Original Research Article

ANTIBACTERIAL PROPERTIES OF SNAIL MUCUS ON BACTERIA ISOLATED FROM PATIENTS WITH WOUND INFECTION

ABSTRACT

Background: Snail mucin has been reported to contain agents with wound healing properties. Mucin obtained from the mucus of snails and epiphgram obtained from species of *Achatina fulica* and *Archachatina marginata* have also been reported to show antimicrobial properties. Snail species are abundantly available and widely consumed as a delicacy across Nigeria.
Aim: To assess the antibacterial effects of mucus secretions from different snail types on bacteria isolated from clinically infected wounds.
Place and duration of study: The study lasted for a period of four (4) months and was conducted at the Microbiology laboratory of The Cross River State University of Technology in Cross River, Nigeria.
Methodology: The in vitro antibacterial potency of snail mucus secretions obtained from *Archachatina marginata* saturalis, *Archachatina marginata* ovum and *Achatina fulica* on bacterial isolates from wound was investigated. The isolates obtained from twenty eight (28) clinical wound samples were *Staphylococcus* spp (24:53.3%), *Pseudomonas* spp (16:33.3%) and *Streptococcus* spp (6:13.4%). The susceptibility of the isolates to snail mucus secretions was assayed on Muller Hilton Agar by the disc diffusion method, using varied mucus/DMSO concentrations of 100%, 80%, 60%, 40% and 20%. The minimum inhibitory concentration and minimum bactericidal concentration of the mucus secretions were also evaluated.
Results: The viscosity of the mucus secretions were rated as *A. marginata* saturalis> *A. marginata* ovum> *A. fulica*, while their colours were yellow, light brown and dark respectively. Results revealed that *Staphylococcus* sp was more susceptible to mucus secretion from the *A. marginata* saturalis (17.4 ± 1.20) than those from *A. marginata* ovum (15.6 ± 1.44) and *A. fulica* (15.4 ± 2.04). The minimum inhibitory concentration of mucus secretions from *A. marginata* saturalis against the test organisms were observed at concentrations of 100% and 20% for *Staphylococcus* sp, 20% for *Pseudomonas* sp and 40% for *Streptococcus* sp respectively. The antibacterial activity of the mucus secretions were observed to be comparable to that of seven (7) different antibiotics used as control.
Conclusion: Snail mucus secretions could be a source for antibacterial agents that can serves as an alternative to the expensive synthetic antibacterial agents used in wound treatment if adequately explored.

Keywords: Snail, mucin, concentration, antibacterial, protein, synthetic, inhibit.

1. INTRODUCTION

The occurrence of antibiotic resistant bacterial pathogens in clinical cases seem to be on the increase on daily basis, a phenomenon which is contributing to the difficulties being faced in the treatment of infections involving bacteria. Having lived for many years, bacterial strains have survived varied environments by developing resistance to new stressors [1]. Hence, the increasing need for the development of new and more effective alternative antibiotics from readily available materials such as antimicrobial proteins produced by some animals, an example of which is mucin produced by snails.

Mucins are a family of large glycosylated proteins (50% w/w carbohydrate). They are a group of nitrogenous substances secreted by mucous glands. They are the major macromolecular components of the mucous secretions that coat delicate epithelial surfaces in animals where they provide protection from microbial and physical damage, and are responsible for the viscoelastic properties of mucous secretions. Some mucins are membrane-bound due to the presence of a hydrophobic membrane–spanning domain that favours retention in the plasma membrane [2]. Snails produce mucin in a very large quantity, which is

often referred to as slime. It has also been documented to contain glycosaminoglycans reported to be of great value in wound healing and repair [3].

A major factor that influences wound healing is bacterial infection. When a wound is infected by bacteria, it produces inflammation and accumulation of fluid which interferes with the healing process [4]. Various bacterial species have been implicated in wound infections, some of which have been identified as *Staphylococcus aureus,* Coagulase-negative Staphylococci, *Escherichia coli, Pseudomonas aeruginosa, Acinetobacter*, *Enterococcus faecalis*, *Proteus* species and *Klebsiella pneumonia* as well as species of streptococcus, with *Staphylococcus aureus* reported as the most predominant isolate [5-9]. These bacteria find their way into broken skin, either as a result of injury, burns or surgery, from skin surfaces of the host and from contaminated surfaces within the environment. *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been reported in various studies to account for 20-40% and 5-15% of nosocomial infections respectively [5]. Studies have unfortunately reported high multiple antibiotic resistance rates displayed by some of these bacteria to commonly administered antibiotics, thereby posing a challenge in the management of wound infections [5,6,8].

Snails produce mucin abundantly in their mucus secretion often referred to as slime, which have been reported to contain antimicrobial proteins [4]. A bactericidal glycoprotein known as achacin, obtained from the body surface mucus of African giant snail has been reported to kill both Gram-positive and Gram-negative bacteria by attacking the cytoplasmic membrane of the cell [10-11]. The use of snail mucin obtained from snail mucus secretions for wound healing has also been documented [12-13]. Since the cost of synthetic drugs is high and snails which produce mucin-containing mucus secretions are abundant in Nigeria, it is therefore essential to explore their potential use as alternative source of antibacterial agent in the control of infections caused by bacteria. This work is aimed at assessing the antibacterial effects of mucus secretions from different snail types on bacteria isolated from clinically infected wounds.

2. MATERIALS AND METHOD

2.1 Collection of snails and extraction of mucus

Three snail types namely *Archachatina marginata* saturalis, *Archachatina marginata* ovum and *Achatina fulica* were purchased from Watt market in Calabar municipality. The snails were handled in accordance with the principles of animal welfare in scientific experiments. The mucus specimens were extracted from the snails by removing the skin from the shell with a sterile sharp-end metal rod into a beaker and the mucus secretions aseptically squeezed out from the soft body. The extracted mucus secretion considered 100% concentration were stored in the refrigerator at 4^{o}C for bacteriological assay.

2.2 Collection of samples from infected wound

Twenty eight (28) clinically infected wound lesions from the wound care unit of the General Hospital Calabar, Nigeria, were aseptically swabbed with sterile swab sticks previously soaked in peptone broth. The samples were stored in an ice packed container as a mixed broth culture and taken to the laboratory for cultural assay.

2.3 Isolation, characterization and identification of wound isolates

Isolation, purification, characterization and identification of bacterial cultures followed the methods described by [5] and [14]. Following collection, the swabs were inoculated on Nutrient agar, MacConkey agar, Mannitol salt agar, Blood agar and Chocolate agar for the isolation of bacteria, using the streak plate method. Culture plates were then incubated at 37^{o}C for 24 hours, after which discrete colonies were further purified by sub-culturing on appropriate media and incubating at 37^{o}C for another 24 hours before characterization. Cultures were Gram stained and characterized based on their cultural, morphological and sugar fermentation reactions on specified media, as well as biochemical reactions such as catalase, oxidase, coagulase, citrate utilization, urease, methyl red, indole, Voges Proskaeur and hemolysis tests.

2.4 Assay of mucus antibacterial activity

2.4.1 Determination of mucus antibacterial activity by disc diffusion method

The antibacterial activity of the mucus preparation was assayed using the disc diffusion method (DDM) as described by [14] and [15] on Muller Hilton agar. In this method, six (6) millimeter diameter discs cut out from No.1 Whatman filter paper, were boiled for 30 minutes to remove any chemical that may inhibit the growth of the microorganisms, and sterilized by autoclaving at 121^{o}C for 15 minutes. The sterilized discs were soaked in a concentration of 100% (v/v) snail mucus. The mucus-impregnated discs were then placed in a water bath at 37^{o}C for 30 minutes to enhance absorption. The mucus impregnated discs were thereafter, air-dried and placed in triplicate on plates already seeded with 1.0 ml of 18 hour old broth culture at 0.5 McFarland standard (1.5 x 10^{8} cfu ml^{-1}) and the discs incubated at 37^{o}C for 24 hours. The zones of inhibition were measured in millimeter as degree of susceptibility of the wound isolates to the mucus formulation and means of the inhibition zones were noted.

2.4.2 Determination of Minimum inhibition concentration

The minimum inhibitory concentration (MIC) was done using mucus formulations with high efficacy against the test isolates by the disc diffusion method, with some modifications [15-16]. To determine the MIC values, paper discs made from filter paper soaked with different concentrations of mucus formulations of 20, 40, 60, 80 and 100 per cent (v/v) were assayed against the bacteria at 0.5 McFarland standard (1.5 x 10^{8} cfu ml^{-1}). The discs containing each mucus concentration was placed equidistant on Muller Hilton agar plates already seeded with the test organisms and incubated overnight at 37^{o}C, after which the zones of inhibition were read. The lowest concentration of mucus formulation which exhibited the largest inhibition zone was interpreted as the minimum inhibitory concentration of the formulation.

2.4.3 Minimum bactericidal concentration as index of growth inhibition

The MBC was assayed at snail mucus concentrations of 60, 80 and 100 per cent (v/v). Equal aliquots of the snail mucus was mixed with equal aliquots of the test organisms at 0.5 McFarland standard and cultured on Muller Hilton agar for at least 18 hours at 37^{o}C. The number of colonies formed were counted and the mean of each duplicate concentration was taken. The lowest concentration capable of reducing bacterial growth on the medium was considered the minimum bactericidal concentration.

2.4 Statistical analysis

Data collected form the results were analyzed using SPSS version 20 (IBM Corp., Armonk, New York). Simple means, percentages and standard deviation were computed as appropriate.

3 RESULTS AND DISCUSSION

The physical properties of mucus secretions from each genera of snail were observed. The extract from *A. marginata* (saturalis) was yellowish in colour while secretions from the *A. marginata* (ovum) and *A. fulica* were light brown and dark in colour respectively. The mucus secretions from *A. marginata* (saturalis) was more viscous (thicker) than that from *A. marginata* (ovum) and *A. fulica* respectively. Mucus from *A. fulica* had the least thickness and was considered to be lighter (Table 1). This study has revealed that the physical characteristics of the three snail mucus secretions used are not the same in terms of colour and viscosity. The viscosity reduced in the order *A. marginata* (saturalis) > *A. marginata* (ovum) > *A. fulica* respectively while the colour varied from yellow in *A. marginata* (saturalis), to brown and dark colours in *A. marginata* (ovum) and *A. fulica* respectively. The differences in these properties may be attributed to differences in the feeding habits of the snail species which in turn affects their nutritional content and composition [17-18]. Feed type has also been reported to affect the composition of both the flesh and haemolymph of snails [23], as well as the volume of mucus they produce [22].

From the 28 clinical wound samples collected (Table 2), *Staphylococcus* sp was the most isolated (53.3%), followed by *Pseudomonas* sp (33.3%). *Streptococcus* sp was the least isolated bacterium (13.4%). The high incidence of *Staphylococcus* sp and *Pseudomonas* sp as well as the presence of *Streptococcus* sp in wounds have also been recently reported by various researchers [19-20]. These

bacteria gain access to wounds from the skin of patients, hospital personnel and other sources within the hospital environment [5, 6, 19]. Selective pressure exerted by antibiotic usage may also have allowed for selection of these bacteria which have been widely reported to display resistance to a spectrum of antibiotics [5]. This observation calls for more strict maintenance of hygiene in wards where patients with wounds are kept in order to control contamination.

The susceptibility of *Staphylococcus* sp, *Pseudomonas* sp and *Streptococcus* sp to the various mucus secretions were tested as presented in Table 3. Results of the susceptibility test carried out revealed that *Staphylococcus* sp was more susceptible to mucus from *A. marginata* (saturalis) (17.4 ± 1.20) than those from *A. marginata* (ovum) (15.6 ± 1.44) and *A. fulica* (15.4 ± 2.04). *Pseudomonas* sp and *Streptococcus* sp were more susceptible to mucus secretions from *A. marginata* (ovum) (19.8 ± 0.88 and 19.3 ± 1.90) than those from *A. marginata* (saturalis) (19.2 ± 1.10 and 18.6 ± 2.14) and *A. fulica* (17.1 ± 1.30 and 17.5 ± 2.72) respectively. Overall, *Pseudomonas* sp was more susceptible to all three mucus secretion than *Streptococcus* sp and *Staphylococcus* spp. This study also revealed that mucous secretions obtained from the three snail types showed varying levels of antibacterial activity on the three test organisms used (*Staphylococcus* sp, *Streptococcus* sp and *Pseudomonas* sp). The mucus secretions also showed an increase in antibacterial activity with increase in concentration, as revealed by the various viable counts observed. The viable counts of each bacterial isolate was least at 100% mucus concentration and highest at 60% mucus concentration for all three types of secretion. Mucus secretion from *A. marginata* (saturalis) and *A. marginata* (ovum) showed more inhibitory activities than that from *A. fulica*. The exact reason for this observation has not be explained by this work, but may not be unrelated to possible difference in the volume of mucin contained in the mucus secretions of the snail species. Further investigation into this, may elucidate the observed differences in their antibacterial activity.

The minimum inhibitory concentration of mucus secretion from *A. marginata* (saturalis) and *A. marginata* (ovum) was determined against *Staphylococcus* sp, *Streptococcus* sp and *Pseudomonas* sp using the disc diffusion method. The MIC for each mucus type was read as the lowest mucus concentration that showed the largest inhibition zone. The minimum inhibitory concentration of mucus secretions from *A. marginata* (saturalis) against the test organisms were observed at mucus concentrations of 100% and 20% for *Staphylococcus* sp, 20% for *Pseudomonas* sp and 40% for *Streptococcus* sp respectively. The least minimum inhibitory concentration was observed in *Pseudomonas* sp at 20% mucus concentration while the highest was observed in *Staphylococcus* sp at 100% mucus concentration. The MIC determined also revealed that mucus secretion from *A. marginata* (saturalis) was more effective against *Pseudomonas* sp (20% concentration) while that from *A. marginata* (ovum) showed higher activity against *Streptococcus* sp (40% concentration). The MIC as well as the zones of inhibitions measured corroborate that antibacterial effect of mucus secretion from *A. marginata* (saturalis) was in the order *Pseudomonas* sp > *Streptococcus* sp > *Staphylococcus* sp. While the MIC revealed more antibacterial activity of *A. marginata* (ovum) mucus secretion against *Streptococcus* sp than *Pseudomonas* sp and *Staphylococcus* sp, the disc diffusion assay revealed more activity against *Pseudomonas* sp than against *Streptococcus* sp and *Staphylococcus* sp.

The minimum bactericidal concentration of the mucus secretions was also determined as the lowest concentration of the mucus secretion that exhibited the largest inhibition zone against the various test isolates (Table 5). The MBC of the mucus secretions were found to increase with an increase in mucus concentration. The viable counts of each bacterial isolate was least at 100% mucus concentration and highest at 60% concentration for all three types of secretion, signifying that the MBC of each mucus type was at 100% concentration. At all concentrations, mucus secretion from *A. fulica* showed more antibacterial activity against *Staphylococcus* sp than *Pseudomonas* sp and *Streptococcus* sp, whereas mucus secretion from *A. marginata* (saturalis) showed more activity against *Pseudomonas* sp than against *Staphylococcus* sp and *Streptococcus* sp at all three concentrations. *Streptococcus* sp was more susceptible to *A. fulica* secretion at 60% concentration and to *A. marginata* (ovum) mucus secretions at 80% and 100% concentration than *Pseudomonas* sp.

The preceding observations (Table 4 and 5) may point to a possible variation in the concentration of the antibacterial factor in snail mucus secretions from the three snail types used in this study. Evidence of antibacterial property in snail mucus as well as mucin obtained from snail mucus have been previously

reported in literature. In a study by [11] and [21], mucous secretion and mucin obtained from *Achatina fulica* showed inhibitory activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. A report by [22] however, did not indicate evidence of antibacterial activity in the mucus of *Archachatina marginata*. In a similar study, [1] reported that epiphgram from normal and albino skinned *Archachatina marginata* showed more antibacterial activity against *Escherichia coli*, *Salmonella* sp, *Staphylococcus aureus* and *Pastueurella* sp than streptomycin. This may suggest the possibility of their mucous secretion being able to inhibit the growth of both Gram-positive and Gram-negative bacteria.

Results of this study also indicate that all three snail mucus secretions showed more inhibitory activity against *Streptococcus* sp at the various concentrations than five (5) out of the seven (7) different antibiotics used as control, except at a concentrations of 100, 80, 60 and 20 for *A*. *marginata* (ovum) mucus (Table 3 and 6). Zones of inhibition displayed by mucus secretion from *A. marginata* (saturalis) against *Staphylococcus* sp, was larger than that of six (6) antibiotics, while only five of the antibiotics showed larger inhibition zones against *Pseudomonas* sp than all three snail mucus secretions at the various concentrations. The study thus further showed that some of the mucus secretions were more inhibitory to the test organisms than some of the commercially available antibiotics used as control. This finding is similar to that showed by epiphgram of normal and albino skinned *Archachatina marginata* [1]. On the contrary, a study by [11] did not report a significant difference in antibacterial activity between mucous secretion of *Achatina fulica* and metronidazole. Snails have some special proteins that aid their survival in the environment and also limit bacterial contamination. According to [21], the antibacterial activity of mucin found in the mucous secretion of *Achatina fulica* is related to antibacterial factors found in its protein moiety rather than to its activity on the cell surface of bacteria. The antibacterial factor might be functioning to protect the wet-skinned animal from external infection and are a component of proteins contained in mucin found in the mucus of snails [1, 21]. The antibacterial protein in the mucus of the giant African snail referred to as achacin, is known to bind both Gram positive and Gram negative bacteria [24-25]. Achacin is a member of the L-amino acid oxidase family and generates hydrogen peroxide to kill bacteria [25]. A research by [26] reported the presence of a high molecular weight lectin, which they designated AfHML (*Achatina fulica* high molecular weight lectin), in the mucus of the giant African snail *A. fulica*. AfHML is secreted from the same collar tissue where achacin is secreted and is believed to accelerate the anti-bacterial activity of achacin by increasing the local concentration of hydrogen oxides in the mucus [26]. A report by [27] stated that the antibacterial factor of snails was a glycoprotein that has two subunits. Digestion with pronase and application of heat up to 75^{o}C for 5 minutes led to the loss of antibacterial activity [27]. This, the researchers reported to mean that the activity of the antibacterial factor of the snails is dependent on protein or the protein moiety of the glycoprotein and must be closely related to the higher-order structures of the protein or to the protein moiety of the glycoprotein. The authors further reported strong growth inhibitory activity of the snail mucus antibacterial factor against both Gram positive and Gram negative bacteria, despite differences in their cell wall structure. According to the authors, it suggests that the key site or the key metabolic step receptive for the antibacterial factor of the snails must be present somewhere in the bacterial cells themselves, namely in the cell walls, cell membranes or the cytoplasm [27].

Table 1: Physical properties of mucus secretions

Snail Sample	Colour	Viscosity
A. marginata (saturalis)	Yellow	+++
A. *marginata* (ovum)	Light brown	++
A. fulica	Dark	+

Legend: +++ Very thick ++ Thick + Light

Table 2: Bacterial isolates from patients with wound infection

Bacteria	No. of samples	Occurrence (%)

Staphylococcus sp	24	53.3
Pseudomonas sp	15	33.3
Streptococcus sp	6	13.4
Total	**45**	**100**

Table 3: Antibacterial properties of various mucus secretions against some bacterial isolates using the disc diffusion method

	Zone of inhibition (mm/mean ± SD)		
Bacterial Isolate	AMs	AMo	AF
Staphylococcus sp	17.4 ± 1.20	15.6 ± 1.44	15.4 ± 2.04
Pseudomonas sp	19.2 ± 1.10	19.8 ± 0.88	17.1 ± 1.30
Streptococcus sp	18.6 ± 2.14	19.3 ± 1.90	17.5 ± 2.72

Values are the means of three replicates

Legend: AMs - *A. marginata* (saturalis); AMo - *A*. *marginata* (ovum); AF - *A. fulica*

Table 4: Minimum Inhibitory Concentration (MIC) of *A. marginata* (saturalis) and *A*. *marginata* (ovum) mucus formulation by disc diffusion method

Test organism	**MIC of AMs (% conc.)**	**MIC of AMo (% conc.)**
Staphylococcus sp	100 & 20	80 & 40
***Pseudomonas* sp**	20	60
***Streptococcus* sp**	40	40

Values are means of three readings

Key: AMs - *A. marginata* (saturalis); AMo - *A*. *marginata* (ovum)

Table 5: Minimum Bactericidal Concentration (MBC) of mucus formulations on viable count of test organisms in culture media ($Log_n cfuml^{-1}$)

Bacteria	**Mucus conc. (%)**	**Snail mucus secretion**		
		AMs	AMo	AF
Staphylococcus spp	60	5.1 ± 0.2	4.9 ± 0.3	4.1 ± 0.2
	80	3.8 ± 0.3	3.6 ± 0.1	2.8 ± 0.3
	100	3.2 ± 0.1	2.8 ± 0.1	2.2 ± 0.4
Pseudomonas spp	60	4.8 ± 0.8	7.2 ± 1.2	5.3 ± 0.6
	80	2.4 ± 0.2	4.9 ± 0.3	4.9 ± 0.2
	100	1.9 ± 0.01	3.7 ± 0.4	3.7 ± 0.3
Streptococcus spp	60	6.6 ± 0.2	5.5 ± 0.3	4.4 ± 0.3
	80	4.8 ± 0.2	3.8 ± 0.3	4.4 ± 0.3
	100	3.9 ± 0.4	3.1 ± 0.1	3.8 ± 0.09

Values are means of three readings ± SD

Legend: AMs - *A. marginata* (saturalis); AMo - *A*. *marginata* (ovum); AF - *A. fulica*

Table 6: Standard antibiotic discs used as control

Antibiotic	**Conc. mg/100ml**	**Inhibition zones (mm) of bacterial isolates**		
		I	**II**	**III**

Amoxylin (AMY)	500	11	13	6
Streptomycin (STR)	500	12	25	16
Chloramphenicol (CHL)	250	10	8	15
Gentamicin (GEN)	280	25	30	10
Pefloxacin (PEF)	500	15	35	8
Cotrimoxazole (COT)	480	10	11	10
Ciprofloxacin (CIP)	500	8	20	3

Legend: I – *Staphylococcus* sp II – *Pseudomonas* sp III –*Streptococcus* sp

4 CONCLUSION

This study reveals the presence of antibacterial factors in the mucous secretions of *Archachatina marginata* (saturalis), *Achatina fulica* and *Archachatina marginata* (ovum). Results showed varied inhibitory and bactericidal potency against *Staphylococcus* sp, *Streptococcus* sp and *Pseudomonas* sp isolated from wounds. Among the three snail types, MIC and MBC values revealed that mucus from *Archachatina marginata* (saturalis) and *Archachatina marginata* (ovum) showed more inhibitory activity against the test organisms than that from *Achatina fulica*. Snail mucus secretions could be a source for antibacterial agents that can serve as an alternative to the expensive synthetic antibacterial agents used in wound treatment if adequately explored.

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