Some Physiological and Toxicological Properties of Snail Mucin Extracted from *Archachatina marginata*

M. U. Adikwu and P. O. Nnamani
Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria

Corresponding Author: Dr. M. U. Adikwu. Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria

Abstract

Snail mucin was extracted from the giant African snail, Archachatina marginata, Fam. Arionidae, by selective washing with distilled water, precipitation with chilled acetone and lyophilization of the aqueous extract. The allergic and toxicological properties (acute and chronic) and the effect of snail mucin on intestinal motility were investigated. The LD_{50} studies showed the material to be safe especially when given via the oral route. The mucin did not cause serious changes in the haematological picture of the animals. However, there was a significant (P > 0.01) fall in the eosinophil level. However, a significant fall in the oesinophil level was noted. Leucopenia was also observed. There were also no significant (P > 0.01) histopathological changes in the liver and kidneys of rats given the snail mucin. There was a mild congestion in the central vein of the liver of the rats that were given 3000-mg/kg body of the snail mucin. The mucin reduced gastric transit time of charcoal meal.

Key words: Physiological, Toxicological Properties, Snail Mucin, *Archachatina marginata*

Introduction

Edible snails play a role in folk medicine, and a study has shown that glandular substances from the edible snails cause agglutination of certain bacteria, and therefore could be of value against whooping cough and some other diseases (Cheney, 1988). Snails produce mucin. Mucin has different roles in animal bodies, some of which are purely protective (Repentigny *et al*, 2000). Mucus O-linked glycoproteins (mucins) are produced and secreted from the mucus cells of salivary glands, oesophagus, stomach, and small and large intestines, as well as the gallbladder and pancreatic ducts. Mucins have a high intrinsic viscosity due to their large size (2×10^6 Da) and extreme hydrophilicity, and they form a gel-like material, which plays an important role in lubrication of epithelial surfaces and host defense. Many species of commensal and pathogenic bacteria have the ability to bind to and/or degrade mucins. The fate of pathogenic bacteria that bind to mucins can include (i) removal with mucus flow, (ii) colonization within the mucus layer, and (iii) penetration of the mucus and adherence to epithelial cells. Binding sites on mucins may compete with receptors on underlying epithelial cells, thereby retarding access of microorganisms to the mucosal surface and favoring their removal thus microorganisms that attack epithelial surfaces must be able to degrade mucin by producing some enzymes known as mucinases and sialidases (Wiggins *et al*, 2001). This research was aimed at extraction of mucin from African giant snail and studying some of its physiological and toxicological properties in animal models.

Materials and Methods

Materials: The following materials were procured from their local suppliers and used without further purification: potassium chloride, copper sulphate, tragacanth powder, sodium chloride, monobasic potassium phosphate, calcium chloride, charcoal meal, Tween 85, safranin red, Gentian violet, malachite green and aluminium oxide (Merck); ethylene-diamine-tetraacetic acid (EDTA). Distilled water was obtained from a batch in our laboratory, which was prepared from an all glass still. All other reagents and solvents were of analytical grade and were used as such.

Snails: The giant African land snails used were procured from Ibagwa-Nkwo market in Nsukka zone of Enugu State, Nigeria. The snails were collected from the wild. A total of 450 snails were procured and used for the extraction of the mucin.

Animals: The animals used were: guinea pigs, mice, Sprague-Dawley, and albino rats of ten weeks old. The animals were procured from the Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka. The weights of the animals were measured with an animal balance (W.B. Nicolson St. Ltd, Glasgow).

Extraction of snail mucin: After procurement, the shells of the snails were knocked open at the apex and a spirally coiled rod inserted to remove the fleshy body from where the excretory parts were removed. The fleshy parts were then placed in 250 ml of water and washed each time until the mucin was completely washed off. These washings were pooled together in a plastic bucket and precipitated using chilled acetone and then lyophilized. The grayish-brown lyophilized flakes of the mucin were pulverized into fine powder using a mortar and pestle and stored in an air-tight container until used. The yield was 1.8 gm per kg body weight of the snails.

Test for allergic properties

Preparation of stock solution: A stock aqueous solution 4 % w/v of the mucin was prepared such that 1 ml of this solution was added to 4 ml of water to give a five-fold dilution as solution A (0.8 %w/v). Similarly, solution B was prepared as a ten-fold dilution of the stock in the same solvent (0.4 % w/v).

Guinea pig wheal test (Local anaesthetic property): A total of four guinea pigs were used. The guinea pigs were prepared 24 h before the test by first dipping in 70 %v/v ethanol and then shaving the hair on the lower back. The skin sensitivity was greatest in the midline and slightly more in the upper part than in the lower part. A sterile sharp 26 G x 3/8 inch needle was used for each injection. The skin on the lower back of the guinea pig was stretched taut by holding the animal with the hand placed around the abdomen and by pulling the skin taut with the thumb and forefinger. The mucin was injected in the same direction as that in which the skin was being held and the needle inserted into the dermis or epidermis. The volume injected (0.1 ml of solution A and 0.2 ml of solution B) were enough to raise a wheal, which was outlined. Five min after the injection, sensitivity of the area was tested by pricking the skin at the injection site with a needle, six times lightly, and, as a control, the skin as far away from the injected site as possible. Six twitches was recorded for this control test, but the responses at the site of the injection wheal indicated the degree of anaesthesia, which is expressed as the number of negative responses, i.e. of failure to twitch; 6/6 indicates maximum anaesthesia and 0/6 indicates no anaesthesia. The test was repeated at 5 min intervals for a period of 30 min after the injection. The total score for each wheal was added up and expressed as the total number of negative responses out of 36 possible. The above procedure was repeated for the other doses (solution A: 0.25, 0.4, 0.5 ml and solution B: 0.5, 0.8 and 1.0 ml) of the stock solution of the snail mucin.

Toxicological studies

LD_{50} determination (acute toxicity study): This was done using Lorke's method (Lorke, 1983). A total of 24 Swiss albino mice were fasted over night although with access to water. They were divided into four groups of six each and their body weights determined to be 20-25 g. The snail mucin was prepared as a suspension. Three groups of six mice were given different doses of the extract in the order of 1.0, 3.0 and 5.0 g/kg body weight of the mice while the fourth group (control) received only water. These high doses were used since earlier smaller doses ranging from 100 mg/kg to 900 mg/kg body weight showed no death. The number of mice that died in each group within 12 h from the time of administration was noted.

Chronic toxicity study

Haematology: The study was carried out on 24 Sprague-Dawley albino male rats of ten weeks old. The 24 rats were weighed individually on the first day and their weight ranged from 85-95 g. They were put into four separate cages such that each cage contained six rats. They were labelled using safranin red for group A, gentian violet for group B, malachite green for group C and charcoal for group D (control). Their blood samples were collected through the orbital sinus of the rats, using capillary tubes and dropped into labelled EDTA-contained bottles with tight closures and shaken well to prevent clot formation. The blood samples were analysed for packed cell volume (PCV) (Akah and Odita, 2001), erythrocyte sedimentation rate (ESR) (Akah and Odita 2001), total leucocytes count (TLC)) (Akah and Odita, 2001) and differential leucocyte count (DLC) (Akah and Odita, 2001) before dosing with the snail mucin.

The snail mucin was prepared as a 2 % suspension. The rats were dosed in such a way that group A was given 750 mg/kg, group B 1,500 mg/kg and group C 3,000 mg/kg. Group D was given only the tragacanth placebo of 500 mg/kg. This was repeated daily for twenty-one (21) days. At the end of the twenty-first day, the blood samples of the rats were withdrawn again and analysed as before.

Histopathology: The rats were then sacrificed and their livers and kidneys removed and preserved in formalin. These tissues were fixed unto slides, and viewed under a microscope and snapped using a special camera (Microphotograph Machine Leica Galen III, Leica Inc., USA). The films were developed to study the effect of the snail mucin chronically on the tissues of the liver and kidneys and to determine any harmful effect the chronic treatment with the muicn had on the tissues of the named organs.

Gastrointestinal motility test in mice: Fifteen albino mice of either sex (20 – 25 g) were randomly divided into five groups of 3 animals per group. The animals were fasted for 24 h prior to the experiment, but had free access to water. One group received Tween 85 (20 ml/kg), the second group received atropine (10 mg/kg) while the remaining 3 groups received different doses of the snail mucin, (100 – 400 mg/kg). All administrations were by the oral route. Five minutes after drug administration, 0.5 ml of a 5 % charcoal suspension in 3 % aqueous solution of Tween85 was administered to each animal orally. The animals were sacrificed 30 mins later and the abdomen opened. The percentage distance travelled by the charcoal plug in the small intestine (from the pylorus to the caecum) in the treatment groups was determined (Akah, 1989; Akah and Offiah, 1992)

Statistical analysis: The statistical analysis was done using a computer programme, statistical Programme for Social Sciences (SPSS Version 7.5) and Microsoft Excel. The mean values of the tests were compared to those of the control groups and the difference regarded at $P < 0.05$ using the Student's *t*-test.

Results and Discussion

Local anaesthetic property and allergic properties: It was shown that snail mucin had a local anaesthetic potential on the guinea pigs especially in a concentrated form 320 mg/ ml. This is shown in Table 1. This effect may be due to the blockage of nerve impulse from the effector site. As the dose of the mucin increased, the wheal size increased and there was a decrease in the sensitivity to skin pricking. Increase in the wheal size is indicative of potential allergic property. Thus parenteral preparations of snail mucin should be discouraged as it has the potential of precipitating allergic (hypersensitivity) reactions.

Table 1: Guinea pig wheal experiment

S/No of Guinea Pigs	Dose of Snail Mucin	Degree of anaesthesia (mg/ml)
1	80	3/6
2	160	4/6
3	240	5/6
4	320	6/6

A new glycosaminoglycan was isolated from the giant African snail *Achatina fulica* (*Kim et al*, 1996). This polysaccharide may be responsible for the local anaesthetic anaesthetic property and other physiological properties exhibited by the snail mucin. However, this will need further studies. The polysaccharide has been shown to possess other physiologic properties (Kim *et al*, 1996). This polysaccharide has a molecular weight of 29,000 Daltons calculated based on the viscometry, and a uniform repeating disaccharide structure of (1→ 4)-2-acetyl,2-deoxy-α-D-glucopyranose (1→4)-2-sulfo-α-L-idopyranosyluronic acid (1→4). This polysaccharide represents a new, previously undescribed glycosaminoglycan. Glycosaminoglycans (GAGs) are a family of linear anionic polysaccharides that are typically isolated as proteoglycans linked to a protein core. The biological functions of proteoglycans, including the regulation of cell growth, result, in large part, through the interaction of the GAG chains in proteoglycans with proteins, such as growth factors and their receptors (Kim et al, 1996). There are two major classes of GAGs: glycosaminoglycans, including heparin, heparan sulphate, hyaluronic acid, and keratan sulfate; and galactosaminoglycans, including chondriotin and dermatan sulfates.

It is related to the heparin and heparan sulfate families of glycosaminoglycans but is distinctly different from all known members of these classes of glycosaminoglycans. Heparin and heparan sulfate have been the subject of intensive study because of their well recognized ability to bind many different proteins that regulate a variety of important biological processes (Lindhart nd Tioda, 1996). Heparin and heparan sulfate GAGs are comprised of alternating 1 → 4 linked glycosamine and uronic acid residues. Heparin sulfate is composed primarily of monosulfated disaccharides of N-acetyl-D-glycosamine and D-glucuronic acid, while heparin is composed mainly of trisulfated disaccharides of N-sulfoyl-D-glucosamine and L-iduronic acid (Lindhart and Tioda, 1996).

The structure of this polysaccharide, with adjacent N-acetylglucosamine and 2-sulfo-iduronic acid residues, also poses interesting questions about how it is made in the light of our current understanding of the biosynthesis of heparin and heparin sulfate. This glycosaminoglycan represents 3 – 5 % of the dry weight of this snail's soft body tissues, suggesting important biological roles for the survival of this organism, and may offer new means of control of this pest. Snail glycosaminoglycan tightly binds divalent cations, such as copper (II), suggesting a primary role in metal uptake in the snail. Finally, this new polysaccharide might be applied, like the *Escherichia coli* K5 capsular polysaccharide, to the study of glycosaminoglycan biosynthesis and to the semisynthesis of new glycosaminoglycan analogs having important biological activities.

The large amount of this GAG found in snail also raises some interesting questions about its biological function(s). Many roles can be proposed for this GAG including: i) binding, uptake, and transport of divalent cation (Kim, et al, 1996); ii) an anti-desiccant; ii) a molecule linked to molecule. The most likely role of snail GAG is its involvement in cation binding. *A. fulica* is a very large gastropod that requires substantial quantities of calcium for its shell. In addition, other divalent ions are critical components of their diets. The blood of snails is blue, as haemocyanin is the copper-based carrier of oxygen in these animals. This shows that snail GAG binds copper (II) much more tightly than heparan sulfate and with about the same avidity of heparin, which has a 3-fold higher level of sulfation. GAGs are known to organize and hold water. Since snails are particularly prone to dehydration, this suggests a second role for this polysaccharide. Snails move on mucus slime through wave-like undulations of their foot muscle. This high molecular polysaccharide is extremely viscous and may represent a component of this slime. Antibiotic properties have been reported for heparin (Lindhart and Tioda, 1996), and *A. fulica* is known to make a bactericidal glycoprotein that is found in its mucus , suggesting that snail GAG may have a protective role. Other interesting questions about the snail GAG is that chemical modification of snail polysaccharide using relatively simple methods, i.e. de-N-acetylation and re-N-sulfation, should lead to a structurally homogenous polysaccharide with the minimum structural features for binding fibroblast growth factors. Most of the physiological effects, including the local anaesthetic property may be related to this polysaccharide, as earlier stated above. However, this statement is still speculative and is a subject for further studies to confirm earlier studies (Kim et al, 1996).

Acute toxicity test: The extract was well tolerated, as the animals did not exhibit any symptoms of overt toxicity. The snail mucin is therefore non-toxic as no animal died. Thus no LD_{50} value could be determined for the snail mucin on the rats.

Haematology: The result of the packed cell volume (PCV) of the blood samples of the rat groups before and after dosing with the snail mucin reveals an insignificant difference ($p < 0.05$) as shown in Fig. 1.

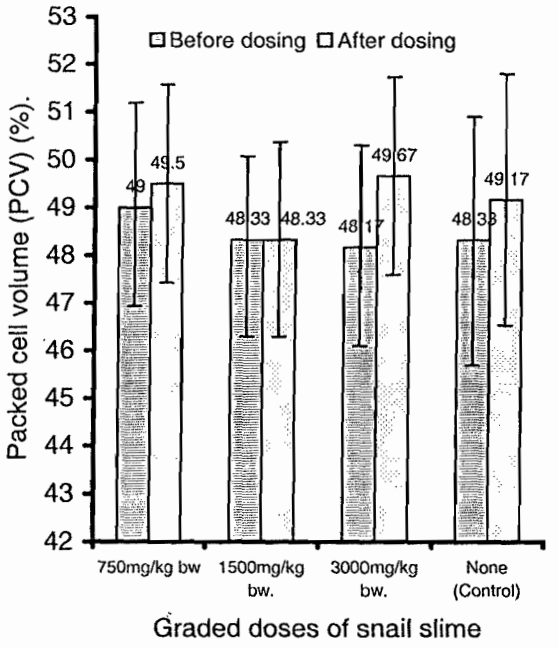

Fig 1. Packed cell volume (PCV) of albino rat groups given graded oral doses of snail mucin extract.

The result of the erythrocyte sedimentation rate (ESR) also reveals an insignificant change ($p < 0.05$) in ESR values after dosing with the snail mucin. The result is presented in Fig. 2. Changes in ESR are an index of damage to vital organs or serious toxicity/infections. The lack of changes in ESR implies that the varied doses of the mucin dispersion caused insignificant alterations in the vital organs- livers and kidneys, of the rats.

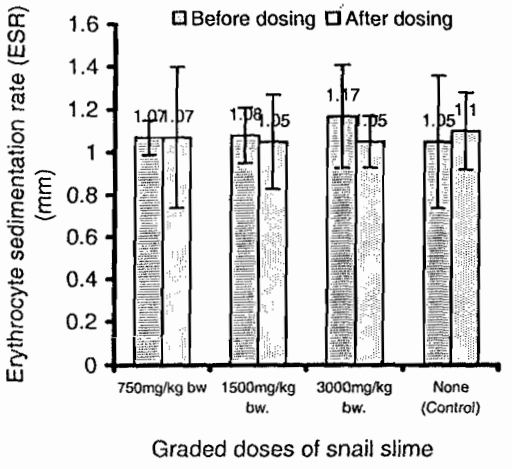

Fig. 2: Erythrocyte sedimentation rate (ESR) of albino rat groups given graded oral doses of snail mucin extract.

The total leucocyte counts (TLC) per microlitre of blood shows that dosing the rats at 750 and 1500 mg/kg had no alteration in the TLC but the 3000 mg/kg dose had a significant fall in TLC ($p < 0.05$) of the rats. This fall is called leucopenia and is probably due to a fall in the absolute monocyte and eosinophil counts (Fig. 3). There was thus no significant effect on the lymphocyte and neutrophil counts of all the rat groups, hence no effect on the immune system.

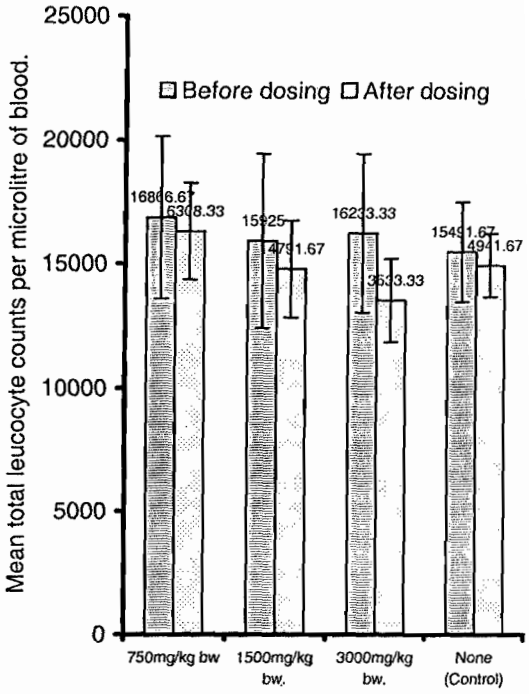

Fig. 3: Total leucocyte counts of albino rat groups given graded oral doses of snail mucin extract.

The result of the absolute lymphocyte count (ALC) per microlitre of blood reveals also an insignificant difference ($p < 0.05$) in the lymphocyte counts of all the rats. This further attests to the fact that there was no effect on the immune system (Fig. 4). Monocytes are known to be involved in phagocytosis of large particles and eosinophils are usually involved in allergic reactions. The significant fall in absolute monocyte count (AMC) of the rats dosed with 3000 mg/kg of snail mucin ($p < 0.01$) could be attributed to the mobilization of these cells for the removal of the snail mucin from the body (Fig. 5).

Fig. 6 shows the result of the absolute neutrophil counts (ANC) per microlitre of blood. This reveals an insignificant fall in neutrophils ($p<0.05$) in the rat groups as shown in the figure.

The eosinophils are involved in allergic response and stress conditions. The very high doses used for the study constituted a stress on the blood system of the rats such that there was significant fall ($P < 0.01$) in the absolute eosinophil counts (AEC) of all the rat groups that received the

snail mucin. The fall tended to be dose-dependent. The extract either constituted a stress or may have induced an allergic response that led to the complete use up of eosinophils.

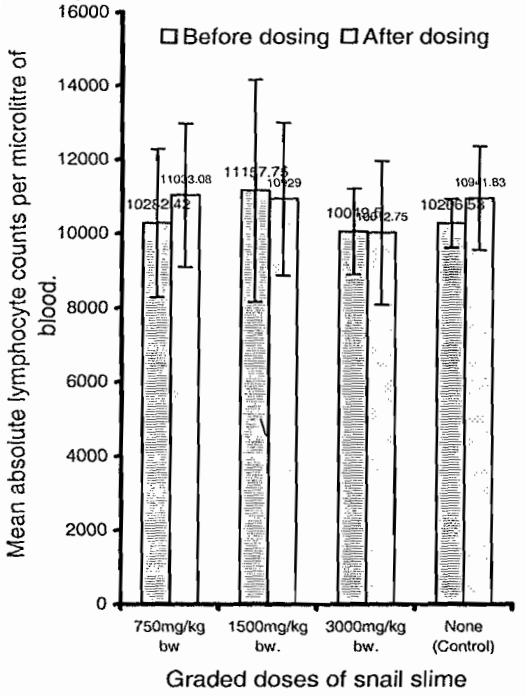

Fig. 4:Absolute lymphocyte counts of albino rat groups given graded oral doses of snail

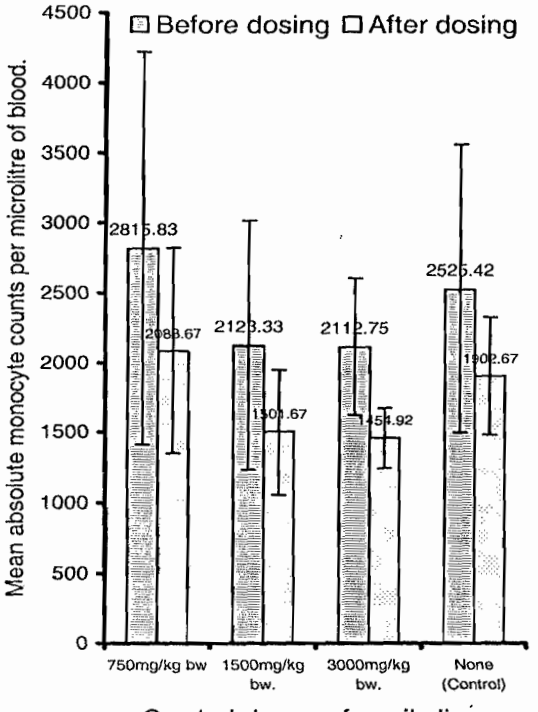

Fig. 5. Absolute monocyte counts of albino rat groups given graded oral doses of snail mucin extract.

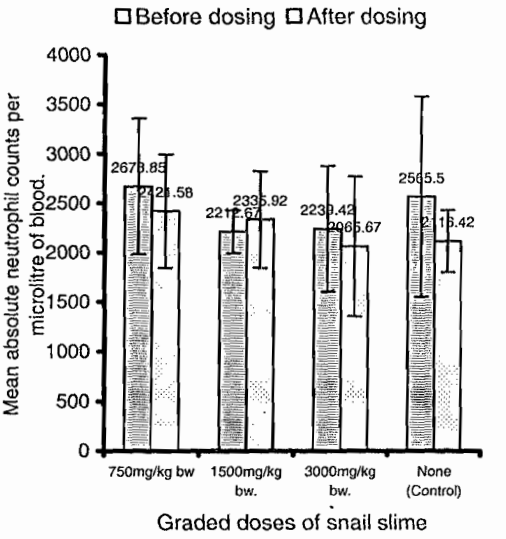

Fig. 6: Absolute neutrophil counts of albino rat groups given graded oral doses of snail mucin extract.

In groups B and C and a reasonably significant fall in the group A rats (Fig. 7). This fall contributed to the low counts obtained in the total leucocyte count of the rat groups otherwise called leucopenia.

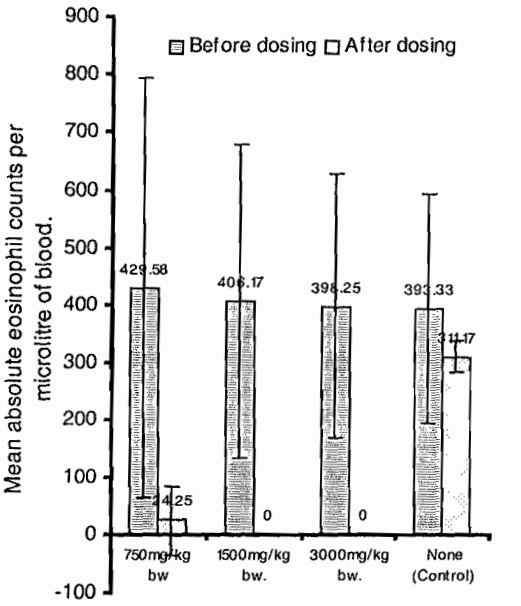

Fig. 7: Absolute eosinophil counts of albino rat groups given graded oral doses of snail mucin extract.

Histopathology: The result of the histopathology shows that there were no significant histopathological changes on the kidney and liver of the rats that were given the snail mucin when

observed under a microscope. The hepatocytes and central veins of the livers of all the rats were intact and normal only for a mild congestion of the central veins of the rats that received 3000 mg/kg of snail mucin. This was probably a sign of increased blood supply needed to detoxify the body of the high dose (3,000 mg/kg) of the mucin extract given to them. The kidneys of all the rat groups were normal. The tubular cells were intact and the glomeruli were normal.

Effect on small intestinal motility: The result of the charcoal meal test is shown in Table 2. The administration of the snail mucin significantly ($p < 0.05$), reduced in a dose-related manner the charcoal meal transit. The inhibition produced by 400 mg/kg of the extract was comparable to that produced by atropine (10 mg/kg). This shows that snail mucin lowers intestinal motility at high doses. The mechanism is however not clear, and needs to be further investigated.

Table 2: Effect of the snail mucin (extract) on small intestinal motility

Drug	Dose (mg/kg)	Distance travelled (%)
Tween 85	20 (ml/kg)	46.2 ± 3.0
Atropine	10	1.0 ± 0.8
Extract	100	25.0 ± 0.8
Extract	200	14.8 ± 2.1
Extract	400	1.1 ± 0.1

P < 0.05

Acknowledgements

One of the authors, Dr. M. U. Adikwu, is grateful to the International Foundation for Science for providing the grant (Grantee F/3560-1) used in this study and to God for creating the snails.

References

Akah, P. A. (1989). "Purgative potentials of *Euphorbia heterophylla*. *Fitoterapia* 60: 45-48.

Akah, P. A and Odita, I. O (2001). Experimental Methods in Physiology and Pharmacology for Medical and Pharmacy Students. ABIC Publishers, Enugu.

Akah, P. A. and Offiah, V. N. (1992). Gastrointestinal effects of *Allamanda cathartica* leaf extract. *Int. J. Pharmacog.* 30: 213-217

Cheney, S. (1988) Reference Branch United States Department of Agriculture, The National Agricultural Library Beltsville, Maryland In: Raising Snails.

Kim, Y. S., Jo, Y. Y., Chang, I. M., Toida, T., Park, Y. and Linhardt, R. J. (1996). A new Glycosaminoglycan from the Giant African Snail Achatina fulica". *J. Biol. Chem.* 271, 11750-11755.

Linhardt, R. J., and Tioda, T. (1996). In: Carbohydrates as Drugs, (Witczak, Z. B., and Nieforth, K. A., eds.) Marcel Dekker, New York, in Press.

Lorke, D. (1983). A new approach to practical acute toxicity testing. *Arch. Toxicology*, 54, 275-287.

Repentigny, L., Aumont F., Bernard K., and Belhumeur, P. (2000). Characterization of binding of *Candida albicans* to small intestinal mucin and its role in adherence to mucosal epithelial cells. Infection and Immunity, 68, 3172-3179.

Wiggins R., Hicks S. J., Soothill P W, Millar M. R. and Corfield, A. P. (2001). Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. Sexually Transmitted Infections 77, 402-408