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L.fulica Mucins: The Novel Source for the Evaluation of Antibacterial Efficacy against Various Pathogenic Bacterial Species.

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Abstract

Lissachatina fulica, the giant African land snail, is one of the most invasive and widely imported land snail species globally. It has been introduced to numerous Asian nations, Pacific and Caribbean islands, and Brazil. L.fulica, secretes mucus to help in crawling and protection because it serves as a lubricant to shield sensitive epithelial body surfaces from various physical and mechanical harms. The mucin that L.fulica releases has attracted attention from all around the world due to its pharmacological similarities to other gastropods. Snail mucins have recently emerged as a treasure mine of new possibilities in the domains of biology, chemistry, biotechnology, and biomedicine. Particular applications for snail mucuses include wound healing, skin care products, surgical glues, and stomach ulcer treatment. Recent advances in integrated omics (genomic, transcriptomic, proteomic, and glycomic) technology have produced more novel biomaterials due to improved characterization of gastropod mucins. Today, antimicrobial resistance caused by microorganisms is one of the gravest medical issues impacting virtually all nations. Newer antimicrobial therapies that lessen this resistance need to be created right away. This research emphasizes on the antibacterial activity of mucin against various bacterial species such as Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Shigella dysenteriae, and Salmonella typhimurium. Therefore, the mucin was extracted by periodically spraying with an acid-stimulating solution i.e., 5% citric acid by diluting with distilled water. The stimulating fluid produced a large amount of mucus without stressing or harming the snail. The antibacterial activity of mucin was detected by using agar disc diffusion method which showed highest zone of Inhibition against Staphylococcus aureus with 25mm and agar well diffusion method showed highest zone of Inhibition against Shigella dysenteriae with 16mm amongst other various Bacterial species mentioned above. Using Bradford's coomassie brilliant blue assay, the protein estimation of mucin was determined. The snail's internal and external shell was examined in order to isolate the microorganism present there. This work is aimed at assessing the antibacterial activity of mucin against various pathogenic bacteria.

Keywords: Agar diffusion, Bacteria, Bradford method, L.fulica, Microflora, Mucin.

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INTRODUCTION

Colossal African land snail, Lissachatina fulica, is regarded as one of the most invasive and extensively imported land snail species globally. It has been introduced to numerous Asian nations, numerous Pacific and Caribbean island nations, and Brazil [1]. L.fulica, a huge African snail, secretes mucus to help in crawling and protection because it serves as a lubricant to shield sensitive epithelial body surfaces from various physical and mechanical harms. Because it has some pharmacological characteristics with other gastropods, the mucin released by L.fulica has garnered international attention [2, 3]. Snail mucins have recently emerged as a wealth of innovative opportunities with a broad range of uses in biology, chemistry, biotechnology, and biomedicine. Snail mucus's have been used specifically as surgical glues, skin care products, wound healing agents, and to treat stomach ulcers. Improved characterisation of gastropod mucins has increased the production of innovative biomaterials thanks to recent developments in integrated omics (genomic, transcriptomic, proteomic, and glycomic) technologies [4]. A fundamental component in cosmeceutical apparel is snail secretion, which is commonly referred to as snail mucus [5]. It was simple to incorporate snail mucus into skincare because of its makeup, which includes several well-known skincare compounds like hyaluronic acid, growth factors, and antioxidants. Glycosaminoglycans, glycoproteins, allantoin, glycolic acid, lactic acid, collagen, and elastin are further components of snail mucin. Because it has a rare

combination of elements that are not normally found in nature, snail mucin is a very special natural product [6].

One of the biggest health issues affecting nearly every country today is antibiotic resistance brought on by bacterial infections. Newer antibacterial treatments that reduce this resistance must so be developed immediately [7]. L. fulica mucus showed encouraging antibacterial efficacy against both Gram-negative bacteria, such as Escherichia coli and Pseudomonas aeruginosa, and Gram-positive bacteria, such as Bacillus subtilis and Staphylococcus aureus [8].To extract snail mucin on a laboratory scale, an acid-stimulating solution in a water spray bottle was sprayed on the snails sporadically for 10 minutes. Five percent of citric acid solution was made using distilled water. Without causing the snail any stress or harm, the stimulating fluid produced a sizable amount of mucus [9]. Antimicrobial activity of mucus that was extracted by the solvent method from the L.fulica was determined by agar disc diffusion method[7].The microgram quantities of snail mucin were measured using the Bradford Coomassie brilliant blue test by detecting absorbance at 590 nm[10].The microflora of the snail's internal and external shell was also isolated by swabbing method.This work is aimed at assessing the antibacterial effects of mucus secretions against several microorganisms along with protein estimations in mucin.

METHODOLOGY

Collection of Snail

The L.fulica (African giant snail) was collected from Maharani Cluster University 39, Off Sheshadri Road, opposite Freedom Park, Racecourse, Gandhinagar, Bengaluru, Karnataka 560001 (latitude and longitude of 12.9789 and 77.5847). The snails were maintained according to the principles under scientific experiments of animal welfare (fig-1) [11].

Figure 1: (a) and (b) L.fulica, (c) Collection of L.fulica and (d) Feeding of L.fulica.

Extraction of mucin

The snails were sprayed sporadically for 10 minutes, with an acid-stimulating solution consisting of 5% citric acid. The solvent solution stimulated the snail to produce mucin without causing any stress or harm to it. It took approximately two hours to complete the extraction cycle. After sufficient filtration with a strainer and additional microfiltration using Whatman filter paper, the mucin were preserved in an appropriate containerand was stored in the refrigerator at 4°C (fig-2) [9,11].

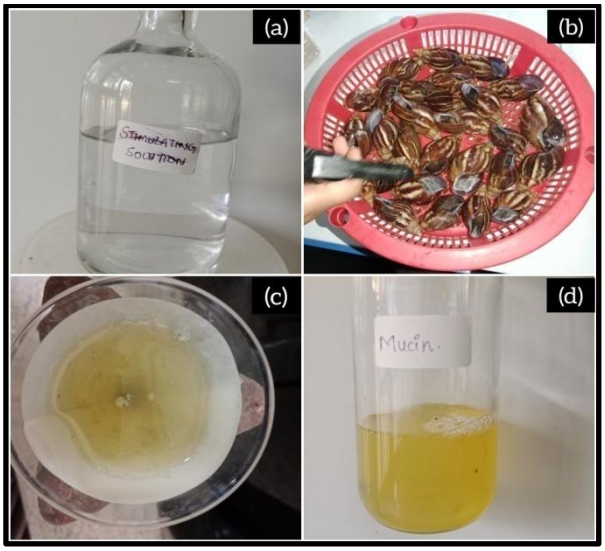

Figure 2: (a) Preparation of stimulating solution, (b) Spraying the stimulating solution on the L.fulica,
(c) Filtration of extracted L.fulica mucin and (d) Filtered mucin preserved at 4°C

Antibacterial Activity

1. Test Bacteria

To investigate the pathogenicity and antibiotic susceptibility of snail mucin against bacterial organisms the pure cultures of bacterial plates were collected from the Department of Microbiology, Maharani Cluster University, Bengaluru.

The organisms that were collected and sub cultured are Bacillus subtilis, Staphylococcus aureus, Pseudomonasaeruginosa, Escherichia coli, Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhimurium.

They were preserved at low temperatures for further research [12, 13].

2. Mueller Hinton Agar (MHA)

Mueller-Hinton agar (MHA) is a growth medium primarily used in laboratories for antibiotic susceptibility testing (table-1), especially the Agar Well diffusion method and Agar disc diffusion method [14].

Ingredient	Amount (in grams)
Starch	1.5g
Beef infusion	2g
Casein hydrolysate	17.5g
Agar	15g
Distilled water	1000ml
pH	7.3+/-0.2

3. Agar diffusion assay

1. Disc diffusion assay

In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism such as Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhimurium. Then, filter paper discs (6 mm in diameter), containing the test compound snail mucin are placed on the agar surface and the filter paper disc

containing Muller Hinton broth was used as a control. The Petri dishes are incubated at 37°C for 24 hours. The diameter of the inhibitory growth zones was then evaluated (fig-3) [16].

2. Well diffusion assay

Agar well diffusion assay was performed. The sterile autoclaved MHA media was poured into the autoclaved plate. Each test organisms such as Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhimurium were spread plated on to the individual solidified media plate. Using the cork borer the wells were made of 1cm in length along with the 4mm in depth. Each plate with one well containing control which was sterile MHA broth and another well contained 100% concentrated mucin. They all were incubated at 37°C for 24 hours and the zone of Inhibition that showed was measured in mm (fig 3) [17].

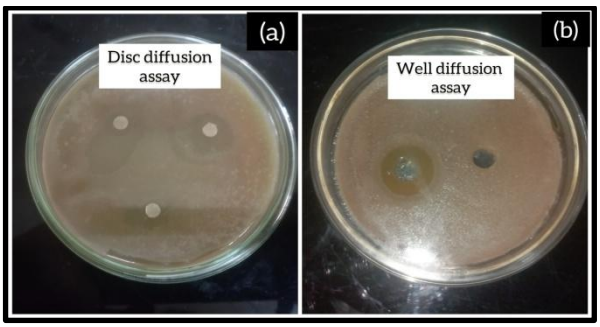

Figure 3: Antibacterial assays

(a) Agar disc diffusion method and (b) Agar well diffusion method.

Protein estimation

A series of 7 clean dry test tubes were taken and labelled as blank, 0.2, 0.4, 0.6, 0.8, 1.0 and unknown , add the BSA(bovine serum albumin) working standard into each test tube ,and make up to 1 ml using sterile distilled water. To the 7th test tube, 1 ml of the test sample such as snail mucin was added. Next, 1 ml of working Bradford reagent was added to all the test tubes, and they were mixed gently. The tubes were then incubated at room temperature for 5 minutes (fig-4). After the incubation period, absorbance was read for all the test tubes at 595 nm. A standard graph was plotted, and the concentration of protein in the snail mucin was calculated based on these readings [18].

Figure 4: Mucin estimation.

(a) Standard Bradford tubes and (b) L.fulica mucin estimation by Bradford method.

Microflora

The swabbing method was used to identify the diversity of bacteria and fungi taxa associated with L. fullica across its outer and inner shells. L. fullica was collected as the first step in studying the skin microbiota. Sterile cotton swabs were taken and swabbed on the inner and outer shell of the L. fullica using a different cotton swab in an aseptic condition. Later, the cotton swab was streaked onto the basal media, such as nutrient agar media for bacteria and potato dextrose agar media for fungi (Table-2 & 3). They were then incubated at 37±2°C for 24 hours to cultivate the bacteria and 25±2°C for a week to cultivate the fungi [19, 20].

Table 2: Composition of nutrient agar [21].

Ingredients	Amount (in grams)
Beef extract	3g
Peptone	5g
Sodium chloride	5g
Distilled water	1000ml
pH	7.0

Table -3: Composition of potato dextrose agar [22].

Ingredients	Amount (in grams)
Potato infusion	200 g
Dextrose	20 g
Agar	15 g
Distilled water	1000 ml
pH	5.6±0.2

RESULTS AND DISCUSSION

Antibacterial activity

The antibacterial activity of L.fulica mucin was performed against various test bacterial organisms by agar well diffusion and agar disc diffusion method and is as tabulated in table-4.

Table-4: Bacterial zone of Inhibition against snail mucin by agar well diffusion and agar disc diffusion methods.

BACTERIA	ZONE OF INHIBITION (in mm)	
	Agar well diffusion	Agar disc diffusion
Bacillus subtilis	15mm	23mm
Staphylococcus aureus	13mm	25mm
Pseudomonas aeruginosa	14mm	22mm
Escherichia coli	No zone of inhibition	No zone of inhibition
Klebsiella pneumoniae	14mm	22mm
Shigella dysenteriae	16mm	21mm
Salmonella typhimurium	15mm	22mm

The antibacterial activity of L.fulica mucin was detected by using agar disc diffusion method which showed highest zone of inhibition against Staphylococcus aureus with 25mm and agar well diffusion method showed highest zone of inhibition against Shigella dysenteriae with 16mm amongst other various test bacteria as mentioned in figure -5 & 6.

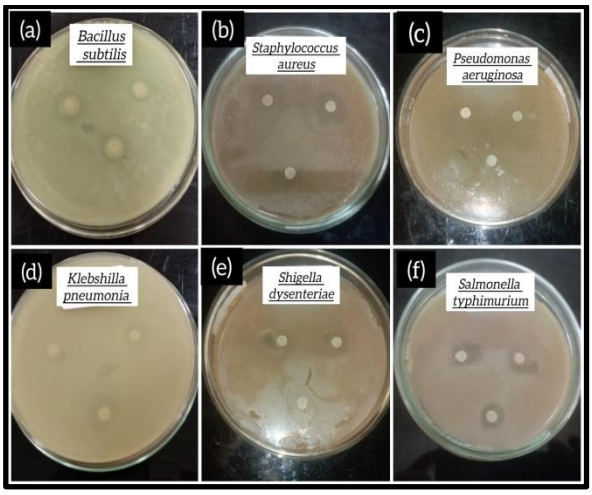

Figure 5: Disc diffusion assay.

(a) Bacillus subtilis, (b) Staphylococcus aureus, (c) Pseudomonas aeruginosa, (d) Klebsiella pneumonia, (e) Shigella dysenteriae and (f) Salmonella typhimurium.

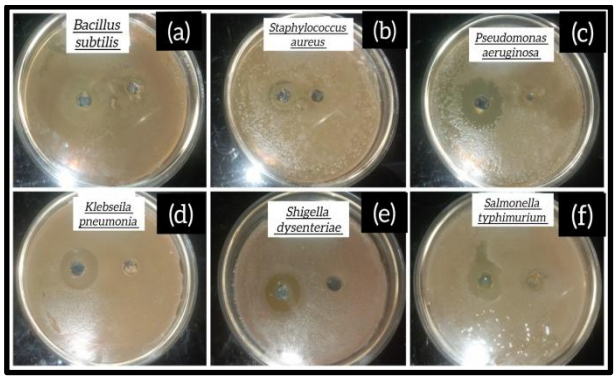

Figure 6: Well diffusion assay.

Bacillus subtilis, (b) Staphylococcus aureus, (c) Pseudomonas aeruginosa, (d) Klebsiella pneumonia, (e) Shigella dysenteriae and (f) Salmonella typhimurium.

Protein estimation by Bradford method

The L.fulica mucin concentrations were measured using the Bradford method. The mucin concentration extracted from L.fulica was 600µg/mL, respectively (Table-5). The protein profile was shown in (fig 7)

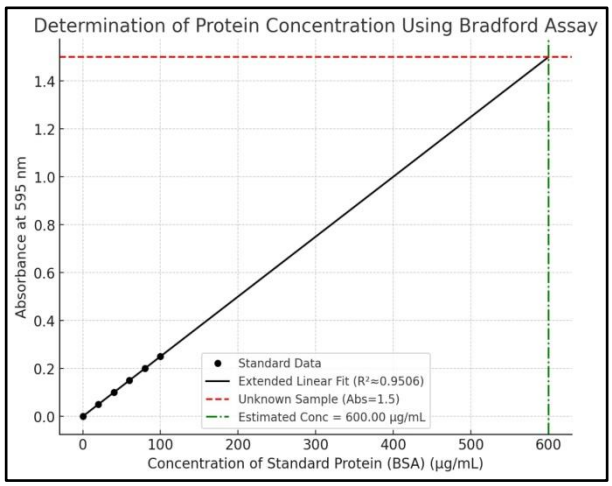

Figure 7: Graphical representation of concentration of standard BSA against absorbance at 595 nm with the L.fulica mucin concentration estimation by Bradford method.

Table 5: Protein Concentration of L.fulica mucin from the standard curve against the concentration of BSA by Bradford method.

***L.fulica* mucin**	**Results**
Mucin concentration	600 µg/mL

Microflora

The L.fulica is in symbiotic association with several bacteria and fungi organisms. The bacteria and fungi present in the head region, internal shell region and the external shell region of L.fulica where isolated by performing skin swab on nutrient agar for bacteria at 37±2°C for 24 hours and potato dextrose agar for fungi at 25±2°C for a week (fig-8 & 9). The macroscopic and microscopic characteristics of isolated microorganisms are as tabulated in table- 6 &7.

Bacteria

Table 6: Isolated bacteria from head region, internal shell region and the external shell region of L.fulica and their characteristics

BODY PART	ORGANISM	MACROSCOPIC	MICROSCOPIC
Head	Streptococci	Small, circular, translucent colonies	Cocci in chains
Internal shell	Gram-negative *Diplobacilli*	Smooth, moist, greyish colonies	Rod-shaped, in pairs
External shell	*Staphylococcus*	Golden yellow, round, convex colonies	Cocci in clusters
	Gram-negative *Coccobacilli*	Small, smooth, slightly raised colonies	Short rods, oval-shaped

Fungi

Table 7: Isolated fungi from head region, internal shell region and the external shell region of L.fulica and their characteristics

BODY PART	ORGANISM	MACROSCOPIC	MICROSCOPIC
Head	Yeast	Creamy, smooth, moist colonies reaching 2-5 mm in 3 days	Oval to round budding cells
Internal shell	*Rhizopus*	Fluffy white colonies, colony diameter up to 60-70 mm in 5-7 days	Large sporangia with rhizoids opposite the sporangiophores
External shell	*Aspergillus*	Colonies ranging in size from 15-20 mm after 5 days to 50-60 mm in 10 days, Slightly umbonate, texture initially hairy which turns velvety after 10 days, regular circular margins, Colour of the colony from top appears dark black due to heavy sporulation, heavy sporulation within a week, colony is cream and wrinkled from reverse after 5 days which later turns darker, with radial sulcation.	Vesicle: globose Conidial heads: Uniseriate Conidia : Globose, rough walled
	Fusarium	Cottony colonies with radial sulcation, 40-60 mm after 5-7 days	Septate hyphae with canoe-shaped macroconidia
	Cladosporium	Colonies are olive-green to olive-brown with a velvety or powdery appearance. Colonies are diffused, and the mycelia form mats	Irregular branched conidiophores, brown to olive-brown conidia, coronate scar structures and conidia in acropetal chains.
	Chrysosporium	Powdery, pale yellow colonies, 20-40 mm in 7-10 days	Single-celled conidia, borne on short conidiophores, often aleuriospores
	Yeast	Creamy, smooth colonies reaching 2-5 mm in 2-3 days	Oval to round budding cells

CONCLUSION

Research on snail mucin is still hindered by numerous barriers, despite the field's increasing interest. Due to their habitat, many snail species that may yield novel mucin discoveries are frequently unreachable. Though the field is still in its early stages, several organizations are looking towards scalable, sustainable methods of creating synthetic mucins. Other species are still ignored, despite the fact that mucins obtained from L. fulica have been thoroughly researched. Mucin derived from many land snails has been extensively utilised in medicine and cosmetics, but some biological activities of the mucin need to be well documented. Nevertheless, most mucin is obtained from land snails, while mucin from freshwater snails has yet to be attended.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

INFORMED CONSENT

Informed consent was not applicable for this study.

ETHICAL STATEMENT

This study was approved by a recognized ethics committee.

AUTHOR CONTRIBUTIONS

Concept and Design: Dr. Kiruthika Panneerselvam and Data Collection, Analysis, and Writing: Aiswarya L., Shabana Begum, and Sumaiya Arishi.

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