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TOXICOLOGICAL EVALUATION OF SNAIL MUCINS BIOPOLYMER FOR POSSIBLE BIOMEDICAL AND PHARMACEUTICAL APPLICATIONS

[1]Alfa, J., [1]Okeke, C. I., [2]Hanifah, A., [3]Okolo, M. O., [3]Saliu, H. M., Musa, A., [3]Agieni G., [4]Romnus, O. C., [5]Augustus, E. and [1,3,5*]Mumuni, M. A

[1]Department of Pharmaceutics and Pharmaceutical Technology, Bingham University, Nasarawa State, Nigeria.

[2]Department of Medical Laboratory Science, School of Medical Laboratory Sciences, Usmanu Danfodiyo University Sokoto, Nigeria

[3]Department of Microbiology, Faculty of Natural Sciences, Prince Abubakar Audu University Ayingba, Kogi state.

[4]Department of Pharmaceutics and Pharmaceutical Technology, Enugu State University of Sciences and Technology, Enugu, Nigeria

[5]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Nigeria

***Author for correspondence**: audu.momoh@unn.edu.ng or momohmumuni123@gmail.com; +2348037784357

ABSTRACT

The safety of mucin being a new entrance as drug carrier and cosmetic adjuvant needs to be evaluated for its biomedical and pharmaceutical applications. The present study investigated the toxicological effect of the extracted snail mucin on the biochemical and hematological parameters in an animal model. The snail mucin was extracted using organic and aqueous solvents, and was evaluated based on physical parameters like pH, taste and odor. The safety of snail mucin after oral administration was evaluated for any changes in biochemical and hematological indices. There were no obvious changes in the physical, hematological and liver enzyme evaluated after dosing the animal with mucin suspension. Taken together, oral administration of various extracts of snail mucin shows no toxicity to any of the parameters evaluated. Overall, the foregoing results suggest that the extracted snail mucin is a potentially safe and promising approach for biomedical and pharmaceutical application.

Keywords: Snail mucin, Toxicity, Biochemical, Hematological

INTRODUCTION

Modification of delivery agents for drug formulation has received much needed boost in recent time as occasioned by COVID-19 pandemic, as dependence on imported excipients is no longer fashionable as every country is battling with their internal needs. From a pharmaceutical point of view, the search for polymer from locally sourced materials was justified and recently received increased attention as to create new entities that are recognized to perform better or different functions compared to the existing materials (Abdullah *et al.,* 2018; Kenechukwu *et al.,* 2017). Chemical or

synthetic based material used in drug delivery have been regarded as potential poison at some point, it is often a matter of doses. More so, some of the most available materials are non-biodegradable, while some have the tendency to interact with the active pharmaceutical agent (API) thereby altering its physicochemical properties (Chinmayee *et al.,* 2015). In some instances, the formulation scientists or pharmaceutical technologists have to carefully and tactically engineer the selection of polymer to avoid its toxicity while maintaining its integrity for effective drug delivery. Despite this approach, residual effects are still considered a challenge to the formulation scientists.

Recently, marine based biomaterials have gained more attention among the researchers in the drug delivery field and very promising results have been established in some quarters. For instance, mucin obtained from African giant snails has formed the frontline agent for drug delivery considering its mucoadhesive potentials and ease of availability (Bansil *et al.*, 2006). Physiologically, the bodies of land snails are characterized by rich mucus which covers their surface, which is said to play a role in preventing the moisture evaporation, helping smooth movements and in protecting the body from mechanical injuries (Amah *et al.,* 2019). This mucous is rich in glycosylated proteins, carbohydrates and is highly viscous, which contributes to the gel-like nature of mucus and also serve as a substrate for polymer attachment through the interaction between the functional groups, hence, forming the basis for their use in drug delivery (Adikwu, 2006).

Scientifically, mucins have been evaluated for any pharmacological properties as well as an inert material for drug delivery (Momoh *et al.,* 2019). Interestingly, aliquot obtained from the coil shell of snails has become an important supplement in daily diets as it claims by the traditional healers to cure diabetes and high blood pressure (Adikwu *et al.,* 2005). Additionally, the potential of snail mucin in cosmetic application as a pigment remover has been reported (Iguchi *et al.,* 1982). In our recent published work, we demonstrated that matrices consisting of snail mucin or snail cyst hybridized with chitosan or polyethylene glycol (PEG) have displayed good and very promising features in drug delivery (Builders *et al.,* 2008). However, despite these impressive capabilities of mucin, some sensitive areas of snail mucin such as mineral and elemental constituents are still begging for more attention so as to ensure its safety in drug carriers for possible human consumption.

Understanding the fact that African giant snail is edible, however, its secretions such as the mucous may contain some toxic substance and could be harmful to human health after consumption, hence the need for its detailed safety evaluation. More so, the general perception that natural products from edible marine creatures of animal origin are non-toxic and without side effects is never true as many toxic substances secreted by these creatures have been proven to be very harmful to humans when consumed (Balabushevich *et al*., 2018; Lawrence *et al*., 2016).

To record the experimental data on the safety of snail mucins, our research group has been focusing on the evaluation of the safety of aqueous and non-aqueous extract of mucin for possible application in biomedical and pharmaceutical application. Thus, this study was designed to prepare some dispersion of the various mucin extracts at different concentrations in aqueous medium by dispersion method. Evidence of the safety of the dispersion on hematological and liver enzyme activity after oral administration on rats was

assessed, while the *in vitro* cells viability study was similarly evaluated.

MATERIAL AND METHODS

Extraction of Snail Mucin (Aqueous and Acetone-based Extracts)

Aqueous extract of snail mucin

Snail mucin (also refers as sialo mucin due to its content in sialic acid) was obtained as previously reported with slight modification (Momoh *et al.,* 2019). Briefly, the mucus secretion of the snail was extracted by gently pressing the snail after it had been removed from its shell until no mucus was secreted. The mucus collected from different snails were pooled together in a container and macerated in water for 24 h at 20 °C to get viscous mucus, which was filtered through a muslin cloth to remove unwanted material. The resultant viscous mucus was then subjected to lyophilization using a lyophilizer (Christ-Alpha 1-2 LD Plus SCIQULP, Germany) at -40 °C. The extracted mucous (aqueous soluble mucin) were collected and pulverized using an end-runner mill (Pascal Engineering Co Ltd, England). The pulverized soluble mucin was collected, labeled as AQ and kept in airtight container for further evaluation.

Acetone-extract of snail mucin

Following the above procedure, chilled acetone was gradually added to the freshly collected mucus and stirred before filtration and drying. The mucin content was then precipitated. The mucin was washed severally with chilled acetone until non-slimy flowing materials were obtained. The resultant precipitate was then exposed to air until all the acetone evaporated and the partially dried residue was lyophilized as stated in the earlier section. The final dried mucin was collected, pulverized using an end-runner mill (Pascal Engineering Co. Ltd, England), labeled AC and stored in an air-tight container. All the extracts were maintained in the temperature control system under standard laboratory conditions necessary to maintain the stability of the mucin.

Characterizations of the Extracted Mucin

Physical evaluation of mucin extracts

The extracts were evaluated by physical appearance, colour, taste, odour and texture. Herein, approximately 1.0 g quantity of the extract were taken and was subjected to colour, odour and texture feeling by an independent observer and the inference regarding the parameters were recorded.

Preparation of mucin dispersion for *in vivo* evaluation

Five different concentrations of aqueous and acetone-based mucin extract were prepared as follows: An approximately of 0.2, 0.4, 0.6, 1 and 2 grams of mucin were dispersed separately in water and homogenized to obtain a dispersion mucin of concentration of 2, 4, 6, 10 and 20 % mg and labeled as AQ1-AQ5, respectively. The same procedure was used in the acetone-based mucin extract and were labeled as AC1-AC5. All the preparations were kept in an air-tight container until used for the study.

Periodic pH evaluation

The pH of the various mucin dispersions was periodically determined by dipping the probe of the meter into a 50-mL beaker containing 20 mL of the sample and the value was taken. All reading was carried out in triplicates and the reading average.

Toxicity evaluation

The effects of the various dispersions of the extracts were examined on the haematological and liver enzyme activities to ascertain the safety of the extracts on the organs of the body after oral administration.

The *in vitro* cell viability study was carried on the mucin extracts.

Protocol for administration of mucin dispersion

A total of fifteen (15) rats of either sex weighing 105-125 g were procured from the Department of Pharmacology and Toxicology, University of Nigeria Nsukka. Animals were housed in standard cages and maintained under standard laboratory conditions. Experimental protocols adopted were based on the Institutional ethical committee for the use of laboratory animals. All the rats used in the study were acclimatized for seven days prior to the study. The rats were randomly divided into three groups of five rats with each group housed in a separate cage. The different concentrations of mucin dispersions were measured 5 mL and were administered orally to the animals. Animals in group I and II received 5 ml of 10 and 20 %w/v of the dispersion according to their body weight, respectively. Rats in group III received 5 ml of distilled water (served as control). All administration was done once daily orally for a period of 14 days. Blood samples were collected from the orbital sinus of the eye with the aid of a capillary tube from the animal 72 hours after the last dose and were used for the hematological and liver enzyme evaluations.

Hematological study

Hematological analysis was performed using an automatic analyzer (Abacus Junior, Model S/N 111244) according to an earlier work with little variation. Herein, a 2 ml portion of the blood samples collected in EDTA was used. The samples were mixed in a Roller mixer at the rate of 30 per minute for 5 min. The blood was analyzed for white blood cells (WBC), red blood cells count (RBC), hemoglobin count (HGB) and platelet counts (PLT).

Liver function test

For the liver enzyme test, dispersion consisting of 10.0 and 20.0 % w/v as stated above were used in the evaluation. Thus, we used two (10.0 and 20.0 % w/v) from each extract, for instance AQ4 and AQ5 for aqueous, while AC4 and AC5 for acetone extract respectively. The hepatotoxicity was evaluated base on the functionality of the liver enzymes using liver enzyme kits and automated machine (Reflotron-Plus machine, model SN747461, Germany) following the manufacturer procedure. The collected blood sample was analysed for changes in the liver enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase or serum glutamic oxaloacetic transaminase (AST or SGOT), and alanine aminotransferase or serum glutamic pyruvic transaminase (ALT or SGPT) using an automated Reflotron-plus machine (model, SN74746). All the tests were done in triplicates and results were averaged.

Cell viability study

The cytotoxicity study on the cells, this was evaluated based on the use of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The protocol described by Zhang and co-workers (Zhang *et al.,* 2013) with slight modifications was adopted. In brief, the HT-29 cells were seeded in 96-well plates (1×10^4 cells/well) with 200 µL of growth medium including 10 % fetal bovine serum (FBS) and placed in a humidified incubator with CO_2 5% for 24 h at 37 °C. The resulting monoclonal was seeded with 250 µL of mucin dispersion as the test sample and distilled water served as control was similarly treated with varying concentration from 500 µg/mL. Thereafter, MTT (15 µL from a 2.5 mg/mL solution) was carefully added dropwise to the well and incubated under the same conditions for 4–6 h. Thereafter, dimethyl sulfoxide (DMSO) was

added to dissolve the formazan crystals and the cell viability was determined by measurement of absorbance at 505 nm using a microplate spectrophotometer (Model 20137, BioTek, USA). Data were expressed as the percentage with reference to control groups.

RESULTS AND DISCUSSION

Mucin as a biomaterial have been used in trials for the delivery of drug molecules and an addictive in cosmetic preparation owing to superior advantages, such as biodegradability, biocompatibility, cost effectiveness, high stability and prolonged release effects (Gubitosa *et al.,* 2020; Wong *et al.,* 2013). Considering the advantage of mucoadhesive property of mucin, it has formed frontline research for various biomedical and pharmaceutical applications. It's generally considered safe owing to the fact that it is sourced from African giant snails. However, the need to evaluate its safety after extraction via aqueous or chemical extraction needs to be established despite the proposed theory that it is from edible snails. Here we evaluate the snail mucin extracted by two different solvents by preparing dispersions of mucin with varying concentrations in water and subject it to physical, hematological and biochemical study.

The pH stability of test mucin samples

The stability of a test for mucin samples for a period of three months was evaluated after dispersing in water. As shown in Fig. 2, both samples (aqueous and acetone extracts) had similar pH stability profiles: However, there was a slight decrease in the pH of the aqueous sample (1b) as compared to acetone sample (1a). These results indicate that the aqueous extract may have some oil or lipid traces in its, and consequently, degradation of the left over trace of oil material could degrade into fatty acid leading to the possible decrease in the pH values. Interestingly, mucin obtained based on acetone as the extractive material show somehow stable pH throughout the period. This indicates that acetone as the extractive removed the oily and fatty content of the mucin (Dhanisha *et al.,* 2016). It was observed that the changes in the pH values were not significant enough to alter its pharmaceutical values. However, to be on the safer side, and for its possible application in drug delivery, the selection should be carefully monitored and where aqueous based mucin is selected, then the formulation scientists need to add stabilizing agents to prevent product degradation.

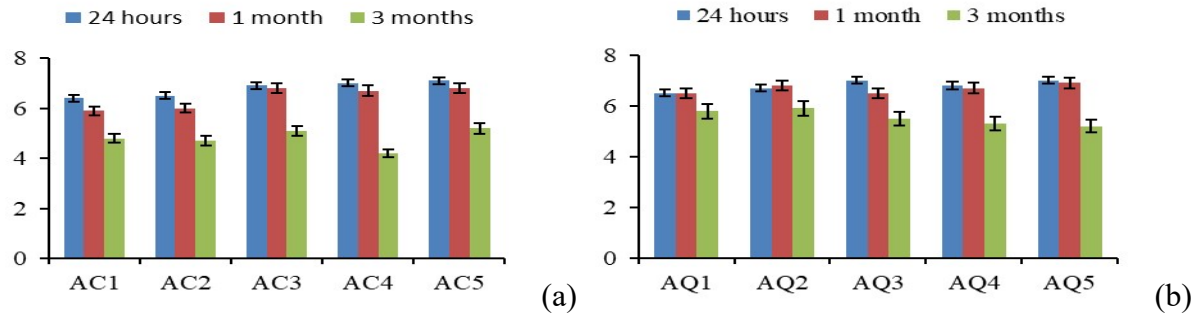

Fig. 1(a, b) Time resolved pH analysis of mucin; (a) AC1-AC5 containing 200, 400, 600,1000 and 2000 mg of acetone mucin extract, and (b) AQ1-AQ5 containing 200, 400, 600, 1000 and 2000 mg of aqueous mucin extract. Note: AC1-AC3, AQ1-AQ3 are sub-acute concentration; while AC4, AC5, AQ4 and AQ5 are acute concentration)

Biochemical analysis

The results of the hematological and liver function test show no significant changes as compared to the standard values. Interestingly, rats administered with water (negative control) shows very similar values to that treated with various mucin extracts, thus indicates no toxic effect observed after the administration of the test agents. However, comparison between the two extracts i.e water extract (WE) and acetone extract (AE), it was observed that the aqueous mucin-based extract was perfectly as good as the standard, indicate no denaturation of the constituents of mucin. This could be ascribed to the fact, that mucous extracted from the snail are completely in it natural forms as there were no chemical added. However, in the area of hematological parameters, there were slight variations in some indices among our measured values compared with established values which were based on international breeders and researchers (Ingle *et al.,* 2011; Perera *et al.,* 2016; Delwatta *et al.,* 2018). The Mean RBC, PCV and WBC and count parameters in the present study were low as compared to the standard values as shown in Table 2. Parameters such as MCHC, MCV were somehow different among the extracts, but the values fall within the acceptable reference values.

Table 1: Effect of (a) Aqueous (b) Acetone Mucin Extracts on Hematological Parameters

Batch[a]	RBC ($\times 10^6$/µL)	HB (g/dL)	WBC (per mm3)	MCV (fL)	MCH (pg)	PLT ($\times 10^5$/ µL)	PCV (%)
200	6.1± 0.1	12.9± 0.9	625± 0.9	78.3 ± 0.9	28.8± 0.1	5.1± 0.2	37.8 ± 0.1
400	4.9± 03	14.3± 0.5	511± 0.1	73.5 ± 2.8	34.2± 0.2	3.7± 0.2	41.3 ± 1.7
600	5.4 ±0.2	11.9± 0.2	625± 0.6	89.7 ± 1.5	36.3± 0.4	2.1± 1.8	39.2 ± 0.9
1000	4.3± 0.5	13.2± 0.9	432± 0.3	63.4 ± 2.1	26.7± 0.8	4.2± 1.1	37.7 ± 0.2
2000	4.9± 0.3	14.6± 0.9	455± 0.1	84.3 ± 3.2	34.4± 0.1	3.0± 0.4	40.3 ± 0.5
Water	5.9± 0.1	11.1± 0.9	585± 0.3	71.9 ± 1.3	33.8± 0.3	4.1± 0.7	42.5 ± 0.4
Ref. value	**3.8-6.68**	**10.4-16.5**	**4400-14 800**	**29.41-123.07**	**18.37-36.98**	**1.7-5.57**	**18-48**

Batch[b]	RBC ($\times 10^6$/µL)	HB (g/dL)	WBC (per mm3)	MCV (fL)	MCH (pg)	PLT ($\times 10^5$/ µL)	PCV (%)
200	5.3± 0.1	13.1± 0.9	695± 0.2	88.0 ± 0.0	38.1± 0.4	4.6± 0.2	39.2 ± 0.3
400	5.6± 0.6	13.8± 0.5	689± 0.3	84.1 ± 2.1	39.2± 0.1	3.9± 0.2	44.3 ± 1.2
600	5.9 ±0.1	10.7± 0.2	702± 0.7	99.2 ± 0.1	39.1± 0.8	3.5± 1.0	43.1 ± 0.3
1000	4.7± 0.2	14.5± 0.9	742± 0.1	83.4 ± 2.1	24.2± 0.2	5.2± 0.1	39.4 ± 0.1
2000	6.1± 0.1	16.0± 0.9	765± 0.1	74.3 ± 0.2	36.3± 0.1	5.0± 0.4	42.6 ± 0.4
Water	5.9± 0.1	11.1± 0.9	585± 0.3	71.9 ± 1.3	33.8± 0.3	4.1± 0.7	42.5 ± 0.4
Ref. value	**3.8-6.68**	**10.4-16.5**	**4400-14 800**	**29.41-123.07**	**18.37-36.98**	**1.7-5.57**	**18-48**

[a]Abbreviations: RBC = red blood cell; HB =hemoglobin; WBC = White blood cells; MCV= mean cells volumen; MCH = mean cell hemaglobin; PLT = platelet; PCV= packed cells volume [b] Batch description: 200, 400, 600 = quantity of mucin administered (subacute concentration of mucin); 1000 and 2000 = amount of mucin administered (acute concentration); water (serve as control); Ref. value =Reference value for the parameters. Note: mucin was measured in (mg); water was measured in (mL).

Table 2. Liver Function Test (LFT)[1] for Aqueous and Acetone-based Mucin Extracts

	Liver function test (aqueous extract)			Liver function test (acetone extract)		
Conc.	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L
1000:	36.2 ±0.01	68.0 ±0.21	121.0 ± 0.11	39.2 ±0.04	98.0 ±0.12	127.0 ± 0.21
2000:	38.5 ±0.13	71.0 ±0.18	119.0 ± 0.52	37.8 ±0.11	69.0 ±0.21	129.0 ± 0.13
Water:	37.0 ± 0.30	70.0 ±0.12	115.0 ± 0.11	37.0 ± 0.30	70.0 ±0.11	115.0 ± 0.11
Reference:	**10 - 40**	**50 -150**	**30 -130**	**10 - 40**	**50 -150**	**30 -130**

[1]Abbreviations: SGPT = serum glutamic pyruvic transaminase; SGOT = serum glutamic oxaloacetic transaminase; ALP = alkaline phosphatase. [2] Batch description: **1000 and 2000 mg of mucin**; water = serves as negative control; Reference = accepted range values for liver enzymes. Note: Reference value and water only applied to liver enzymes study.

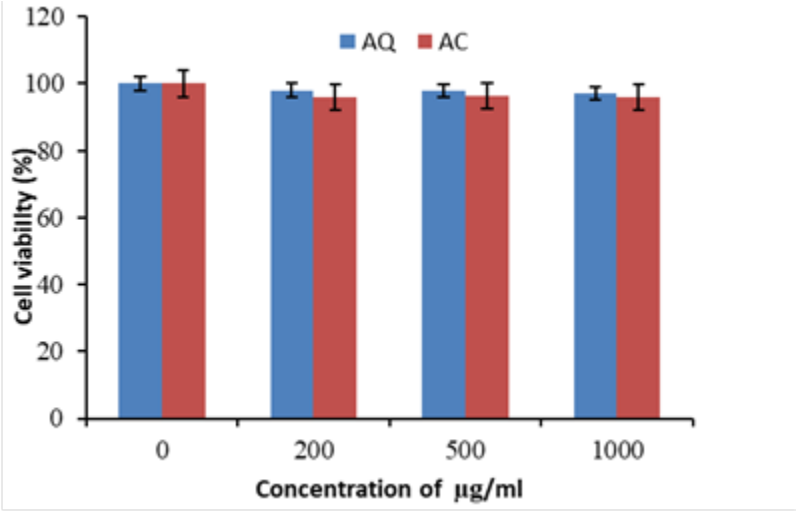

Fig.5. Cell viability profile at different concentration of mucin. Abbreviations: AQ = aqueous extract and AC = acetone extract. Data are presented as mean ± SD (n= 5). Note: mucin is measured in mg.

Cell cytotoxicity as a function of toxicity

The potential application of the different mucin extracts in biomedical fields has been evaluated by evaluating the cytotoxicity on the HT-29 cells. The cell viability of the plain extracts was determined using MTT assay. Three concentrations (200, 500 and 1000 mg) of the mucin extracts were used in the studies and the cells without treatment with mucin were similarly investigated as the negative control. As shown in Fig. 5, the cell viability of the both extracts of mucin were very high and over 90% even at the concentration of 1000 µg/mL indicating that mucin extracted with acetone and aqueous do not significantly affect the viability of the cell at the concentration tested. There was no significant difference between the two extracts. These results demonstrate that the mucin as drug carriers have no obvious cytotoxicity noted and could be used as a possible pharmaceutical application in drug delivery.

Conclusion

Based on our present findings as per its safety, and considering the advantages of mucin as reported in previous drug delivery study with respect to biodegradability, biocompatibility, high stability, high drug encapsulation efficiency, cost-effectiveness and prolonged drug release effects, mucin being a natural polymer would have a good place in pharmaceutical industry. The extracted mucin based on (aqueous and acetone) from snails were subjected to toxicological evaluation for possible pharmaceutical and biopharmaceutical application. Both extracts show no significant difference in all their evaluations. Parameters such hematological and liver enzyme activities are within the standard range and show similar values as per the control batches. However, there is slight variation in the pH value obtained after three months of the dispersion, but not enough to discredit the extract. There were no observable changes in the physical parameters assessed, as both mucins showed no toxicity as per the viability of the cells tested. Based on our current findings, mucin extracted from snails is safe and could serve as pharmaceutical excipient or an adjuvant in drug delivery.

Declaration of Competing Interest

The authors have no conflict of interest regarding this research paper.

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