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Antimicrobial Activity and Characterization of *Annona Muricata* Linn (Annonaceae) Leaf- Loaded Chitosan Nanoparticle against Cancer Associated Microbes

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Abstract:

Background: *Chitosan have been used to transport drug into an acidic environment where the chitosan packaging will degrade, releasing the drug onto the desired environment.*

Objective: *This research was aimed at evaluating antimicrobial properties as well as characterization of Annona muricata loaded chitosan nanoparticles on selected cancer associated microbes in Nigeria.*

Methods: *Annona muricata chitosan nanoparticles (AMCS), a Nano-seized drug carriers, were prepared by ionic gelation of chitosan and sodium tripolyphosphate (TPP) anions. Antimicrobial properties and characterization of A. muricata chitosan formulation of leaf methanol extract were evaluated on ten cancer associated microbes; Bacillus subtilis NCTC8239,Salmonella typhi ATCC9184,Staphylococcus aureus NCTC6571,E.coli NCTC10418,Staphylococcus aureus (clinical isolate), MRSA (clinical isolate), Streptococcus pyogenes (clinical isolate), Klebsiella pneumonia ATCC 10031, Candida albican ATCC 19231 and Staphylococcus aureus NCTC 6571 using agar well diffusion method.*

Results: *AMCS exhibited great antimicrobial properties against all the microbes which were significantly different from that of the broad spectrum antibiotic ceftriaxone 10 mg; USP. Compatibility of A. muricata loaded chitosan nanoparticles as revealed by atomic force and transmission electron microscopes showed that chitosan nanoparticles are effective drug carriers for both antimicrobial and anticancer agents.*

Conclusion: *The study showed that chitosan loaded plant extract of Annona muricata is a sure promise towards treating some infectious diseases such as cancer and microbial infections since it demonstrated more potency on the microbes than a first line antibiotic.*

Keywords: *Antimicrobial, chitosan nanoparticles, Annona muricata, electron microscopes*

1. INTRODUCTION

Annona muricata is a members of the family of custard apple tree called Annonaceae and species of the genus *Annona* known mostly for its edible fruit Annona. *Annona muricata* produce fruit that are usually called soursop due to its slightly acidic taste when ripe [1]. The fruit is juicy, acidic, whitish and aromatic with abundant seeds. The average weight of 1000 fresh seeds is 479g and have an average oil content of 24% [2]. The creamy and delectable flesh of fresh fruit consist of 80% of water, 1% protein, 18% carbohydrate and fair amount of Vitamin B, B2 and C and Potassium and dietary fiber. Other common names of *Annona muricata* include Hausa name (Gwanda Masar); as a result of its widespread cultivation, many local names have developed for the fruit. In English, it is most widely known as a Graviola or soursop as well as a custard apple, especially in India and Australia (custard apple also refers to *Annona reticulata*, a closely related species). Common species in the Annona family include *A. squamosa*, *A. reticulata* and *A. crassiflora* [3].

Chitosan are nanoparticles formed by dissolving or treating shrimp and shells of crustaceans with alkali sodium hydroxide. Based on nanotechnology definition a nanoparticles is a small object that behaves as a whole unit with respect to its transport and properties. They are particles of any shape with diameter of 1 x 10^9 and 1 x 10^7 ranges. Chitosan have been used to transport drug into an acidic environment where the chitosan packaging will degrade releasing the drug onto the desired environment [4]. chitosan properties also allows it to be used in transdermal drug delivery therefore it

is to used extensively in drug delivery [5]. Chitosan nanoparticles can be obtained by deacetylation of chitin, a naturally occurring and abundantly available (in marine crustaceans) biocompatible polysaccharide. Chitosan nanoparticles are a drug carrier with wide development potential and have an advantage/controlled drug release which improves drug solubility, enhances efficacy and reduces drug toxicity because of their small size or microscopic nature of size, they are capable of passing through barriers *in vivo* (such as blood-brain barrier) and delivering drug to the lesion site to enhance efficacy [6]. Many cancers such as ovarian cancer, breast, colon cancer, and liver cancer are often associated with microorganisms especially bacteria, thus promoting metastasis of cancer cells [7]. This research was aimed at evaluating antimicrobial properties as well as characterization of *Annona muricata* leaf extract loaded chitosan nanoparticles on selected cancer associated microbes in Nigeria.

2. MATERIALS AND METHODS

2.1. Materials

Chitosan with degree of deacetylation and average molecular weight 86 % and 110 k Da respectively (Sigma Aldrich, Ltd, Canada), methanol extracts of leaves of *Annona muricata*, glacial acetic acid (0.25% v/v), TPP (0.84% w/v), 0.1M NaOH, acetone, phosphate buffer (PBS) solution pH7.4, etc were used. All other reagents and solvents used were analytical grades.

2.2. Methods

2.2.1. Collection and Preparation of Plant Materials

The fruits of *A. muricata* were collected from savannah woodland at Takum L.G.A, Taraba State 60 km from Bali and identified at the herbarium unit of the Department of Biological Science Ahmadu Bello University Zaria, where a voucher number was deposited for the plant. The plant part was air dried for two weeks, and pounded using an electronic blender into fine powders.

2.2.2. *Extraction of plant materials*

200 g of the powdered part was weighed using electronic scale balance into three separating funnels respectively. 500 mL of methanol containing 200 mL of water was poured into the samples using cold maceration techniques. The set up were left to stand for 48 hours to enable the constituents dissolve thoroughly in methanol. The filtrates were collected into a beaker and subsequently transferred into evaporating dish in order to concentrate them. The extract was weighed and percentage yields was calculated and then stored in desiccators for further use.

$$\textit{Percentage yield} = \frac{\textit{weight of extract}}{\textit{Weight of ground plant}} \times 100$$

2.2.3. Preparation of chitosan Nanoparticle

Chitosan (CS) nanoparticles were prepared by ionic gelation of CS and sodium tripolyphosphate (TPP) anions. CS was dissolved in aqueous solution of glacial acetic acid (0.25% v/v) at concentration of 1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml. 5mL of 0.84% w/v TPP was added under magnetic stirring at room temperature in drop wise using 10 ml syringe needle into 10mL CS solution containing 10 mL of extract. The pH of the solution was adjusted by adding 0.1M NaOH solution to the CS complex and stirred for 30 min on magnetic stirrer. CS complex was centrifuge at 12000 rpm for another 30 min and decanted. The supernatant was kept, and CS complex was washed with 20 ml acetone, and dried in an oven at 40^0C overnight for onward usage. Five formulations were performed for the plant extract (leaves only).

2.2.4. Characterization of Nanoparticles

% Entrapment: Re-dispersed CS nanoparticle was centrifuged at 1500 rpm for 40 min at 25^0C to separate the free drug in the supernatant. The concentration of extract was determined using UV-Spectrophotometer (Model 840, Japan) at 266nm after dilution with PBS (pH 7.4) . Calculated by the formula:

$$\% \text{ entrapment efficiency (\%EE)} = \frac{\text{experimental drug content}}{\text{Total drug content}} \times 100$$

Yield of Nanoparticles: Nanoparticles were freeze dried, collected and weighed. Yield of nanoparticles was calculated using:

$$\% \text{ Yield} = \frac{\text{weight of dried CS}}{\text{Initial dry weight of starting material}} \times 100$$

Values were in triplicate, i.e. weight of CS, extracts and CS loaded chitosan.

Particle Size Of Nanoparticle Using Zeta Sizer: CS nanoparticle was diluted with 0.1M KCl and then placed in an electrophoresis cell with an electric field potential of 15.4 v/cm applied using zeta sizer (Malvern zeta sizer 3000HS, UK). Analysis was done after three consecutive readings. Photon correlation spectroscopy (PCS) was thereafter used to determine particle size distribution by adding the particle dispersions into the sample dispersed unit and stirred to reduce the aggregation between the nanoparticles. The average volume to mean particle size was measured after three readings.

In vitro drug release study: To study *in vitro* drug release, dialysis tube with an artificial membrane was used. Prepared CS nanoparticles were re-dispersed in 5 ml PBS (p H 7.4) and subjected to dialysis by immersing the dialysis tube to the receptor compartment containing 150 mL PBS. The medium in the receptor was agitated continuously using magnetic stirrer, and maintained $37^0C \pm 1^0C$. 5 ml of the sample was then removed from the receptor compartment at intervals of 5 min for 8h and 5 mL fresh buffer was replaced each time. Amount of drug released was determined using spectrometric method at 266 nm [8].

Kinetic Modeling: To understand the kinetic and mechanism of drug release, the result of *in vitro* drug release study of CS nanoparticles were fitted with various kinetic equations i.e. zero order and first order [8].

Morphological study of CS nanoparticles: Morphology of CS nanoparticles were determined to examine the particle size and surface morphology using transmission epifluorescence microscope (500,000x objective, Olympus) and inverted biological electron microscope at scanning view. CS nanoparticles were dissolved in 5 mL diethyl pyrocarbonate treated water and mount in osmium tetra oxide, then viewed. Various photographs of CS nanoparticles under each microscope were taken to compare the morphology and sizes.

Stability studies: Formulations 2 (FM-2) was divided into three sets of samples and stored at 4^0C in refrigerator for 12 h and transferred to a 75 % relative humidity controlled oven overnight. After 60d reading, drug contents of all samples were determined by the method as in drug content described earlier on.

2.2.5. Evaluation of Antimicrobial Activity

Antimicrobial activity of AMCS was determined using a modified Kirby-Bauer [11] disc diffusion method. Briefly, 100 µL of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10^8 cells ml^{-1} for bacteria, or 10^5 cells mL^{-1} for fungi. 100 µL of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. The extract was tested using 6 mm sterilised filter paper discs. Discs were impregnated with 10 µL of the test sample, allowed to dry and placed onto inoculated plates.

The plates were allowed to stand at 4 °C for 2 hours before incubation with the test microbial agents and were incubated at 30 ° C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *B. subtilis*, *E. coli*, *S. pyogenes* and *S. aureus* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. *C. albican* inoculated plates were incubated at 25 ° C for 48 hours then the zones of inhibition were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this report. Standard discs of ceftriaxone 10mg/ml was obtained from Oxide Ltd. and served as positive controls for antimicrobial activity. For fungi, nystatin discs (100 µg, Oxide Ltd.) were used as a positive control. Filter discs impregnated with 10 µL of distilled water were used as a negative control.

Statistical Analysis

Results were analyzed as means ±SEM for replicate reading as well as one-way analysis of variance (ANOVA) using SPSS software version 20.

3. DISCUSSION

The antimicrobial activity of the *A. muricata* loaded chitosan nanoparticles (AMCS) was further investigated by bacterial growth time course assays in the presence and absence of the extract. The concentration of AMCS used in these assays was 41.7 mg/ml. *A. muricata* leaves CS nanoparticles was able to significantly inhibit all the microbes growth within 1 h indicating a rapid antimicrobial action. *E. coli NCTC10418* (table 4) growth was the least inhibited by AMCS fruit extract, in agreement with previously reported results [9-15].

Precisely speaking, these studies and previous studies within this laboratory are the first studies that have demonstrated the antimicrobial properties of *A. muricata* leaves CS nanoparticles. Both Gram-positive and Gram-negative bacteria were susceptible to AMCS greatly when compared with ceftriaxone 10mg,USP. The broad range of microbial susceptibilities is dose dependant in the formulations (table 1-3 and fig. 1a,b) and it indicates the potential of AMCS as a surface disinfectant as well as for medicinal purposes and possibly as drug carries to inhibit microbial growths in cancerous cells, and stop metastasis of cancer cells to neighboring tissues as often seen in cancer patients[16-21]. Drug releasing ability of AMCS is controlled as the contents were not released at the same time (tables 2 and 3), which gives the formulations a greater antimicrobial potentials over the standard antibiotic(table 4).

4. RESULTS

Table1. *Formulation and Characterization of A. Muricata Loaded CS Nanoparticles*

Batch code	Amount of CS (mg)	Drug: Polymer	Particle size (nm)
FM-1	10	1: 1	400 ± 5.04
FM-2	10	1:2	441 ± 4.20
FM-3	10	1:3	509 ± 8.90
FM-4	15	1:4	524 ± 10.50
FM-5	15	1:5	668 ± 10.70

Results are mean ± SEM, FM 1-5 are formulations for Annona muricata loaded CS

Table2. *% Entrapment and % Drug Release*

Batch code	% Entrapment	Cumulative % drug released in 2½ h (%)
FM-1	58.75 ± 0.23	85.43
FM-2	62.38 ± 0.56	82.6
FM-3	68.80 ± 0.58	78.34
FM-4	72.50 ± 0.42	76.03
FM-5	78.85 ± 0.37	74.64

**Results are mean ± SEM, FM 1-5 are formulations for Annona muricata loaded CS*

Table3. *Correlation Coefficients According to Different Kinetic Equations*

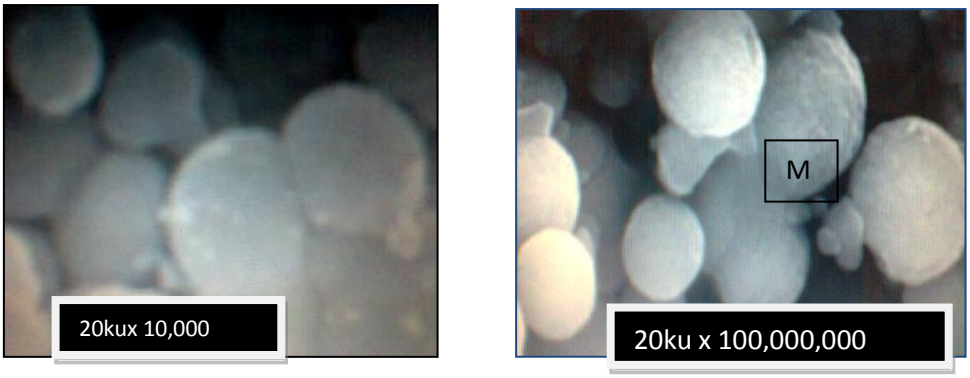
Batch code	% Cumulative drug released	Zero order	First order
FM-1	85.43	0.794	0.982
FM-2	82.6	0.903	0.991
FM-3	78.34	0.793	0.988
FM-4	76.03	0.826	0.965
FM-5	74.64	0.791	0.981

**FM 1-5 are formulations for Annona muricata loaded CS*

Table4. *Diameter Zone of Inhibition of Ethanol of Annona Muricata Extract Loaded Chitosan Nanoparticles (Mm) Against Microbes*

Test microorganism	Extract/Chitosan (AMCS) (mm)	Standard Antibiotic (Ceftriaxone,10mg USP) (mm)
Bacillus subtilis NCTC 8230	24.0±0.12	22.0 ± 0.08
Salmonella typhi ACTC 9184	32.5±0.10*	29.0 ± 0.09*
Staphylococcus aureus NCTC 6571	31.5±0.18*	14.0±0.07
Escherichia coli NCTC 10418	22.0± 0.08	21.5±0.10
Staphylococcus aureus (CI)	40.0 ±0.21*	13.0± 0.07
MRSA (Clinical isolate)	46.56 ±0.22**	16.04±0.10
Klebsiella pneumonia ATCC 10031	25.98±0.12	14.10±0.07
Candida albican ATCC 19231	20.18±0.09	18.78±0.10
Staphylococcus pyogenes (CI)	28.12±0.14	17.90±0.08
Staphylococcus aureus ATCC 13704	32.20±0.18*	14.66±0.06

*Results are means ±SEM for triplicate readings, AMCS(Annona muricata chitosan nanoparticles), MRSA(Methicillin Resistance Staph. aureus), CI(Clinical isolates), *P ≤ 0.05, **P ≤ 0.01; One-way ANOVA; statistical significant difference*



a. TEM image b. IBEM image

Figure1. *TEM/IBEM Images of a. Muricata Loaded CS Nanoparticle; TEM (Transmission Epifluorescence Microscope), IBEM (Inverted Biological Electron Microscope), M (Microspheres)*

Antibacterial activities of *Annona muricata* chitosan nanoparticles were high on the selected microorganisms than ceftriaxone a first line antimicrobial agent. The resistance (MRSA) strain was affected most by AMCS. See table 4:

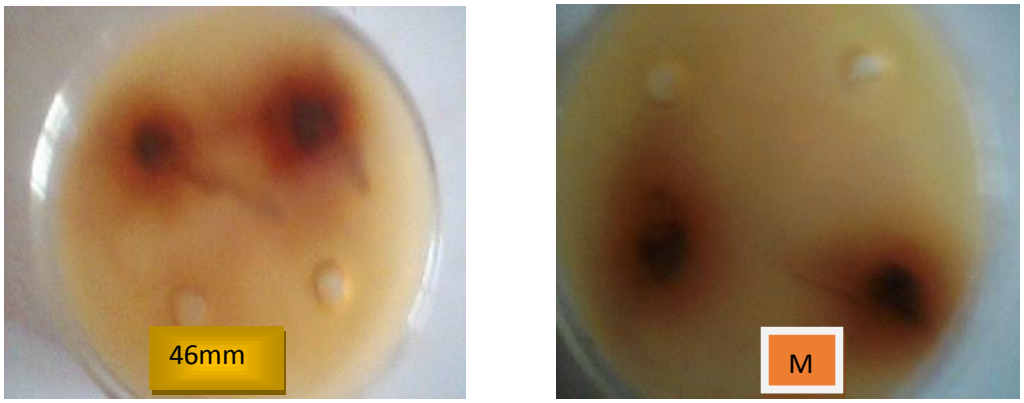


Figure2. *Chitosan Nanoparticles Disc Zone of Inhibition Against Microbes; M (Zone Of Inhibition)*

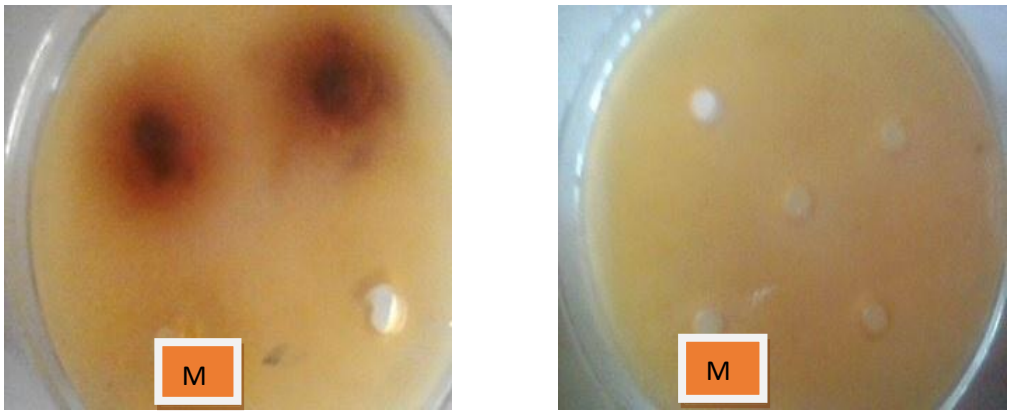


Figure3. *Diameter Zone of Inhibition of Standard Drug; Ceftriaxone, 10 Mg, USP, M(Zone of Inhibition)*

5. CONCLUSION

The results obtained showed that *Annona muricata* loaded chitosan nanoparticles from the leaves have antimicrobial effect on tested organisms which are causative agents of metastasis in cancer cells. Hence, chitosan loaded plant extract from the leaf could be a source of synthetic drug carrier for antimicrobial and other diseases caused by the species of microorganism.

In the table 1, as the concentration of polymer increase, the particles size also increase. This is very consistence with shapes as seen in scanning electron and atomic force microscopes in the figure 1 below.

Cumulative drug release by chitosan was not holistic. This makes chitosan nanoparticles as efficient drug delivery tool in cancer tissues. See table 2 and 3 below: Entrapment percentage increased from

FM1-5 while cumulative drug released decreased. The reason is that since chitosan are well absorbed by the endoplasm of humans, radical release of their contents is not possible to achieve the desired effect.

The spherical nature of the microspheres showed that the plant extract and chitosan mixed properly and were very compatible. See figure 1.

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Conflict of Interest

We have no conflicts of interest.

Research Locations

Department of Pharmaceutics and Microbiology, University of Ghana School of Pharmacy, Legon Accra and Biology Laboratory, Science Laboratory Technology Department, Federal Polytechnic, Bali, Nigeria.

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