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Phytochemical Content and Anti-Inflammatory Activity of Chitosan Nanoparticles Loaded with *Annona muricata* L. (Annonaceae) Stembark Extract in Wistar Rats

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Abstract

The stembark of Annona muricata L. has been used by some communities in Nigeria for the treatment of pain, inflammation, cancer, fevers and other diseases for many decades. This present study was carried out to evaluate the phytochemical contents as well as the anti-inflammatory activity of A. muricata methanol stembark extract in carrageenan and formalin-induced Wistar rat models. The preliminary phytochemical screening of A. muricata methanol stembark extract (AMSE) was determined following standard methods while the acute oral toxicity test of AMSE was determined in five Wistar rats of opposite sex using the OECD guideline 425. The anti-inflammatory activity of the extract was evaluated using carrageenan-induced rat paw oedema. The results of the phytochemical screening showed the presence of flavonoids, alkaloids, tannins, saponins, and cardiac glycosides. GC-MS analysis of methanol stembark extract showed the presence of many compounds including phytol, oleic acid, oleyl alcohol, 2-pentadecanol, n-hexadecanoic acid, etc. Oral acute toxicity of AMSE showed that extract was well tolerated by the animals at the dose of 5000 mg/kg body weight after one week. Neither signs of toxicity nor mortality were witnessed in the animals, especially within the first 24 hours of oral toxicity testing. The anti-inflammatory evaluation of stembark extract at the doses of 300, 600 and 1200 mg/kg body weight (i.p.) showed a dose-dependent decrease in rat's paw oedema in both carrageenan-induced rat paw oedema. These decreases were significant ($p < 0.05$) when compared to that of the standard drug (Diclofenac sodium 10 mg/kg; i.p.). The results from the study showed that chitosan NPs loaded with A. muricata methanol stembark extract possessed more anti-inflammatory activity than the crude extract by suppressing the production of pro-inflammation mediators. The study justifies the acclaimed use of A. muricata stembark extract in traditional medicine for the treatment of inflammation.

Keywords: *Annona muricata*, Phytochemicals, Anti-inflammatory, Carrageenan.

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INTRODUCTION

Annona muricata L. (Annonaceae) commonly called soursop is a lowland tropical fruit-bearing tree belonging to the family Annonaceae (Brasil *et al*., 2008). The family is one of the largest families in the Order Magnoliales consisting of approximately 130 genera and 2,300 species (Kim *et al*., 1998). The plant is an evergreen, small and upright tree that grows up to a height of 9.1 meters. Its whitish aromatic prickly fruit is very sweet about 30 cm long and often soft when ripped. In Nigeria, the plant is called Soursop (Pigin English), *Chop-chop* (Igbo) and *Gwanda Masar* (Hausa). All the parts of the plant are used in traditional medicine for treating various diseases as anticancer, analgesic, antipyretic, anti-inflammation, antihypertensive, antidiabetic agents, etc. The biological activity of the plant was due to the presence of secondary metabolites such as alkaloids, flavonoids, saponins, tannins and cardiac glycosides (Komansilan *et al*., 2012). Despite the popular uses of the plant in ethnomedicine, there are none or few entries for the anti-inflammatory activity of chitosan NPs loaded with *Annona muricata* stembark extract.

Chitosan is a biodegradable linear polymer which ranges from 1-1000 nm in size made from the exoskeletons of crabs, insects, crayfish and prawns (Raval *et al*., 2010). It is a biocompatible polymer regarded as safe for human dietary use and approved for wound dressing applications. Chitosan has been used as a carrier in polymeric nanoparticles for drug delivery through various routes of administration. It has chemical functional groups that can be modified to achieve specific goals, making it a polymer with a tremendous range of potential applications (Selomulya *et al*., 2011). Chitosan nanoparticle is the most important derivative of chitin, derived from crustacean shells such as those from prawns or crabs, as well as from the cell walls of fungi produced by removing the acetate moiety from chitin (Mahmoud, 2010). The ability of chitosan NPs to deliver drugs specifically to tissues or organs without causing any harm to other healthy tissues or organs of the body makes it an excellent drug delivery agent in both *in vitro* and *in vivo* models (Vllasaliu *et al*., 2013).

This present study was carried out to evaluate the phytochemical contents of stembark extract and the anti-inflammatory activity of chitosan NPs loaded with methanol stembark extract of *Annona muricata in vivo* rat models.

METHODS

Collection, identification and preparation of plant material

Fresh stembarks of *Annona muricata* were freshly collected from a compound in Takum, Taraba State, Nigeria. The plant was identified and authenticated by Dr. Cletus A. Ukwubile of the Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, Nigeria. A voucher specimen number of UMM/FPH/ANO/001 was deposited in the herbarium of the Department of Pharmacognosy. The stembarks were then air-dried under shade for two weeks until a constant weight was obtained and then reduced in fine powder using an electronic blender (M500, China). The powdered stembark was weighed and stored in a clean sample bottle for further use (Egua *et al*., 2014).

Extraction of plant material

Powdered stembark of *A. muricata* weighing 1.5 kg was extracted using 5 L of absolute methanol (Sigma Aldrich, St. Lous Mo, USA) by cold maceration technique for 72 h. The filtrate was concentrated *in vacuo* using a rotary evaporator at 64.7 ^{o}C (ThermoFisher, UK) to obtain a dark-brown extract weighing 125 g (% yield was 8.33). The extract was stored in a clean sample bottle and kept in a desiccator for further use (Dai and Mumper, 2010).

Phytochemical analysis of stembark methanol extract (AMSE)
The preliminary phytochemical analysis of the stembark methanol extract was carried out following standard procedure to determine the presence of some secondary metabolites such as alkaloids, tannins, flavonoids, saponins, anthraquinones, steroids and phytosterols *(Abubakar et al*., 2023).

GC-MS analysis of AMSE
The phytoconstituents of compounds present in the crude methanol extract of stembark were evaluated in Agilent Technologies 7890A gas chromatography (GC) coupled to a mass spectrum detector (MSD) (Agilent Technologies, USA). The carrier gas was helium with column velocity flow of 1.0 mL/min, ion-source temperature was 250 °C, interface temperature was 300 °C, operating pressure was 16.2 psi, out time was 1.5 min, injection temperature was 300 °C in split mode at 1 µL injector, while the temperature of the column was initially 50 °C for 5 min and raised to 250 °C at the rate of 20 °C/min for 5 min (Ukwubile *et al*., 2019). The total elution time was 25 min, and each compound was calculated in terms of relative abundance, peak areas and retention times while the identification of compounds was done by comparing with data from the NIST library (Olivia *et al*., 2021).

Experimental animals
Forty (40) Wistar rats of the both sexes weighing between 50 and 80 g were purchased from PJ Rat Farm Ltd, Jos, Nigeria. The animals were housed separately in aluminium cages and allowed to acclimatise in the laboratory for two weeks with free access to water and food. The approval for the use of these animals was given by the research ethical committee of PJ Rat Farms with approval number PJ/106/2022.

Acute oral toxicity of AMSE in rats
The median lethal dose (LD_{50}) of the extract was determined using the OECD GUIDELINE 425. Five Wistar rats of opposite sex were randomly selected and administered with a maximum dose of 5000 mg/kg body weight orally. The animals were observed for signs of toxicity, especially within the first 4 h and then observed for 14 days. The animals were later sacrificed at the end of the study since no signs of toxicity were witnessed (Khalifa, 2022).

Preparation of chitosan NP-loaded *A. muricata* stembark extract
Chitosan nanoparticle was prepared by ionic gelation of sodium tripolyphosphate (TPP) in a ratio of 4:8 (extract: chitosan). The prepared chitosan NP was prepared and characterized according to previously described procedures with modification(Desai and Park, 2005).

Evaluation of anti-inflammatory activity of AMSE
Carrageenan-induced paw oedema rat model
Paw oedema was induced by injecting 0.1 mL of 1% w/v carrageenan dissolved in 1 % carboxymethylcellulose (CMC) into the sub-plantar region of the left hind paw of each rat (Yimer et al., 2020). The animals were then grouped into five groups of five rats each as shown below:
Group I was the normal control group which received 10 ml of distilled water orally,
Group II was the positive control group which received 20 mg/kg diclofenac sodium orally,
Group III received 20 mg/kg CSAMSE orally,
Group IV received 600 mg/kg AMSE orally,
Group V received 1200 mg/kg AMSE orally.

The diameter or volume of the paw oedema was measured after administration by oral gavages in 60, 120, 180, and 240 min using a Vernier caliper. Percentage inhibition of paw oedema by the extract was then calculated using the formula below:

$$\% \ inhibition = \frac{Pc\text{-}Pt \ x \ 100}{Pc}$$

where Pc denotes the paw diameter or volume of the control group, and Pt denotes the paw diameter or volume of the treated groups.

Formalin-induced paw oedema rat model

The rats were grouped similarly as described in the above carrageenan-induced model except that 0.2 mL of 2 % (v/v) formalin which was freshly prepared in 20 mL of distilled water was used as an oedematogenic agent. Paw oedema diameter or volume was measured before and after the injection of formalin in the sub-plantar region. Measurement of paw volume or diameter was made at fixed times for seven consecutive days using the Vernier caliper (Yimer *et al*., 2020).

Statistical Analysis

The data obtained were expressed as mean ± SD (n = 5). Statistical significance between the treated group and control group was determined at the value of $p < 0.05$ using split plot ANOVA followed by Dunnett's post hoc test. Analysis was carried out using SPSS statistical software version 23.

RESULTS

Phytochemical analysis of AMSE

The % yield of the methanol stembark of *A. muricata* was calculated to be 8.33 % while the qualitative phytochemical analysis revealed the presence of saponins, tannins, alkaloids, cardiac glycosides, phenols, flavonoids, acetogenins, fats/oils and triterpenoids/steroids. The quantitative analysis further showed that acetogenins, alkaloids, fats/oils and tannins occurred in the highest amount (Table 1).

Table 1: Phytochemical contents of *A. muricata* methanol stembark extract

Constituent	Inference	Amount/unit
Acetogenins	+	25.64 %
Saponin	+	2.5 %
Anthraquinones	-	Na
Cardiac glycosides	+	2.8 %
Flavonoids	+	51.33 mg/g
Tannins	+	20.58 mg/g
Alkaloids	+	25.64 %
Fat/oils	+	12.08 %
Steroids/Triterpenes	+	14.01 %

+ denotes present, - denotes absent, and Na denotes not applicable.

GC-MS analysis of A. muricata methanol stembark extract

The GC-MS analysis showed that the methanol stembark extract contains mainly various alkaloids, fatty acids and annonaceous acetogenins as well as phytol. The alkaloids and annonaceous acetogenins have the highest peak area or abundance (Table 2).

Acute oral toxicity study

The result showed that the extract did not produce any sign of toxicity and mortality after 14 days of acute intoxication of 5000 mg/kg body weight maximum dose. It showed that the extract was well tolerated by the animals at this dose because an $LD_{50} \geq 5000$ mg/kg is of biological unimportant (Khalifa, 2022).

Table 2: GC-MS analysis of *A. muricata* methanol stembark extract.

Compound	PA (%)	RT (min)	Formula	Class
Anomuricine	1.29	17.15	$C_{17}H_{17}NO$	Alkaloid
Anomurine	3.21	18.01	$C_{20}H_{25}NO_4$	Alkaloid
Stepharine	12.11	16.23	$C_{18}H_{19}NO_3$	Alkaloid
Atherospermine	5.04	18.05	$C_{20}H_{23}NO_2$	Alkaloid
Coreximine	1.33	17.25	$C_{19}H_{21}NO_4$	Alkaloid
Coclaurine	6.88	19.01	$C_{17}H_{19}NO_3$	Alkaloid
Reticuline	16.44	19.05	$C_{19}H_{23}NO_4$	Alkaloid
Epoxymurin A & B	13.97	18.12	$C_{35}H_{62}O_3$	Acetogenins
Oleic acid	3.28	19.96	$C_{20}H_{36}O_2$	Fatty acid
7-tetradecenol (Z)	9.38	19.74	$C_{14}H_{26}O$	Fatty acid
Phytol	5.60	19.52	$C_{20}H_{40}O$	Diterpene
Hexadecanoic acid E-ester	1.30	18.52	$C_{18}H_{36}O_2$	Fatty acid
n-hexadecanoic acid	7.24	18.08	$C_{18}H_{32}O_2$	Fatty acid
Oleyl alcohol	6.25	16.75	$C_{18}H_{36}O$	Fatty alcohol
2-pentadecanol	3.96	10.11	$C_{15}H_{32}O$	Fatty alcohol
1,2-benzene-Octyl ester	2.72	17.21	$C_{20}H_{30}O_4$	Fatty acid

PA denotes peak area; RT denotes retention time.

Preparation and characterization of chitosan NP-loaded AMSE

The result showed that prepared chitosan NP loaded with AMSE has a moderate size of 225 ±1.04 nm, higher drug release profile, and entrapment efficiency. The release pattern was sustained following first-order kinetic (Table 3).

Table 3: Preparation and characterization of chitosan NP-loaded AMSE

Parameter	Value (unit)
Yield	56.04 %
Particle size	225 ± 1.04 nm
Zeta potential	26.15 ± 2.11 V
CDR (6 h)	96.24 %
Drug entrapment	64.12 ±4.02 %
Swelling index	55.14 ± 2.14 %

CDR denotes cumulative drug release in 6 hours, n = 3 for replicate experiment

Evaluation of anti-inflammatory activity of AMSE

The results in Figures 1 and 2 below showed the extract possessed dose-dependent anti-inflammatory activity in both carrageenan and formalin-induced paw oedema in rat models. However, the highest percentage inhibition of paw oedema diameter or volume was witnessed in the group treated with chitosan NPs loaded with *A. muricata* methanol stembark extract (CSAMSE) with a value of 78.24 %. This result was comparable to that of the standard drug diclofenac sodium ($p < 0.05$) within the study periods of 60, 120, 180 and 240 min for the

carrageenan-induced model and 1st, 2nd, 3rd and 4th day in formalin-induced model respectively.

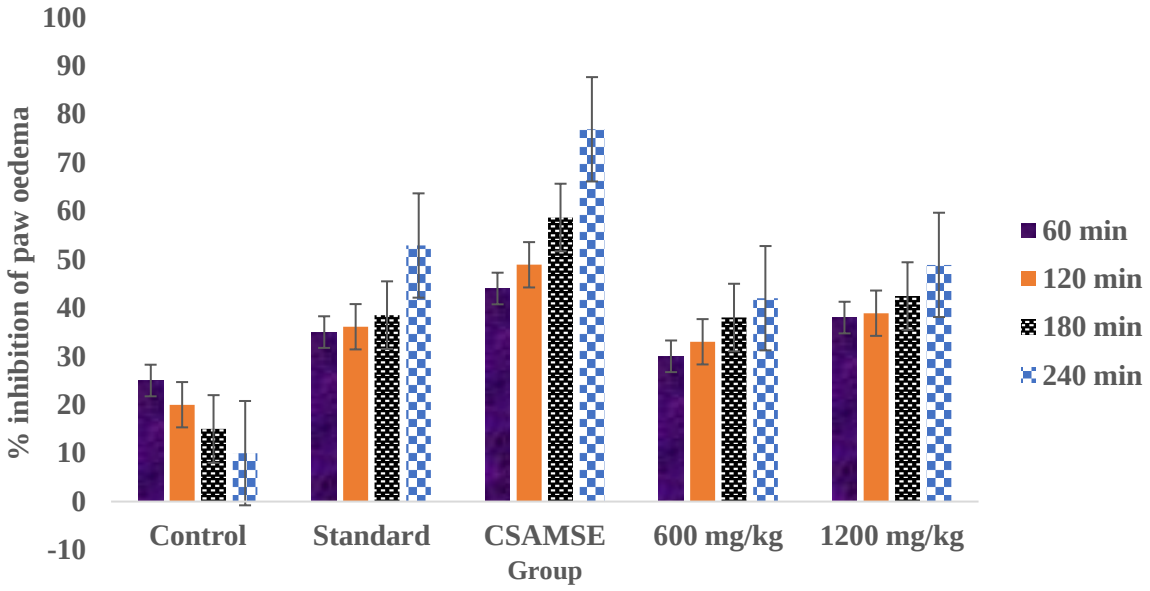

Figure 1: Effect of A. muricata on carrageenan-induced paw oedema in rats. Results are mean ±SD (n = 5). P < 0.05 (split plot ANOVA) was taken as statistically significant when compared with the control group.

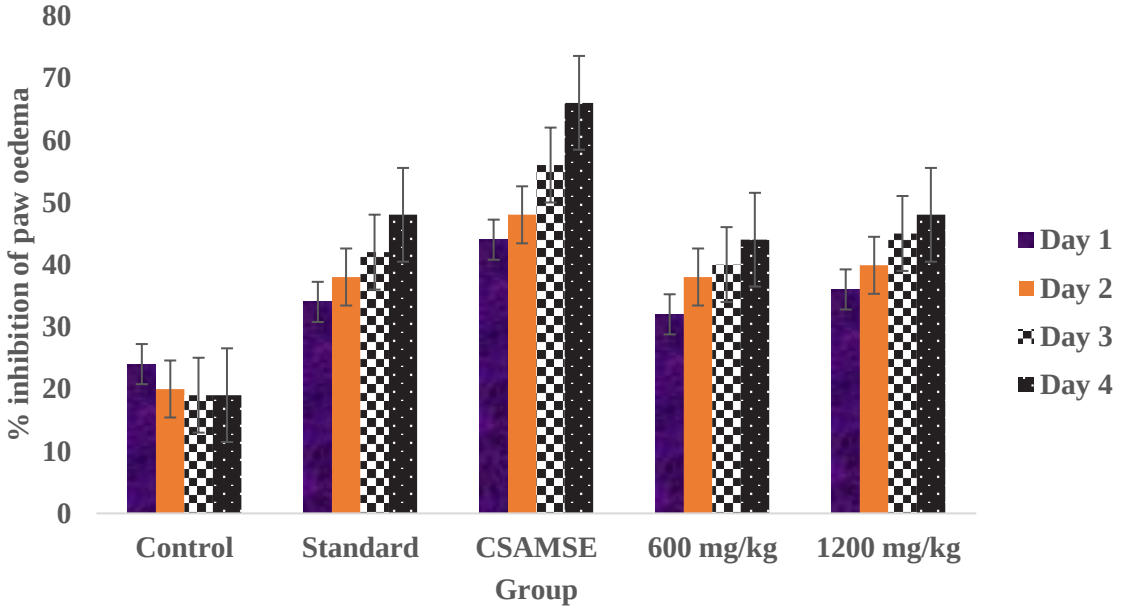

Figure 2: Effect of *A. muricata* on formalin-induced paw oedema in rats. Results are mean ±SD (n = 5). P < 0.05 (split plot ANOVA) was taken as statistically significant when compared with the control group.

DISCUSSION

Medicinal plants are major sources of drug agents with various pharmacological potentials due to the presence of secondary metabolites. They are very crucial for use in human healthcare and play vital roles in the healing process because plants contain different types of phytoconstituents (Alamgir, 2017). These plants have been used in Nigeria's healthcare for decades. Studies have shown that plant-based medicines are safe for use in disease conditions

(Sofowora *et al*., 2013). The world market value of medicinal plant products across various countries exceeds $100 billion per annum due to the activities of traditional medicine practitioners. According to the World Health Organization (WHO) Expert Group, traditional medicine is the total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing (Sofowora *et al*., 2013).

In the current study *A. muricata* stembark methanol extract revealed the presence of various secondary metabolites such as alkaloids, flavonoids, tannins, cardiac glycosides, saponins and phytosterols. These findings were in agreement with previous works on the plant by other researchers (Torres *et al*., 2014). These secondary metabolites play a crucial role in the human healthcare system. For example, some compounds revealed by the GC-MS such as acetogenins, fatty acids, alkaloids, saponins, flavonoids and terpenes were reported to be used as anti-inflammatory agents, analgesics, anticancer and antioxidants (Alamgir, 2017). The roles of these metabolites were not different from those in the current study. Similarly, many plant products were considered safe for use in healthcare if they did not produce any sign of toxicity at a maximum dose of 5000 mg/kg body weight (Kpemissi *et al*., 2020). The study showed that the extract was well tolerated in the rats at a dose of 5000 mg/kg with no sign of toxicity or mortality after the study period. This finding also supported many previous works on *A. muricata* stembark extract where it concluded that the plant is very safe (Taylor and Noller, 2005).

Despite the numerous advantages of using medicinal plants in healthcare, there are disadvantages such as the delayed process in disease treatment, poor drug delivery system, uneven distribution of drugs within body tissues, lack of modern ways in traditional medicine and unhygienic mode of preparation (Jones *et al*., 2006). In other to overcome some of these challenges, chitosan nanoparticles are now being used as carriers for many drugs. This will help to deliver drug agents specifically to the site of disease, minimize uneven biodistribution of drugs to both disease and healthy tissues and efficiently target drug agents (Javid *et al*., 2013). In this study, chitosan loaded with A. muricata stem bark extract effectively decreased the paw oedema diameter or volume of the rats better than samples delivered directly into the paw oedema tissues of rats. The particle size, entrapment efficiency, cumulative drug release, swelling index and sustained release pattern of formulated chitosan nanoparticles were responsible for the observed biological activity of the chitosan-loaded extract. This is because these parameters are essential characteristics of chitosan nanoparticles as carriers for any drug agent. The particle size must fall between 1 and 1000 nm, with a minimum zeta potential of not more than 30 V (Vllasaliu *et al*., 2013). The study showed that the characterized chitosan nanoparticles meet all the criteria as an excellent nanocarrier for the anti-inflammatory agent used in the current study.

It has been reported that carrageenan-induced inflammation is one of the suitable methods to evaluate the acute anti-inflammatory effects of a drug agent. The initial phase of inflammation takes place within one hour of injection of carrageenan in the sub-plantar region of the animal which is partly because of the trauma of injection as well as the secretion of histamine and serotonin pro-inflammation markers (Sharma *et al*., 2020) As seen from the study, there was significant inhibition of paw oedema, volume by chitosan loaded stembark extract (CSAMSE) at 240 min when compared to other treatment groups as well as the control. Therefore, it can be concluded that chitosan loaded with stembark extract of *A. muricata* showed potential inhibition of pro-inflammatory mediators such as histamine, cyclogenase and serotonin

thereby preventing the production of prostaglandins (PGAs) which facilitate inflammation (Borquaye *et al*., 2020).

Similarly, formalin-induced paw oedema is another suitable method to assess the chronic anti-inflammatory effects of drug agents (Sulaiman *et al*., 2009). In the current study, administration of CSAMSE inhibited formalin-induced paw oedema in rats than any other treatment group as well as the normal control group. The study further confirmed chitosan nanoparticles as a potential carrier for anti-inflammatory drugs of plant origin and an excellent drug delivery tool. In conclusion, the study showed that *A. muricata* methanol stembark extract possessed anti-inflammatory activity in a dose-dependent fashion due to the presence of many phytoconstituents. However, this activity was more pronounced in the group treated with chitosan nanoparticles loaded with *A. muricata* methanol stembark extract.

CONCLUSION

The current study has shown that the highest anti-inflammatory activities were obtained in chitosan nanoparticles loaded with *A. muricata* methanol stembark extract than any other group. Results from the current study also showed the extract contains many metabolites which are responsible for the observed biological activity. Further study is suggested to isolate and elucidate the structure of bioactive compounds responsible for the observed biological activity of the plant.

Declaration of competing interest

We have none to declare.

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