017).

Quantitative phytochemical analysis of *A. muricata* methanolic leaf extract by GC-FID: GC-FID is one of the contemporary methods used to detect and isolate phytoconstituents in plants (Tedone *et al*., 2014; Ogbuagu *et al*., 2019). The results from the GC-FID quantitative analysis are presented in Figure 2 and Table 2 below.

The result shows that the most prominent phytochemicals found in AME belong to the broad class, flavonoids, a class of polyphenolic compounds representing 47.60 % of the phytochemicals. They include Epicatechin (32.79 µg/ml), Flavone (10.51 µg/ml), Flavonones (7.55 µg/ml), Kaempferol (9.04 µg/ml), Naringin (9.36 µg/ml), Catechin (10.1 µg/ml), Flavan -3-ol (3.80 µg/ml), Proanthocyanin (2.06 µg/ml) and Rutin (13.54 µg/ml). Epicatechin (32.7852 µg/ml or 15.80 %) was the flavonoid with the highest concentration while Proanthocyanin (2.06 µg/ml or 0.99 %) was the flavonoid with the lowest concentration.

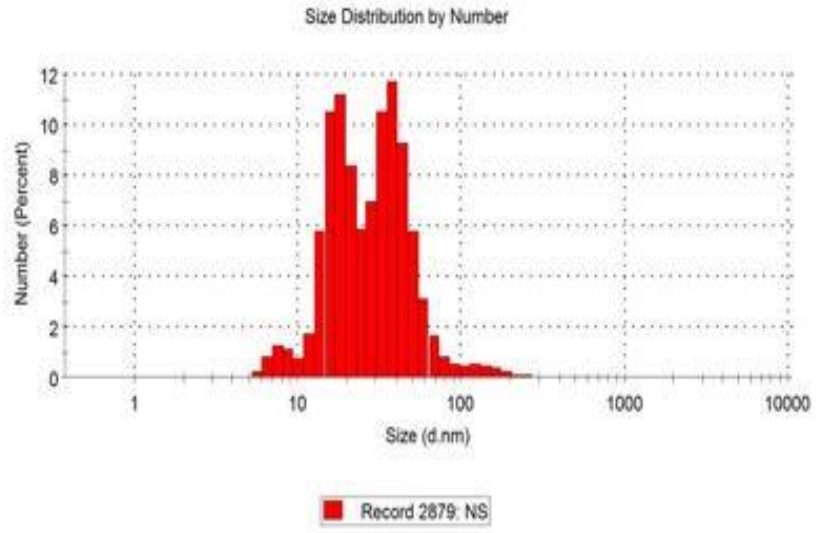

Figure 1:
Particle size analysis of AME-nanosuspension

Table 2:
Phytochemical composition of AME by GC-FID

Class of Phytochemicals	Phytochemical	Retention	Area	Height	Concentration (µg/ml	Composition (%)
Flavonoids (47.60%)	Epicatechin	34.6	5855.8934	152.417	32.7852	15.8032
	Flavone	32.996	14116.1752	362.657	10.5116	5.0668
	Flavonones	20.313	12759.8375	321.421	7.5508	3.6397
	Kaempferol	25.65	10042.0106	256.66	9.0406	4.3578
	Naringin	17.966	11341.843	289.103	9.3574	4.5105
	Catechin	2.39	12314.1358	315.451	10.1	4.8684
	Flavan -3-ol	4.12	6419.9277	165.611	3.8038	1.8335
	Proanthocyanin	42.276	3490.1614	89.393	2.0617	0.9938
	Rutin	6.016	18188.0636	463.276	13.5437	6.5284
Tannin (2.725%)	Tannin	7.47	8464.0412	216.081	5.6525	2.7246
Alkaloids (23.58%)	Ephedrine	44.17	10528.0402	268.71	32.3742	15.6051
	Lunamarin	0.266	3591.7626	129.473	8.2695	3.9861
	Spartein	10.366	19600.635	498.4	8.2734	3.9880
Cardiac glycosides (3.79%)	Cardiac glycoside	12.97	6239.826	159.347	7.868	3.7926
Saponins (1.67%)	Sapogenin	15.46	4968.7504	126.85	3.4726	1.6739
Steroids (6.28%)	Steroids	22.73	9574.5704	243.861	13.0275	6.2796
Cyanogenic glycosides (5.21%)	Cyanogenic glycoside	27.536	11491.6099	293.434	10.812	5.2116
Anti nutrients (6.33%)	Phytate	29.86	5480.1714	139.875	7.1817	3.4617
	Oxalate	36.876	6991.6694	178.122	5.9504	2.8682
Other phenolics (2.81%)	Resveratol	39.2	10236.2838	260.67	5.8221	2.8064

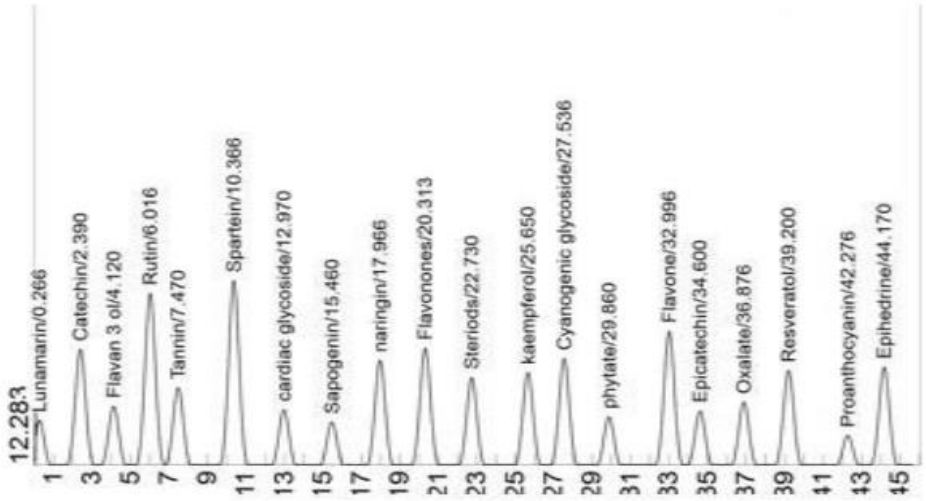

Figure 2:
Chromatogram of the GC-FID analysis

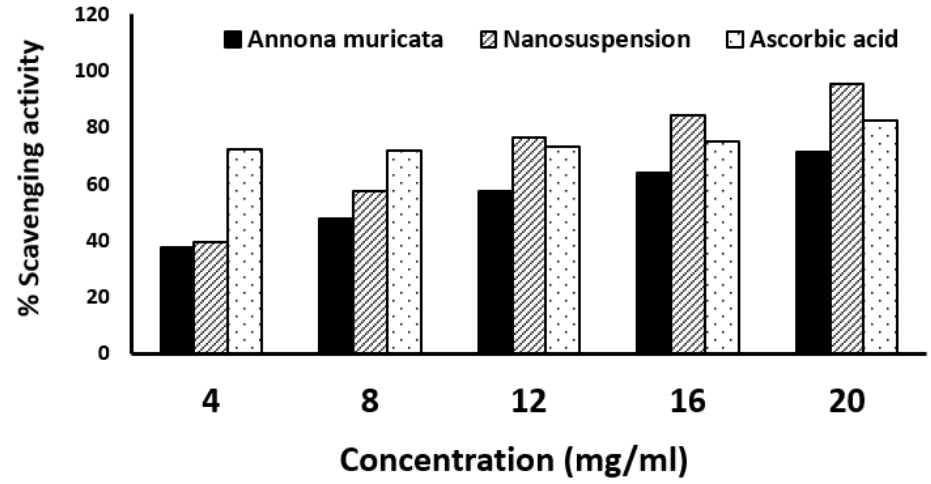

Figure 3:
Percentage DPPH scavenging activity of AME crude and AME-nanosuspension

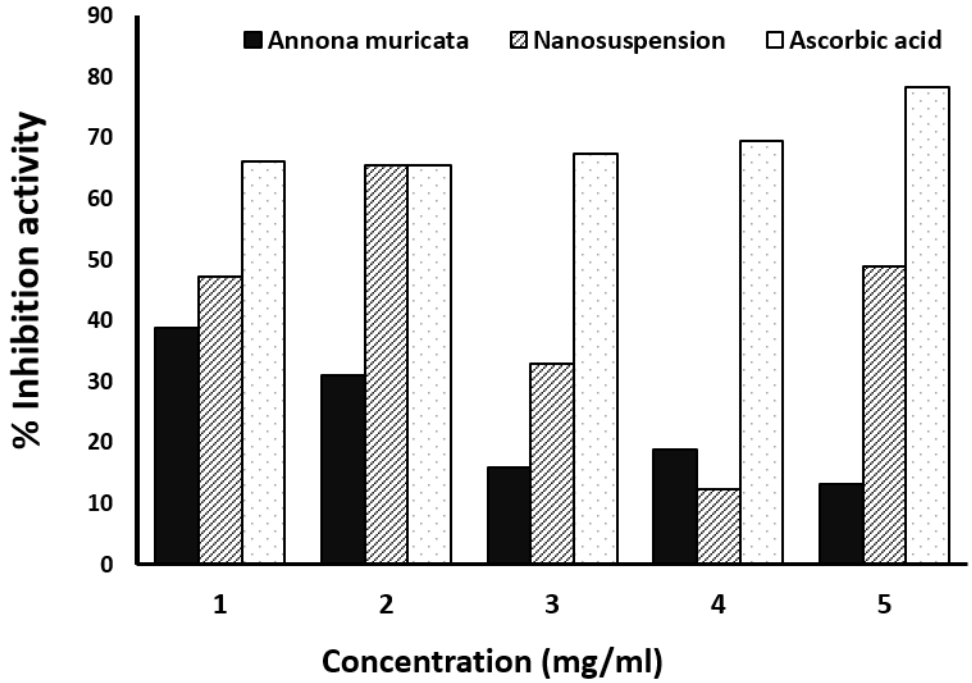

Figure 4:
Percentage ferric reducing antioxidant power of AME-crude and AME-nanosuspension

The alkaloids are next to the flavonoids representing 23.58 % with the following phytochemicals: Ephedrine (32.37 µg/ml), lunamarin (8.27 µg/ml) and spartein (8.27 µg/ml). Other classes of phytochemicals detected comprise tannins (2.73%), cardiac glycosides (3.79%), saponins (1.67%), steroids (6.28%), cyanogenic glycosides (5.21%), anti-nutrients (6.33%) and other phenolics (2.81%).

The phytochemicals in *A. muricata* detected by GC-FID in this study are the same as those detected in a study by Onyeike *et al*., (2023) but differ markedly from the phytochemicals detected in another study by Onuah *et al*., (2019) with the inclusion of quinine, morphine and ribalinidine. This variation could be attributed to the environmental factors which usually cause variation in components of herbal materials.

Radical scavenging activity (DPPH and FRAP): The result of the DPPH radical scavenging activity is represented in Figure 3 while the result of ferric reducing antioxidant power (FRAP) is represented in Figure 4. The DPPH radical scavenging activity was concentration dependent. At 12, 16, and 20 mg/ml the AME-nanosuspension gave higher antiradical activities than the AME crude and the standard (ascorbic acid). The FRAP activity was not concentration dependent. The observed antiradical activity may be due to the presence of high amounts of polyphenolic compounds and flavonoid present in the sample. It can be implied from the result that the formulation of AME-crude into AME-nanosuspension augmented the antioxidant activity of AME significantly ($p<0.05$). This significant antioxidant activity may be due to the improvement of the solubility and absorption of the phytoconstituents. AME-nanosuspension compared favourably with the standard antioxidant Ascorbic acid and surpassed it at higher concentrations as seen in the DPPH assay in Figure 3 below.

It has been reported that flavonoids and phenolics are free radical scavengers that prevent oxidative cell damage and have strong anticancer activities and they might induce mechanism that affect cancer cells and inhibit tumor invasion (Pourmorad *et al*., 2006; Ugwu *et al*., 2013; Rafat *et al*., 2008). These activities could be attributed to their ability to neutralize and quench free radicals (Omale and Okafor, 2008).

In conclusion, this study revealed that the methanolic extract of *Annona muricata* leaves are rich in phytochemicals as detected and quantified by GC-FID. *Annona muricata* leaves are a repository for free radical scavenging molecules such as alkaloids, tannins, terpenoids, phenolic acids, flavonoids and other metabolites, which are basically rich in antioxidant activities. The use of nanotechnology can resolve some of the inherent biopharmaceutical shortcomings of herbal materials and serve as a veritable tool for optimizing the bioactivity of the extracts in herbal formulation.

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