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Preparation of Chitosan Nanoparticle from Crab Shells and Evaluation of Analgesic and Anti-inflammatory Activities of Chitosan-loaded *Haematostaphis barteri* Hook. f. Stembark Extract

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ABSTRACT

Haematostaphis barteri Hook. f. is a tree belonging to the Family *Anacardiaceae* whose morphological parts have been used for decades for treating various diseases in Northern Nigeria. This study aimed to prepare chitosan nanoparticles from crab shells and evaluate the analgesic and anti-inflammatory activities of chitosan loaded with stembark extract from *H. barteri*. Phytochemical screening of extract, preparation of chitosan, and evaluation of analgesic and anti-inflammatory activities were carried out following standard procedures. The qualitative phytochemical analysis of the extract revealed the presence of alkaloids, saponins, tannins, flavonoids, and cardiac glycosides while steroids, triterpenes and anthracenes were absent. The LD₅₀ of the crude methanol extract in Wistar albino rats was determined to be greater than 5000 mg/kg body weight and after 14 days of single dose oral administration. No signs of toxicity were recorded. Prepared chitosan nanoparticle loaded with extract has a yield of 26.54 %, particle size of 210 ± 1.01 nm, zeta potential of 25.4 ± 1.02 V, drug entrapment efficiency of 68.4 %, cumulative drug release of 88.24 % and swelling index of 58.14 %. The extract produced a dose-dependent analgesic and anti-inflammatory activity. The chitosan-loaded extract produced the highest inhibition of writhing and paw oedema diameter in the rats, which was significant (p < 0.05) when compared with the normal controls and standards. In conclusion, the study showed that chitosan-loaded stembark extract from *H. barteri* possessed more potential analgesic and anti-inflammatory activities than those delivered without chitosan nanoparticles. The study further affirms the potential usefulness use of the stembark extract for the treatment of pain and inflammation.

Keywords: Analgesic; Anti-inflammatory; Chitosan nanoparticle; Crab shells; *Haematostaphis barteri*

INTRODUCTION

Medicinal plants are very important for humans and livestock as food, medicines and shelter. For many years, plants have changed human cultural practices and in turn, humans have changed plants' diversity by domesticating and translocating various plant species to prevent them from extinction (Alamgir, 2017). Many plants have been used in traditional medicine as treatments and remedies for various diseases such as inflammation, pains, diabetes, cancer, tumours, hypertension, ulcers, etc., due to the presence of secondary metabolites like alkaloids, saponins, flavonoids, tannins, cardiac glycosides, triterpenoids and phytosterols, and one of such plant is *Haematostaphis barteri* Hook. f.

Haematostaphis barteri commonly called 'blood plum' belongs to the family *Anacardiaceae*. It is a woody plant

that is mostly found in west tropical African countries like Nigeria, Ghana, Ivory Coast, Senegal and other countries like Sudan, South Africa and Congo (Boampong, 2015). It is locally called 'Tsamiyar Lamarudu' in Hausa language. The leaf of the plant contains phytoconstituents such as saponins, tannins, alkaloids, flavonoids (Ezekiel *et al.*, 2016). Its stembark extract has been acclaimed to be used in ethnomedicine for the treatment of inflammations (both acute and chronic), pains, ulcers, diabetes, anaemia, typhoid and malaria fevers (Samadori *et al.*, 2017).

Despite the enormous benefits derived from medicinal plants and their products, there still exist some problems which are associated with the use of plants in human healthcare. These problems include toxicity of some plant parts, uneven distribution of drugs (either from plants or synthetic), inaccurate targeting of disease tissues or organs

by therapeutic agents, negative effects of the drugs on healthy tissues or organs and delayed treatment periods of medicinal plant extracts (Alamgir, 2017). Therefore, to overcome these setbacks and improve the potency of therapeutic agents thereby lowering the side effects, better targeted therapeutic measures as carriers that can deliver drugs specifically to disease tissues or organs are needed, and one such is chitosan nanoparticles (CSNPs). Nanotechnology as a science has provided suitable means for targeted as well as sustained-release of drugs in a controlled manner (Soutter, 2013).

Drug delivery system refers to a method of drug delivery that specifically targets organs, tissues, subcellular organs and cells using different types of carriers called nanocarriers for controlling the release of drug contents as well as absorption to improve their pharmacological potentials, enhance bioavailability and biodistribution as well as eliminate their toxic side effects (Vilasaliu *et al.*, 2013). It has been reported that polymer-based drug delivery systems will improve greatly the current treatment of diseases like pains, inflammations, sexually transmitted infections and cancers because of their ability to navigate through many of the body's biological barriers unnoticed. Among these polymers are chitosan nanoparticles which is a natural polysaccharide ranging from 1-1000 nm in size made from shells of crabs, shrimps, crayfish and chitins of insects like grasshoppers (Desai and Park, 2005).

Chitosan nanoparticle is chemically composed of β -(1,4)-linked N-acetyl glucosamine units obtained by the deacetylation reaction of chitin using strong alkalis such as sodium hydroxide at 50 °C. It has numerous advantages over other polymers used in drug delivery such as biodegradable, bioavailable, target specificity, and non-toxic (Younes and Rinaudo, 2015). Chitosan has been used as a carrier for various drugs such as anticancer, antitumor, antibacterial, antifungal and antidiabetic agents (Javid *et al.*, 2013).

This study was carried out to prepare chitosan nanoparticles from crab shells and evaluate the analgesic and anti-inflammatory activities of chitosan nanoparticles loaded with methanol stembark extract of *Haematostaphis barteri*.

MATERIALS AND METHODS

Collection, Identification and Preparation of Plant Material

The bark of *Haematostaphis barteri* Hook. F. was collected from a forest in Maiduguri in June 2022. The plant was identified by Dr. C.A. Ukwubile of the Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, Nigeria. A voucher specimen number of UMM/FPH/ANC/005 was deposited at the herbarium of the Department of Pharmacognosy. The stembark was air-dried under shade for two weeks until constant weight was obtained. The stembark was then reduced into fine powder using an electronic blender (Model: P500, Japan), weighed and 1000 g of it was extracted using 2.5 Litres of absolute methanol (Sigma

Aldrich, St. Louse Mo, USA) by cold maceration technique for 72 hours (State *et al.*, 2023). The filtrate was then evaporated in *vacuo* using a rotary evaporator (ThermoFisher, UK) at 64.7 °C to yield a dark-green gel-like extract with a percentage yield of 14.2 %. The extract was then stored in a sample bottle after weighing for further usage.

Phytochemical Screening of Extract

The qualitative phytochemical screening of *H. barteri* methanol stembark extract was carried out to detect the presence or absence of some secondary metabolites such as alkaloids, flavonoids, saponins, tannins, triterpenes/steroids, cardiac glycosides, anthracenes, etc. using standard procedures previously described (Baeshen *et al.*, 2023).

Preparation and characterization of chitosan nanoparticle loaded with H. barteri stembark extract

Briefly, crab shells were collected from a stream bank in Jaji Igabi Local Government Area, Kaduna State. They were cleaned from dirt and meat left on the skin. The shells were washed thoroughly with water several times and dried in an oven at 40 °C for 3 hours and coarsely ground using an electronic blender. Chitin was then isolated from crab shells using deproteination, demineralization and decolourization processes by adding 100 mL of NaOH to the content at 100 °C for 5 h. The solution is separated from the residue and the residue is neutralized by washing with distilled water several times. The residue (chitosan) was then dried in an oven at 60 °C for 4 hours. After drying, the residue is finely ground and sieved with a size of 20 mesh. Exactly 70 g of the chitosan powder was dissolved in 100 mL of deionized water and 40 g of plant extract was added (Ukwubile, 2015). The content was stirred using a magnetic stirrer at 3000 rpm for 30 min. A cross-linker sodium tripolyphosphate (TPP) was then added dropwise under constant magnetic stirring. The content was stirred for another 10 min, and the suspension was then centrifuged to separate the nanoparticles from unreacted chitosan and TPP. Finally, the chitosan NP pellets were then suspended in water and freeze-dried to obtain the formulated chitosan nanoparticles (Kumar *et al.*, 2011). The formulated microsphere was then characterized in terms of %yield, particle size, % entrapment efficiency, morphology, swelling index and drug release (Guo *et al.*, 2020).

Characterization of formulated drug-loaded chitosan nanoparticles

The percentage yield and entrapment efficiency were calculated concerning the initial and final weights of the drug, zeta potential was determined using the Malvern Zetasizer equipped with high-resolution fluorescent lamps (Malvern apparatus, USA), the pH was determined using the pH meter (ThermoFisher Tech., UK), morphology was carried out using the scanning biological microscope, drug release was evaluated using the dialysis tubation method and the swelling index was determined using the final and

initial volumes of drug-loaded chitosan NPs in measuring cylinder (Guo *et al.*, 2020).

Experimental animals

Sixty (60) Wistar albino rats weighing 100-150 g were purchased from PJ Rats Farm Ltd, Jos, Nigeria. They were allowed to acclimate for one week in the laboratory at 15 ± 1 °C temperature, 45 ± 5 % humidity, and 12 h light/dark light cycle before use. The rats were allowed free access to food and water.

Acute oral toxicity testing

An acute oral toxicity study was conducted using the Organization for Economic Co-operation and Development (OECD) 425 test guidelines in rats' method which emphasized the use of fewer animals. Briefly, five Wistar rats of opposite sex weighing between 100-150 g were randomly selected and administered a limit dose of 5,000 mg/kg body weight (b.w.) extract orally. The rats were monitored for signs of toxicity for 24 h with much emphasis on the first 4 h and then for 14 days. The animals were sacrificed at the end of this period (Khalifa, 2022).

Evaluation of analgesic activity

The acetic acid-induced writhing method was used. Briefly, the rats were randomly grouped into five groups of five rats per group as follows:

Group I was the normal control which received 10 mL of distilled water orally,

Group II was the positive control which received 50 mg/kg b.w. Ibuprofen orally,

Group III received 100 mg/kg b.w. chitosan-loaded H. barteri stembark extract (CSHBE) orally,

Group IV received 200 mg/kg b.w. H. barteri stembark extract (HBE) orally,

Group V received 400 mg/kg HBE orally.

After 30 min, 0.1mL of 1% acetic acid (v/v) solution was administered (i.p.) to the animals. They were then observed for abdominal writhing after a 5-minute cut-off time at 30, 60, 90 and 120 min by turning them upside down and viewing them using a magnifying lens (Lima Bezerra *et al.*, 2022). The inhibition of writhing was calculated from the formula below:

$$\% \text{ inhibition} = \frac{\text{N\textsubscript{o} of writhing in control group} - \text{N\textsubscript{o} of writhing in treated group}}{\text{N\textsubscript{o} of writhing in the control group}} \times 100$$

Evaluation of anti-inflammatory activity

The carrageenan-induced paw oedema in the rat model was used. The rats were grouped as described above. They were then injected 0.1mL carrageenan in 1 % normal saline

solution into the sub-plantar region of the right hind paw. The paw oedema diameter was then measured using a Vernier caliper at 0, 1, 2, 3, and 4th hours (Yimer *et al.*, 2020). The inhibition of paw oedema diameter (mean) was calculated from the formula below:

$$\% \text{ inhibition} = \frac{\text{N\textsubscript{o} of paw diameter in control} - \text{N\textsubscript{o} of paw diameter in treated}}{\text{N\textsubscript{o} of paw diameter in control}} \times 100$$

Ethical Statement

The handling of these animals strictly follows the ethical guidelines for the use of animals in research. The approval for the use of these animals was issued by the Animal in Research Committee of the PJ Rats Farm Ltd, Jos, Nigeria with approval number PJR/0056-RT/2023.

Data Analysis

The data obtained from the study were subjected to analysis of variance (split plot ANOVA) followed by Dunnett's post hoc test. Data were expressed as mean \pm SD. The value of $p < 0.05$ was considered statistically significant. Data analysis was carried out using SPSS version 23 statistical software.

RESULTS

The phytochemical analysis of the methanol stembark extract revealed the presence of carbohydrates, alkaloids, saponins, tannins, flavonoids, cardiac glycosides, triterpenes, and steroids while anthraquinone was absent (Table 1). Furthermore, the prepared chitosan nanoparticle showed interesting characteristics such as minimal particle size of 201 ± 1.01 nm, zeta potential of 25.4 ± 1.02 V, cumulative drug release of 88.24 %, swelling index of 58.14 % and entrapment efficiency of 68.4 % (Table 2). The result also revealed that chitosan nanoparticles loaded with stembark extract (CSHBE) released more drugs at a more acidic pH (3.5) following first-order kinetic (Figure 1). The morphology of formulated chitosan nanoparticles was round as revealed by scanning biological microscope (Figure 2).

From the acute oral toxicity testing, the extract did not produce any sign of toxicity and mortality after two weeks of extract administration of 5000 mg/kg dose. There was no body itching, reddish eyes, reduced food intake, etc. The extract was well tolerated in the rats at the maximum dose investigated suggesting the safety of the extract. The study also showed that chitosan-loaded extract and drug significantly reduced the number of writhing in the analgesic study and inhibition of paw oedema diameter in carrageenan-induced paw oedema. In all, a dose-dependent activity was observed in groups treated with *H. barteri* stembark extract but chitosan-loaded extract produced better activities (Figures 3 and 4).

Table 1: Phytochemical constituents of *H. barteri* methanol stembark extract

Constituent	Tests	Observation	Inference
Carbohydrate	Molisch	Blue-black	+
	Barfoed's	Blue	+
Saponin	Frothing	25 min	+
	Haemolysis	Bursting of red blood cell	+
Anthraquinones	Bontrager	No colour change	-
	Mod. Bontrager	No colour change	-
Cardiac glycosides	Keller- kiliani	Brown ring	+
	Kedde's	Red colour	+
Flavonoids	Shinoda	Red colour	+
	NaOH	Yellow colour	+
Tannins	Ferric chloride	Pink colour	+
	Lead subacetate	Red ppt	+
	Goldbeater's	Black colour	+
Alkaloids	Dragendorff's	Orange ppt.	+
	Mayer's	Creamy ppt.	+
	Wagner's Test	Cloudy ppt.	+
Triterpenes/Steroids	Salkowski	Red colour	+
	Lieberman-Burchard	Reddish-brown	+

Note: + means present or detected, and - means absent or not detected.

Table 2: Preparation and characterization of chitosan NP-loaded *H. barteri* extract

Parameter	Value (unit)
Yield	26.54 %
Particle size	210.0 ± 1.01 nm
Zeta potential	24.4 ± 1.02 V
CDR (6 h)	88.24 %
Drug entrapment	68.40 ± 2.01 %
Swelling index	58.14 ± 1.24 %

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

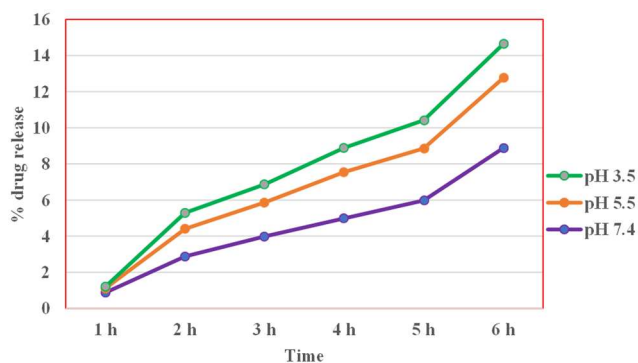


Figure 1: Effect of various pH on amount of drug released by formulation code CSHBE4 in 6 hours as obtained from in vitro release study.

DISCUSSION

The presence of certain metabolites in the methanol stembark extract of *H. barteri* was responsible for the observed biological activities of the plant. This is because metabolites such as alkaloids, flavonoids and cardiac glycosides have been reported to exert analgesic and anti-inflammatory effects by inhibiting pro-inflammatory factors like cyclooxygenase enzymes, histamines, serotonin and prostaglandins (Alamgir, 2017). Also, the study has shown that administration of the extract at a maximum dose was very safe since a median lethal dose

greater than 5000 mg/kg b.w. was biologically unimportant (Khalifa, 2022).

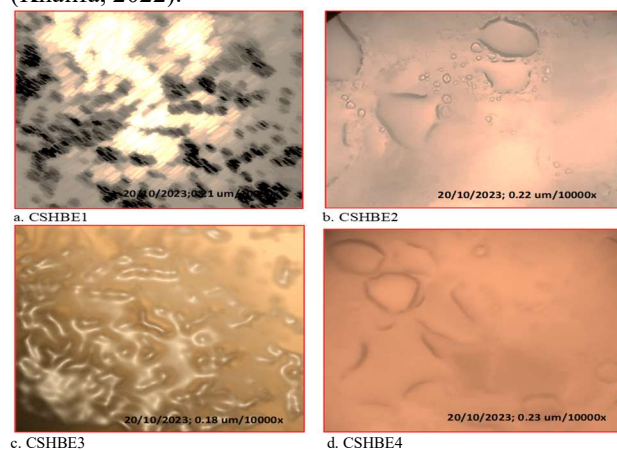


Figure 2: Morphology of various (CSHBE1 to CSHBE4) formulated chitosan nanoparticles loaded with *H. barteri* stembark extract as viewed by scanning biological microscope. Note: Formulation code CSHB1 was not cross-linked with sodium tripolyphosphate (TPP), codes CSNBE2, CSHBE3 and CSHBE4 were cross-linked with TPP. Formulation code CSHBE4 was selected for the current study.

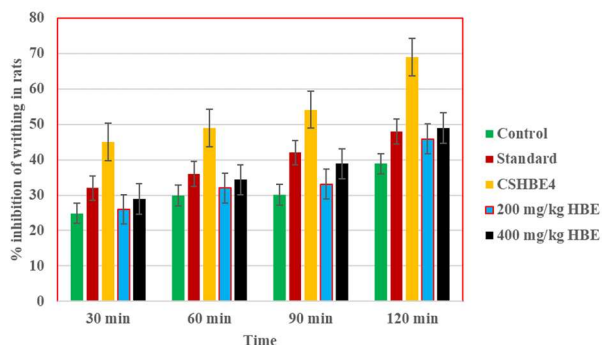


Figure 3: Effect of *H. barteri* methanol stem bark extract on acetic acid-induced abdominal writhing in rats. Results are mean \pm SD (n = 5). The value of $p < 0.05$ was statistically significant when compared with the control using split plot ANOVA followed by Dunnett's post hoc test.

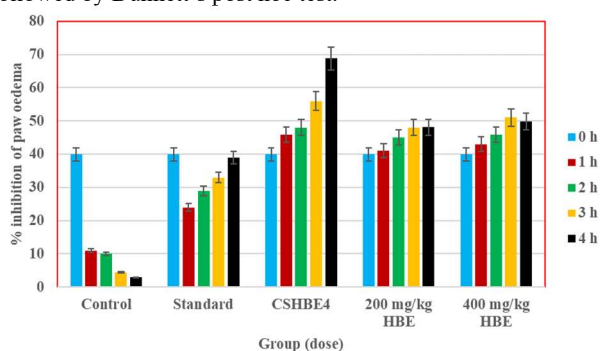


Figure 4: Effect of *H. barteri* methanol stem bark extract on carrageenan-induced abdominal paw oedema in rats. Results are mean \pm SD (n = 5). The value of $p < 0.05$ was statistically significant when compared with control using split plot ANOVA followed by Dunnett's post hoc test.

Chitosan nanoparticles are characteristically useful in drug delivery because of their small particle sizes, which provide a large surface-to-volume ratio, and physicochemical properties that may differ from that of their other nanoparticles (Guo *et al.*, 2020); thus, chitosan is generally used in biomedical applications and, particularly, as vehicles for drug and gene delivery into tissues and organs (Jha and Mayanovic, 2023). In this study, the formulated chitosan nanoparticles-loaded *H. barteri* extract characterized possessed a small particle size of less than 1000 nm, high drug entrapment efficiency, round-shaped morphology and release profiles. These parameters were responsible for the ability of nanoparticles to effectively deliver drug agents due to their ability to pass through the bloodstream unnoticed by endothelial cells in the body (Desai and Park, 2005). Similarly, the ability of chitosan nanoparticles in the current study to maintain a sustained release drug delivery at acidic conditions (pH 3.5) from the *in vitro* study conferred it with potential therapeutic potential over other treatment strategies as seen from the study. Chitosan nanoparticles are increasingly being used as carriers for various drugs as delivery agents with zero side effects (Guo *et al.*, 2020). This is similar to the current study where it was used to deliver analgesic and anti-inflammatory agents.

An acetic acid-induced abdominal writhing test was used to detect the peripheral analgesic effect of the stem bark extract due to its ability to detect the analgesic activity of

medicinal plants and other drugs at doses which remain inert for other assays (Fyad *et al.*, 2020). The acetic acid-induced writhing test is a recommended model for evaluating the peripheral analgesic effect of plant extracts and drugs (Yimer *et al.*, 2020). In this study, intraperitoneal injection of acetic acid results in irritation and stimulation of the peritoneal cavity of the rats which triggers the synthesis and release of some endogenous pro-inflammatory mediators such as serotonin, prostaglandins, histamine and bradykinins (Yimer *et al.*, 2020). These pro-inflammatory mediators triggered chemically-induced visceral pains marked by constriction of abdominal muscles (writhing), that is why the acetic acid-induced writhing test was considered a model of visceral pain (Yimer *et al.*, 2020). In the present study, chitosan nanoparticles loaded with *H. barteri* showed the highest inhibition of abdominal writhing when compared with other treatments. This is due to the aforementioned characteristics of the formulated chitosan nanoparticles in the current study.

Carrageenan-induced paw oedema is a model which is used to assess the anti-inflammatory effects of plant extracts and products as well as to evaluate the mechanisms involved in inflammatory activity. This method is a phlogistic, non-antigenic agent and is devoid of noticeable systemic effect and believed that the experimental model showed a high degree of reproducibility in acute phase inflammation (Yimer *et al.*, 2020). In the current study, induction of acute inflammation by sub-planar injection of carrageenan in the hind paw of the rats produced a dose-dependent reduction in paw oedema volume or diameter with increasing time. A much greater reduction of paw oedema volume was witnessed in a group treated with chitosan nanoparticles loaded with *H. barteri* stem bark extract when compared with other treatment groups. The result obtained showed that there was a statistically significant difference ($p < 0.05$) between the chitosan nanoparticles-loaded extract and the control group. The ability of the chitosan-loaded extract to greatly inhibit paw oedema volume was due to inhibition of the synthesis of inflammation mediators (Yemitan and Adeyemi, 2017).

Conclusion

The study showed that *Haematostaphis barteri* methanol stem bark extract possessed potential analgesic and anti-inflammatory activities in rats due to the presence of phytochemicals in the extract. However, biological activities were observed in chitosan nanoparticles-loaded extract due to the characterized parameters seen in the nanoparticles making it an excellent carrier of analgesic and anti-inflammatory agent. It is recommended that the potential of the methanol stem bark extract of *H. barteri* should be further evaluated to isolate the bioactive compound(s) responsible for the observed biological activities in the current study for potential drug discovery.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

UCA: Designed the study, experimented, interpreted data, and manuscript draft; **IEO:** Performed experiment, provided chitosan source, and analyzed data; **MHH** and **IBB:** Performed experiment, reviewed manuscript, and performed literature search. All authors read and approved the final manuscript for submission.

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