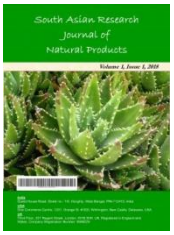

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Antimicrobial Potential of the Crude Extracts and Peptide Fractions of Two Marine Molluscs: *Tympanatonus fuscatus* Var Radula (Linneaus) and *Pachymelania aurita* (Muller)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors EQ, OO and AS designed the study, wrote the protocols, managed the literature searches and performed all analysis. Author EQ also wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This study was aimed at evaluating the antimicrobial potential of the alcohol and aqueous extracts as well as peptide fractions of *T. fuscatus* and *P. aurita.*
Place and Duration of Study: Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.
Methodology: The antimicrobial activity of the whole body aqueous and acetone-methanol extracts of *T.fuscatus* Var Radula and *P.aurita*, collected from the Niger-Delta region of Nigeria,

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were evaluated based on inhibition zone diameter using the agar well diffusion method against ten bacterial isolates and *C. albicans.* These organisms were further used in the TLC bioautography experiment. The peptide fraction from the organic extracts of both organisms was obtained by Molecular sieve chromatography on Sephadex LH20. Peaks obtained were pooled and further analysed on TLC. A simple contact TLC bioautographic procedure was used to detect the number of antibacterial and antifungal peptides present in the extracts of both *T. fuscatus* and *P. aurita*.
Results: The aqueous extract of both *T. fuscatus* and *P. aurita* had no antimicrobial effect against the test microorganisms whereas the acetone-methanol extract showed broad-spectrum antibacterial activity against five bacterial isolates at the highest concentration (100 mg/ml). It also showed inhibition against *C. albicans* at this concentration (100 mg/ml).
All the peptides exhibited bactericidal activity against the five test bacterial isolates and bacteriostatic activity against *C. albicans*. This activity was denoted by inhibition of growth in the region in which the peptides on the TLC plate made contact with the agar containing the isolates.
Conclusion: Further studies to effectively separate these peptide fractions into individual peptides and further investigate the antimicrobial activity of the individual peptides is required.

Keywords: Antimicrobial; crude extracts; Tympanatonus fuscatus; Pachymelania aurita.

1. INTRODUCTION

In general, marine molluscs are soft bodied and sessile and often live in microbe-rich habitats. Hence, molluscs are often exposed to pathogens and do not often possess a proper physical barrier against microbial infection. This suggests that molluscs must have evolved alternative biological defense strategies and systems, including the secretion of mucus containing a range of different antibacterial, antifungal, antiparasitic and antiviral secondary metabolites to protect themselves against an onslaught of microbial invasion from their environment [1,2]. One of the defense strategies used by mollusc, and indeed, a host of other organisms is the production of antimicrobial peptides (AMPs) also known as host defence peptides (HDPs). AMPs play key roles in innate immunity and they had been observed in a wide variety of organisms in the last few years. Research has been focused on the discovery and exploitation for health benefits of AMPs and other peptides with antimicrobial activity. This is partly driven by the need for new antibiotics, which is in turn due to the emerging threat of antibiotic resistance.

AMPs are ribosomally synthesized from proteinogenic amino acids. They are short, generally positively charged, potent, broad spectrum antibiotics and have been demonstrated to kill both gram positive and gram negative bacteria [3]. Unlike antibiotics, which target specific cellular activities, AMPs target the lipopolysaccharide layer of cell membrane, which is unique to microorganisms. The high cholesterol level and negative charge of the bacterial membrane ensure that eukaryotic cells are usually not targets of many AMPs [3,4,5]. Another important feature of AMPs is their fast killing ability. Some AMPs can kill in seconds after the initial contact with cell membrane [4]. In addition to their role as endogenous antibiotics, some AMPs contribute to inflammation as well as exhibit immunomodulatory activities [3]. Hence, these AMPs act indirectly to kill microorganisms by modulating the host defense systems. Some other AMPs kill bacteria by inhibiting some important pathways inside the cell such as DNA replication and protein synthesis [4].

Their preferential attack on the cell membrane or cell wall of bacterial and fungi ensure that AMPs should not cause widespread resistance. In cases where specific protein targets are involved, the possibility exists for genetic mutations and bacterial resistance. However, evidence suggests that this is a rare event which can be overcome by subtle structural modifications made to the AMP [5].

Other activities that AMPs have been shown to possess include: anti-biofilm, wound repair and anticancer activities. They are also known to play a role in regulation of the adaptive immune system [2,6]. Hence, several AMPs are currently being evaluated in clinical trials, not only as novel antibiotics [5], but also as new pharmacological agents to modulate the immune response, promote wound healing, and prevent post-surgical adhesions.

This study was undertaken to evaluate the antimicrobial potential of the crude extracts as well as the peptide fraction of *Tympanatonus fuscatus* Var Radula and *Pachymelania aurita*.

Tympanatonus fuscatus and *Pachymelania aurita* are two of the most common mollusc species which inhabit the mangroves of the Niger Delta region of Nigeria although they are also found along the coast of West Africa as well as in Angola and Gabon. Their flesh is used in the preparation of delicacies and although related species feature in a range of traditional natural remedies, including wound healing and stomach upsets, and have been evaluated for their antimicrobial properties, these two species have not been assessed to determine if they possess antimicrobial activity. This is the first known study to investigate the antimicrobial potential of whole body extracts of *T. fuscatus* Var Radula and *P. aurita*, obtained from the Niger Delta region of Nigeria.

2. MATERIALS AND METHODS

2.1 Microorganisms

Microbial isolates were obtained from the National Collection of Industrial Food and Marine Bacteria (NCIB), UK and the American Type Culture Collection, ATCC, Rockville, MD, USA. The bacteria strains used for this study were: *Proteus vulgaris* (NCIB 67); *Pseudomonas aeruginosa* (NCIB 950); *Bacillus subtilis* (NCIB 3610); *Staphylococcus aureus* (ATCC 43300); *Escherichia coli* (NCIB 86); *Micrococcus luteus* (NCIB 196); *Klebsiella pneumoniae* (NCIB 418); *Clostridium sporogenes* (NCIB 532); *Bacillius stearothermophilus* (NCIB 8222); *Serratia marcescens* (NCIB 1377) while the yeast strain used was *Candida albicans.*

2.2 Sample Collection

Live *Tympanotonus fuscatus var radula* and *Pachymelania aurita* were purchased from the Oron Beach Market, Oron, Akwa Ibom State, Nigeria (GPS coordinates: 4°49´37.6´´ N 8°14´04.4´´ E). The molluscs were washed thoroughly to remove mud and then deshelled to collect both their flesh and hemolymph.

2.3 Preparation of Acetone-methanol Extracts

The alcohol extracts of *T.fuscatus* and *P. aurita* were prepared using the method described by Eghianruwa et al. [7]. 200 g of mollusc flesh in its hemolymph was macerated using a blender and extracted twice with 1 L acetone for both cycles. Each cycle of extraction with acetone was carried out at room temperature for 12 hrs with constant stirring using a magnetic stirrer and the homogenate was filtered using a muslin cloth. After acetone extraction, the biomass residue of the sample was subjected to two cycles of extraction using a total of 1500 ml of methanol. The Acetone and methanol fractions were combined and concentrated by evaporation using a rotary evaporator at 40ºC then stored at 4ºC.

2.4 Preparation of the Aqueous Extracts

200 g of mollusc flesh in its hemolymph was homogenized with 2000 ml of Phosphate buffered saline; PBS, pH 7.2 (0.1 M Sodium chloride in 0.025 M Sodium dihydrogen orthophosphate with 0.1 M PMSF) using a blender. The homogenate was left to extract for 48 hours at 4ºC after which it was centrifuged at 10,000 g using a cold centrifuge, freeze dried and stored at 4^{o}C.

2.5 Sensitivity Test

The antimicrobial activity of the extracts was carried out using the agar-well diffusion method as described [8] with some modifications. The bacterial strains used were first grown on nutrient agar for 18hrs before use. The turbidity of the 18 hr old culture was adjusted to 0.5 McFarland Standards (106 cfu/mL) in sterile normal saline. The inoculum was then seeded onto sterilized Mueller-Hinton agar using a sterile swab stick. Wells were made in the seeded plates using a sterile 6mm cork borer. The wells were filled up with known concentrations of the extracts (25, 50 and 100 mg/ml) using a micropipette. Care was taken to avoid spillage of the extract onto the surface of the medium. The plates were allowed to stand on the work bench for 1hr to allow proper inflow of the extract solutions into the medium before incubating in an incubator at 37°C for 24 hr following which the plates were observed for zones of inhibition. The effects of the extracts of *T. fuscatus* and *P. aurita* on the bacterial strains were compared to a standard antibiotic (Streptomycin). Water and a mixture of acetone-methanol (1:1) were also used as control in the experiment to confirm that any activity observed is as a result of the extract and not the solvents used in extraction.

2.6 Molecular Sieve Chromatography on Sephadex LH20

The peptide fractions from the alcohol extracts of *P. aurita* and *T. fuscatus* were obtained via molecular sieve chromatography on a Sephadex

LH20 column. Sephadex LH20 resin (40 g) was swollen at room temperature in 200 ml of absolute methanol for 5 hrs. The slurry was stirred every hour during this time and fine particles were removed by decantation. The slurry was packed into a column (10 × 1.5 cm) according to instructions contained in the Pharmacia laboratory techniques manual. The packed column was equilibrated with 300 ml PBS, pH 7.2. Crude aqueous extract (2.5 ml) of either *P. aurita* and *T.fuscatus* were applied on the column and eluted with 1 column volume of methanol and fractions (1 ml) were collected at a flow rate of 10 ml/hr. Peptide-containing fractions were detected by means of TLC.

2.7 TLC Bioautography

Analytical thin layer chromatography was used to detect the peptide containing fractions from the Molecular sieve experiment using the method as described by Osoniyi and Onajobi [9]. The TLC was carried out on aluminium-backed silica 60 F254 gel plates (10 cm X 8 cm), using a solvent system of butanol: acetic acid: water (3:1:1) as the mobile phase while the detection stain was 0.2% Ninhydrin in ethanol. Plates were activated in an oven at 100-120°C for 1 hr and allowed to cool before use. The solvent system was prepared fresh 15 mins before each run, placed in the tank, swirled and allowed to saturate the tank before the run. 5 µl of each fraction obtained from the molecular sieve procedure was spotted on the plate and allowed to dry before placing in the tank for the run. After the run, the plate was air dried, sprayed with the ninhydrin stain and then dried in the oven at 100°C for 15 mins to allow for colour development. Plates used in bioautography were not sprayed with the stain.

The bioautography assay was carried out in order to determine which peptide band exhibits antimicrobial activity. Agar plates inoculated with the microbial strains were prepared as described above for the sensitivity testing with the exception of the holes. The alcohol extracts of either *P. aurita* or *T. fuscatus* were run on a TLC as described above. The unstained TLC plates were placed on the Agar plates and the plates were allowed to stand on the work bench for 1 hr to allow proper diffusion of the peptides on the TLC plates into the medium before incubating in an incubator at 37°C for 24 hr following which the agar plates were observed for zones of inhibition. The plates were incubated for a further 24 hr after which the TLC plates were removed with a pair of forceps. After the removal of the TLC plates, the agar plates were incubated for another 48 hrs to check for microbial growth.

3. RESULTS AND DISCUSSION

The results showing the length of the zones of inhibition of the various bacterial species by the extracts are shown in Table 1. The aqueous extracts of *P. aurita* and *T. fuscatus* do not exhibit any antimicrobial activity against the organisms tested, even at the highest concentration tested (100 mg/ml). Alcohol extracts of both *P. aurita* and *T. fuscatus*, on the other hand demonstrated antimicrobial activity. A zone of inhibition measuring approximately 8 mm was observed in the culture of *S. aureus* and *B. stereothermophilus* when treated with 25 mg/ml of the alcohol extract of *P. aurita* (PAAC). At 100 mg/ml, longer zones of inhibition were observed in the cultures of *S. aureus* and *B. stereothermophilus*, *M. luteus*, *C. sporogenes* and *K. pneumoniae*. Zones of Inhibitions were also observed in the cultures of *M. luteus, C. sporogenes, K. pneumoniae, B. stereothermophilus* and *S. aureus*, treated with the alcohol extract of *T. fuscatus* (TFAC) but only at a concentration of 100 mg/ml of extract. Zones of inhibition (12 mm) were also observed in the culture media of *C. albicans* treated with the crude alcohol extracts of *P.aurita* and *T. fuscatus* at a concentration of 100 mg/ml of extract. Hence, the bioautography experiments were carried out using cultures of *M. luteus, C. sporogenes, K. pneumoniae, B. stereothermophilus*, *S. aureus* and *C. albicans*.

The results of the molecular sieve chromatography of the crude alcohol extract of *P. aurita* and *T. fuscatus* is illustrated in Fig. 1. Three peptide peaks were detected by spotting each fraction in TLC. Each peak was observed to contain several peptide molecules (Fig. 2). However, peak 2 obtained from both extracts (PAAC and TFAC) appear to possess similar peptides.

Resolved but unstained TLC plates of the analysed peptide peaks were placed in cultures of *M. luteus, C. sporogenes, K. pneumoniae, B. stereothermophilus*, *S. aureus* and *C. albicans*. After 24 hrs of the start of the bioautography experiment, no microbial growth was observed under the plates (Fig. 3). Slight zones of inhibition were observed in the cultures of *M. luteus* and *Bacillius Stearothermophilus* treated with TFAC. After 48 hrs, the TLC plates were removed and observed for microbial growth

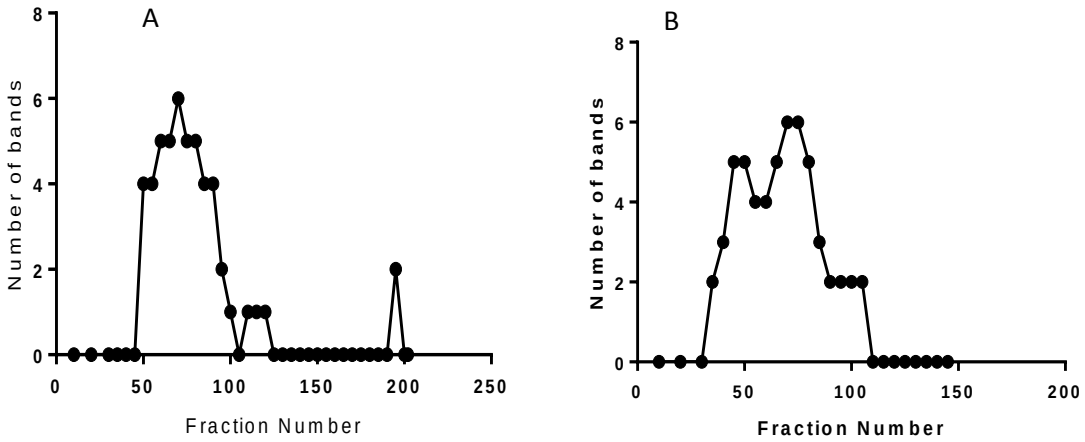

Fig. 1. Elution profile from thin layer chromatography of fractions obtained from molecular sieve chromatography of the alcohol extract of *P. aurita* (A) and *T. fuscatus* (B) on Sephadex LH20. Eluant for the molecular sieve chromatography was methanol. The major peaks were pooled separately and used for the bioautography experiment

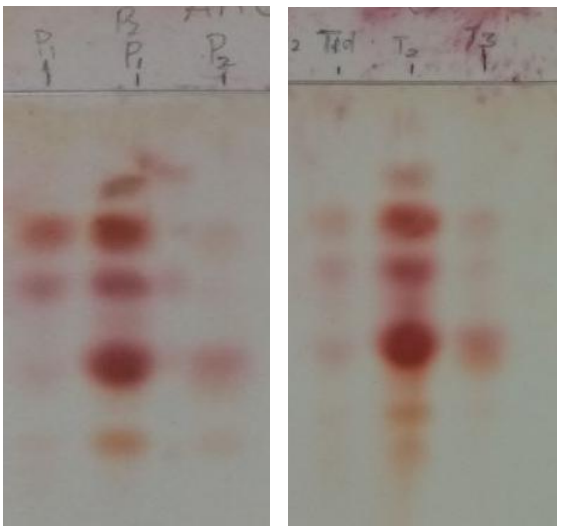

Fig. 2. TLC chromatogram of the three peaks pooled from the molecular sieve chromatography separation of the alcohol extract of *P. aurita* (P1-P3) and *T. fuscatus* (T1-T3). Replicate plates, which was not stained after the TLC run, was used for the bioautography experiments

(Fig. 4). Microbial growth was observed in the area previously covered by the TLC plate in the culture for *C. albicans*, although the growth was slight and not as profuse as in the rest of the culture. The culture dishes were incubated further for 48 hrs after the removal of the TLC plates (Fig. 5). Slight microbial growth was observed in most of the cultures albeit less than in the rest of the culture plate.

To survive their microbe-rich environment, molluscs must have evolved alternative biological defense strategies and systems, including the secretion of mucus containing a range of different antibacterial, antifungal, antiparasitic and antiviral secondary metabolites to protect themselves against an onslaught of microbial invasion from their environment [1,2].

The observation, from this study, that only the alcohol extracts exhibited antimicrobial activity against any of the isolates tested, are in line with evidence from previous studies which have reported that the compounds responsible for antimicrobial activity are mainly non-polar in nature [10,11], hence alcohols (especially methanol) are a better solvent system for more reliable extraction of antimicrobial molecules

Table 1. Zones of inhibition values (mm) from the sensitivity testing of the crude aqueous and alcohol extracts of *P. aurita* and *T. fuscatus*

Isolates	PAAC			TFAC			Control Streptomycin (1 mg/ml)
	25 mg/ml	50 mg/ml	100 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml	
P.vulgaris (NCIB 67)	0± 00	0± 00	0±00	0 ± 00	0 ± 00	0±00	25± 00
Ps. Aeruginosa (NCIB 950)	0±00	0 ± 00	0±00	0± 00	0 ± 00	0±00	25± 0.6
B.subtilis (NCIB 3610)	0±00	0 ± 00	0±00	0± 00	0 ± 00	0±00	27± 0.4
S.aureus (ATCC 43300)	8±1.3	12± 0.7	12±1.4	0± 00	0 ± 00	11.5±0.7	27± 1.7
E.coli (NCIB 86)	0±00	0 ± 00	0±00	0± 00	0 ± 00	0±00	30± 00
M.luteus (NCIB 196)	0±00	0 ± 00	12±1.4	0± 00	0 ± 00	12.0±1.4	30± 00
K.pneumoniae (NCIB 418)	0±00	0± 00	11.5±0.7	0± 00	0 ± 00	11.5±0.7	25± 1.3
C.sporogenes (NCIB 532)	0±00	0 ± 00	11.5±0.7	0± 00	0 ± 00	11.0±00	25± 0.7
B.stereothermophilus(NCIB 8222)	8± 0.6	12 ± 00	10.5±0.7	0± 00	0 ± 00	10.5±0.7	30± 00
S.marcescens(NCIB 1377)	0±00	0± 00	0±00	0± 00	0 ± 00	0±00	25± 00
C.albicans	0±00	0±00	12±00	0±00	0±00	12±00	25 ±00

Values are expressed as mean ± SEM, n=2. Streptomycin (1mg/ml) was used was the standard control

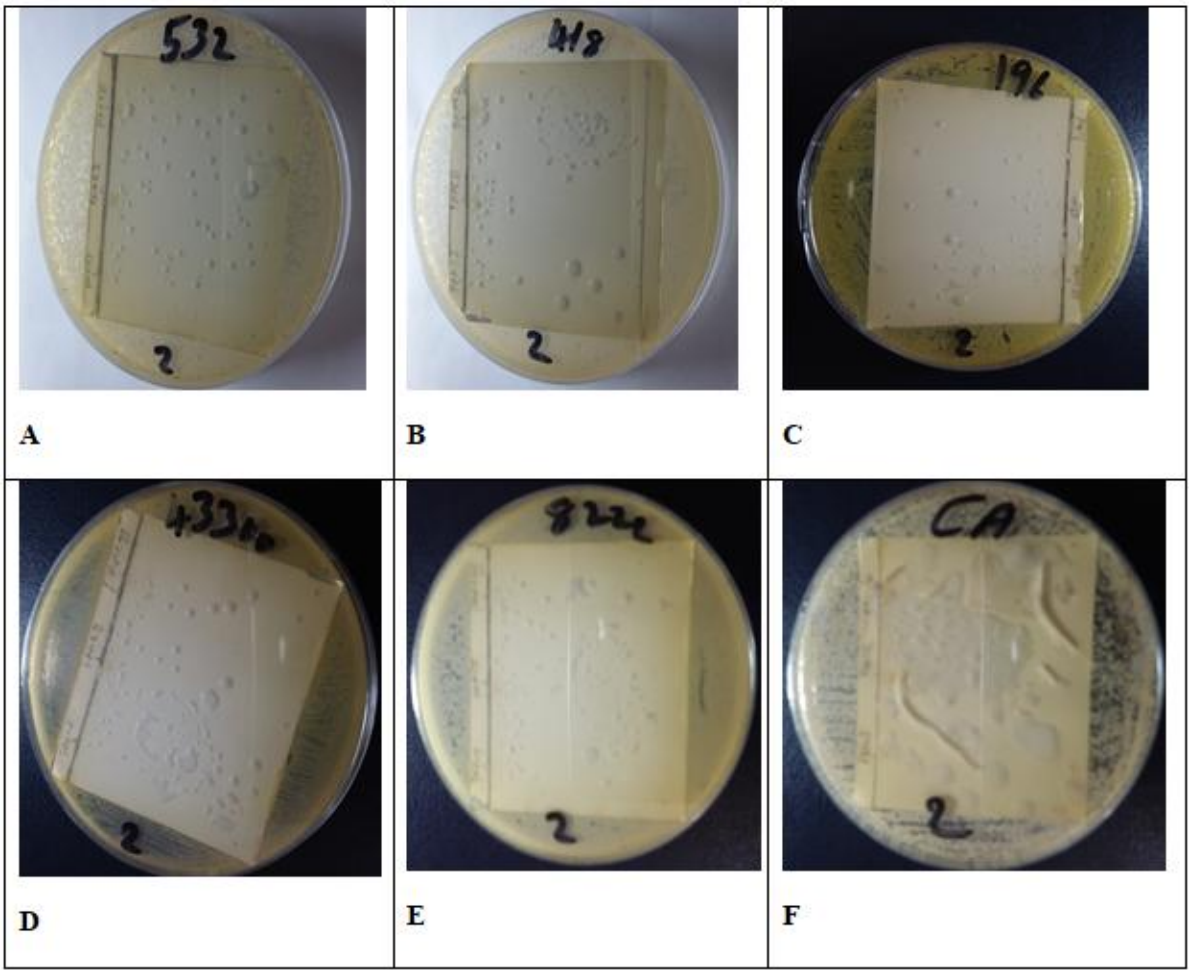

Fig. 3. Stage 1 of the contact bioautography experiment (24 hrs after the start of the experiment) of the peptides obtained from molecular sieve of the crude alcohol extract of *P.aurita* on sephadex LH20. The Agar plates were inoculated with the microbial strains: A = *C. sporogenes* (532), B = *K. pneumoniae* (418), C = *M.luteus* (196), D = *S.aureus* (43300), E = *B. stereothermophilus* (8222) and F = *C. albicans* (CA). Similar results were observed for *T. fuscatus*

from natural sources compared to other solvents [12,13,14]. Indeed, in the case of marine organisms, non-polar antimicrobial molecules would be most desirable as these molecules would not easily interact with the surrounding water environment and be washed easily off the organism.

In isolating molecules with antimicrobial activity, bioautography is a very useful technique as the Rf of the active compounds can be used in bioassay guided fractionation instead of requiring labour intensive determination of activity of several fractions [11,15]. This also ensures that the molecule isolated at the end is the same one that was present in the extract and not an artefact of the isolation procedure [11]. In contact bioautography, antimicrobial agents diffuse from a developed TLC plate to an inoculated agar plate [15]. The complete lack of bacterial growth in the area in contact with the peptides from the TLC plates indicates that all the peptides present on the chromatogram from the extracts exhibit antibacterial activity which can be said to be bactericidal in nature. The peptides also exhibited antimicrobial potential against *C. albicans* but the slight but uniform growth observed suggests that the peptides also exhibited a fungistatic, rather than fungicidal activity against *C. albicans*. After removal of the TLC plates, the isolates were still incubated for a further 48 hrs. At this time, slight microbial growth was observed in the area of the of the Agar plate that had been in contact with the

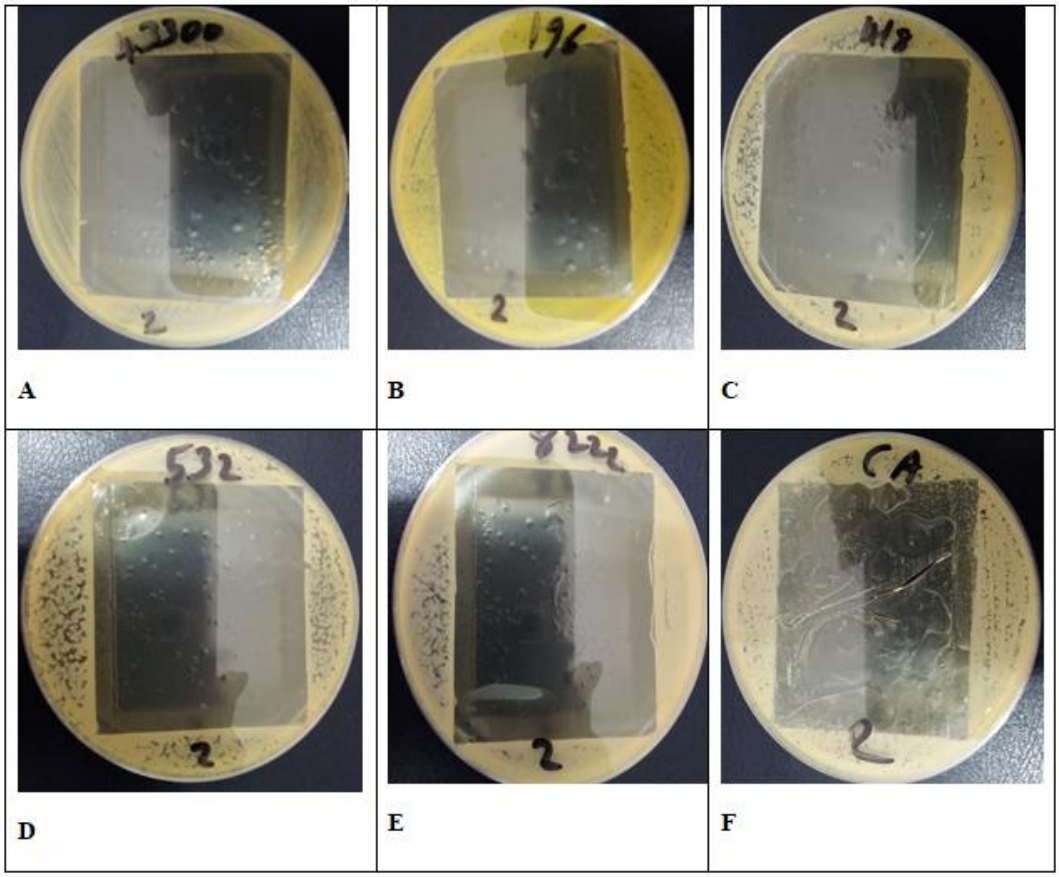

Fig. 4. Stage 2 of the contact bioautography experiment (48 hrs after the start of the experiment) involving the peptides obtained from molecular sieve of the crude alcohol extract of *P. aurita* on sephadex LH20 and peaks resolved on TLC. The Agar plates were inoculated with the microbial strains; A = *S. aureus* (43300), B = *M. luteus* (196), C = *K. pneumoniae* (418), D = *C. sporogenes* (532), E = *B. stereothermophilus* (8222) and F = *C. albicans* (CA). Similar results were observed for *T. fuscatus*

peptides. As the growth of the microbes in this area was not as profuse as other areas of the plate, this implies a sustained antimicrobial activity of the peptides.

One of the defence strategies used by molluscs, and indeed, a host of other organisms is the production of antimicrobial peptides (AMPs) also known as host defence peptides (HDPs). AMPs play key roles in innate immunity. Unlike antibiotics, which target specific cellular activities, AMPs target the lipopolysaccharide layer of cell membrane, which is unique to microorganisms. The high cholesterol level and negative charge of the bacterial membrane ensure that eukaryotic cells are usually not targets of many AMPs [3,4,5]. Hence, Selectivity is a very important feature of the antimicrobial peptides and it can guarantee their function as antibiotics in host defense systems. Another important feature of AMPs is their fast killing ability. Some AMPs can kill in seconds after the initial contact with cell membrane [4]. In addition to their role as endogenous antibiotics, some AMPs contribute to inflammation as well as exhibit immunomodulatory activities [3]. Hence, these AMPs act indirectly to kill microorganisms by modulating the host defense systems. Some other AMPs kill bacteria by inhibiting some important pathways inside the cell such as DNA replication and protein synthesis [4]. Their preferential attack on the cell membrane or cell wall of bacterial and fungi ensure that AMPs should not cause widespread resistance. In cases where specific protein targets are involved, the possibility exists for genetic mutations and bacterial resistance. However, evidence suggests that this is a rare event which can be overcome by subtle structural modifications made to the AMP [5].

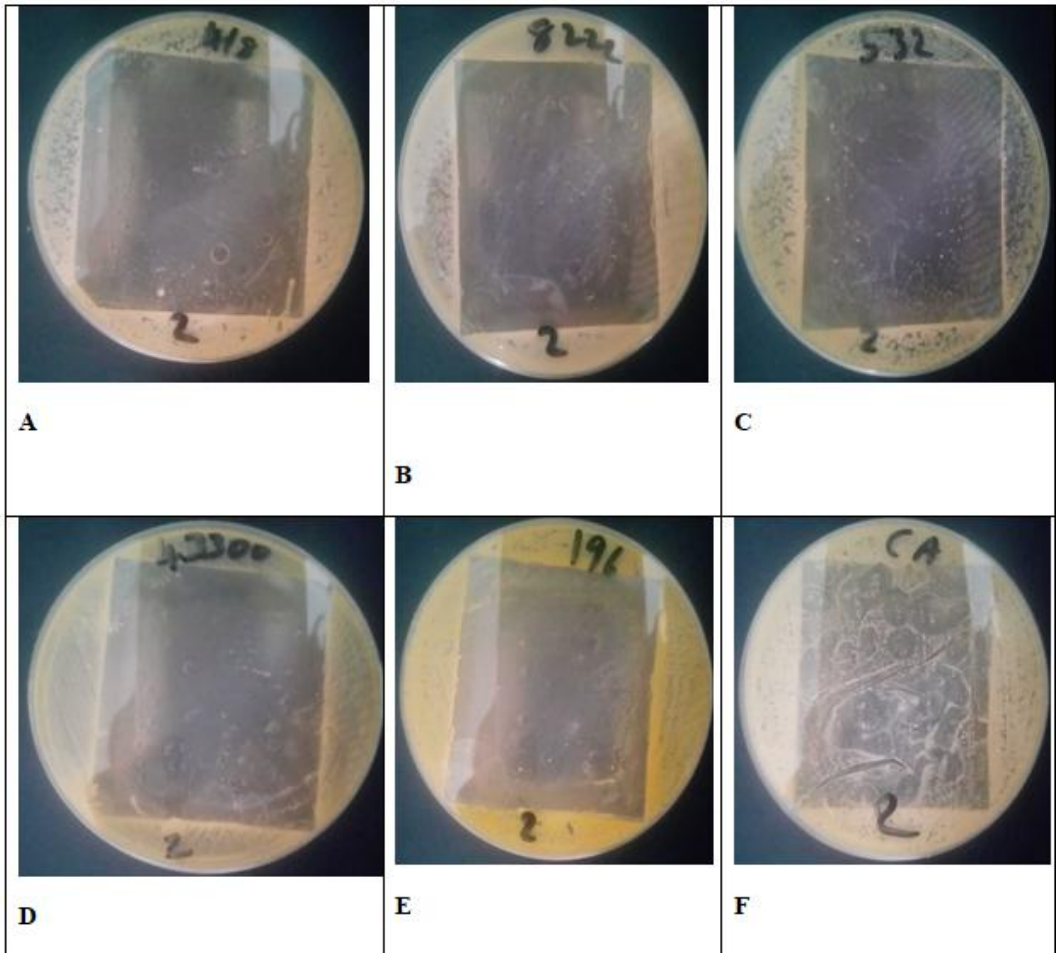

Fig. 5. Stage 3 of the bioautography experiment (96 hrs after the start of the experiment) involving the peptides obtained from molecular sieve of the crude alcohol extract of *P. aurita* on sephadex LH20 and peaks resolved on TLC. The Agar plates were inoculated with the microbial strains; A = *K. pneumoniae* (418), B = *B. stereothermophilus* (8222), C = *C. sporogenes* (532), D = *S. aureus* (43300), E= *M. luteus* (196) and F = *C. albicans* (CA). Similar results were observed for *T. fuscatus*

Generally, AMPs are only effective against one class of microorganisms (e.g., bacteria or fungi). However, there are some notable exceptions like indolicidin, which can kill bacteria, fungi, and viruses (HIV) [4]. The AMPs from the alcohol extract of *P.aurita* and *T.fuscatus* have been shown in this study to be effective against both bacteria and fungi species albeit by different mechanisms.

4. CONCLUSION

The results demonstrate that the acetone-methanol extract of *T. fuscatus* and *P. aurita* possess multiple peptides with antibacterial and fungistatic properties which may be useful as antimicrobial agents in new drugs for therapy of infectious diseases. Further studies to isolate each individual peptide for further studies is required.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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