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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF SOME TERRESTRIAL GASTROPODS FROM EGYPT AGAINST *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI*

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ABSTRACT

Terrestrial invertebrates are subjected to a wide range of microbial infections throughout their life. Therefore, they have powerful antimicrobial agents. The present study was designed to evaluate the antibacterial efficiency of different extracts of terrestrial gastropods against two pathogenic bacteria: *Escherichia coli* and *Staphylococcus aureus*. Phosphate buffer saline (PBS), acetone, and methanol were used to extract viscera, haemolymph, and mucus from the snails, *Helix aspersa* and *Eobania vermiculata*, and the slug, *Deroceras reticulatum*. All extracts from haemolymph and mucus of the three snails did not inhibit the growth of the selected pathogenic bacteria. However, the methanolic extract of *E. vermiculata* viscera inhibited the growth of *S. aureus* at 50% concentration; and the inhibition zone reached 12.3 ± 0.6 mm. In addition, the methanolic extract of *H. aspersa* viscera inhibited the growth of *S. aureus* at 100 and 50% concentrations up to 19.3 ± 0.6 and 18.0 ± 0.1 mm, respectively, and induced a moderate decrease in the growth of *E. coli* at 100% concentration. In addition, scanning electron microscopy (SEM) assured damaging impacts of the methanolic and PBS viscera extracts of the two snails "*H. aspersa* and *E. vermiculata*" on both *E. coli* and *S. aureus*. In conclusion, the antibacterial properties of the methanolic viscera extracts of the terrestrial gastropods may encourage the discovery of new and safe antibiotics from animal origin.

INTRODUCTION

Molluscs are widely distributed throughout the world and have many representatives such as slugs, land snails, clams, squids, and octopods[1]. The garden snail, *Helix aspersa*, has several pharmacological activities that are useful in the biomedical area[2]. Molluscan bioactive compounds exhibited antitumour, antibacterial, and antiviral properties[3,4]. Different snail crude proteins from the freshwater and land snails were previously evaluated for their antimicrobial activity[5]. The most active crude proteins were from the land snail, *Cryptozona bistrialis*, which was capable of completely inhibiting the development of the

pathogenic bacteria *Staphylococcus aureus*, *Micrococcus luteus*, and *Pseudomonas aeruginosa*, as well as the pathogenic fungi *Candida albicans*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, and *Mucor racemosus*[5]. Some marine molluscs have antimicrobial proteins, which contain a variety of antimicrobial and antioxidant compounds[6]. In addition, many of the bioactive compounds such as peptides, sterols, nitrogenous compounds, and fatty acid derivatives were isolated from the molluscan protein-rich meat[6]. The antibacterial glycoprotein, achacin (140-160 kDa), which isolated from the land snail, *Achatina fulica*, inhibited the growth of Gram-positive bacteria, *S. aureus*[7]. Glycans, peptides, glycopeptides, and proteins were also isolated from the haemolymph of the garden snail, *Helix lucorum*[8]. In addition, the antibacterial substances in the mucus of *H. aspersa* ranged from 30-100 kDa, and exhibited activity against *S. aureus*[9].

Many authors explored the bioactive compounds from different parts of the land snails[1,10-13]. El Mubarak *et al*.[10] isolated allantoin and glycolic acid from the mucus of *H. aspersa*, which showed therapeutic properties for human skin. Snail mucus also used in repairing ulcers, and its bioactive compounds-derived drugs can be used in creams to ease scars and skin abrasions[1]. The most antimicrobial peptides were found in the haemolymph of the invertebrates, with activity against bacteria, virus, and protozoa[11]. Several proline-rich antimicrobial peptides were isolated from the haemolymph of *Rapana venosa* and *Helix lucorum* snails that showed strong antibacterial activities against Gram-positive and Gram-negative bacteria[12]. The mini-mum inhibition zone was 2 mm against *S. aureus* in the crude methanolic extract of the gastropod, *Babylonia spirata*[13]. Scanning electron microscopy (SEM) considered as an important tool to directly detect the changes of cell morphology and membrane integrity. Many authors used this technique to describe the antibacterial activity of the plant extracts; for example, Kaya *et al*.[14] used SEM to detect shrinking and degradation of the cell walls of *Staphylococcus aureus* and *Escherichia coli* treated with chloroform, acetone, and methanolic extracts of *Ocimum basilicum*. Ramli *et al*.[15] also reported rupture of the cell wall and leakage of the cytoplasm from treated bacteria with ethanolic extract of *Syzygium polyanthum L*. (Salam) leaves. In addition, the treatment of *S. aures* by the ethyl acetate extract of the fungus, *Nigrospora sphaerica*, caused shrinkage of the cell surface, irregular shape, and cleavage of the cell envelope[16]. Therefore, the present study aimed to evaluate the potential antibacterial properties of different tissue extracts of different gastropods against bacterial strains, *S. aureus* and *E. coli*, using the agar well diffusion method and SEM.

MATERIAL AND METHODS

Animals

The terrestrial gastropod snails used in the present study were *H. aspersa*, *Eiobania vermiculata*, and the slug, *Deroceras reticulatum*. They were collected from the infested ornamental and grass plants. The gastropods were maintained under laboratory conditions at room temperature and 80% relative humidity.

Animals' maintenance

Wild individuals (10-15, average weight 4.0 ± 0.5 g/snail and 2.0 ± 0.7 g/slug) were placed in glass containers measuring (15 cm width × 15 cm height × 22 cm length). The containers were filled with sandy loam. The lid of the containers was a muselin material for ventilation. The gastropods were daily supplied with fresh lettuce (*Lactuca sativa*) leaves and water for soil humidity. The remaining food and faecal matter were removed at the end of every other day. The gastro-pods were acclimatised under laboratory conditions for at least four weeks before being used in the screening tests.

Haemolymph, mucus, and tissue extraction

Haemolymph samples were collected from snails and slug from the cephalic sinus using a syringe needle (21 gauge). Mucus production was stimulated by gently holding the snails and slugs. It was collected by plastic Pasteur pipette and pooled into one aliquot. The mucus was left to settle and diluted with solvents. The soft bodies of gastropods were rinsed with distilled water and let to dry, then removed from the shells and dissected. The internal viscera were cut into small pieces and homogenised with different solvents such as phosphate buffer saline (PBS), acetone, and methanol, then were kept on ice[13,9]. Homogenates were centrifuged at maximum speed in the cooling centrifuge, and the supernatants were kept on ice till the inculcation of samples.

Extraction of bioactive compounds

The extraction was classified into three trials using three solvents. In the 1st trial, the viscera tissue extract was carried out by PBS, and was used at concentrations 100%, 50%, and 25%. The PBS mucus extract (highly viscous) was used at concentrations 50%, 25%, and 12.5%, while the PBS haemolymph extract was used at concentrations 100% and 50%. In the 2nd trial, the viscera tissue extract was done by acetone, and was used at concentrations 100% and 50%, while the acetone mucus extract was used at concentration 50% only. In the 3rd trial, the viscera tissue was extracted by methanol, and was used at concentrations 100% and 50%, while the methanolic mucus extract was used at concentration 50% only.

Antibacterial assay

Gastropod crude extracts of haemolymph, mucus, and viscera were tested for inhibition of growth of the bacterial strains, *S. aureus* (ATCC6598, American Type Culture Collection, Manassas, VA, USA) and *E. coli* (ATCC8739). Bacterial strains from stock cultures were kept in the refrigerator, and inoculated into sterilized Muller Hinton agar plates. The antibacterial activity was determined by the agar well diffusion method[17]. The nutrient agar plates were swabbed with the respective 24 hours broth culture of the used bacteria species, and kept for 15 minutes in a laminar chamber. Wells (5 mm) were cut in the agar media for the inculcation of 100 µL gastropod extracts. The controls were used with/without solvents to assess their effect on bacteria. The plates were incubated at 37°C for 24 hours. A standard antibiotic (amoxicillin, 1000 µg/mL) was used to confirm the antibacterial activity of the extracts. The diameters of inhibition zones were the area devoid of bacterial growth around the wells, and were measured as a distance (mm) and a percentage (%) of the total diameter of the bacterial growth on growth media (Figure 1) as the following:

Inhibition of growth (%) = (total diameter of the Petri dish (mm) / diameter of the inhibition zone (mm) against the tested organism)

Scanning electron microscopy (SEM)

The antibacterial activity of the PBS and methanol extracts of *H. aspersa* and *E. vermiculata* viscera against both *S. aureus* and *E. coli* was further observed by using the SEM examination. The bacterial inoculums (100 µL) was inoculated into a flask containing 18.9 mL of Mueller Hinton broth and incubated on a shaker, 150 rpm for 18 hours at 37°C. After incubation period, 1 mL of each extract was added to the bacterial culture. At the same time, the bacterial culture was added to another flask without extract as a control. Then, the flasks were incubated at 37°C at 150 rpm for 12 hours. After the incubation time, the pellets of the treated bacterial culture were fixed in 2.5% glutaraldehyde (v/v) buffered with 0.1 mol sodium phosphate buffer (pH = 7.2). For the post-fixation step, samples were suspended in 1% osmium tetroxide in 0.1 mol phosphate buffer (pH = 7.2) for one hour, then centri-fuged, and the supernatant was discarded. Thereafter, the pellet was dehydrated using ethanol series for 10 minutes.

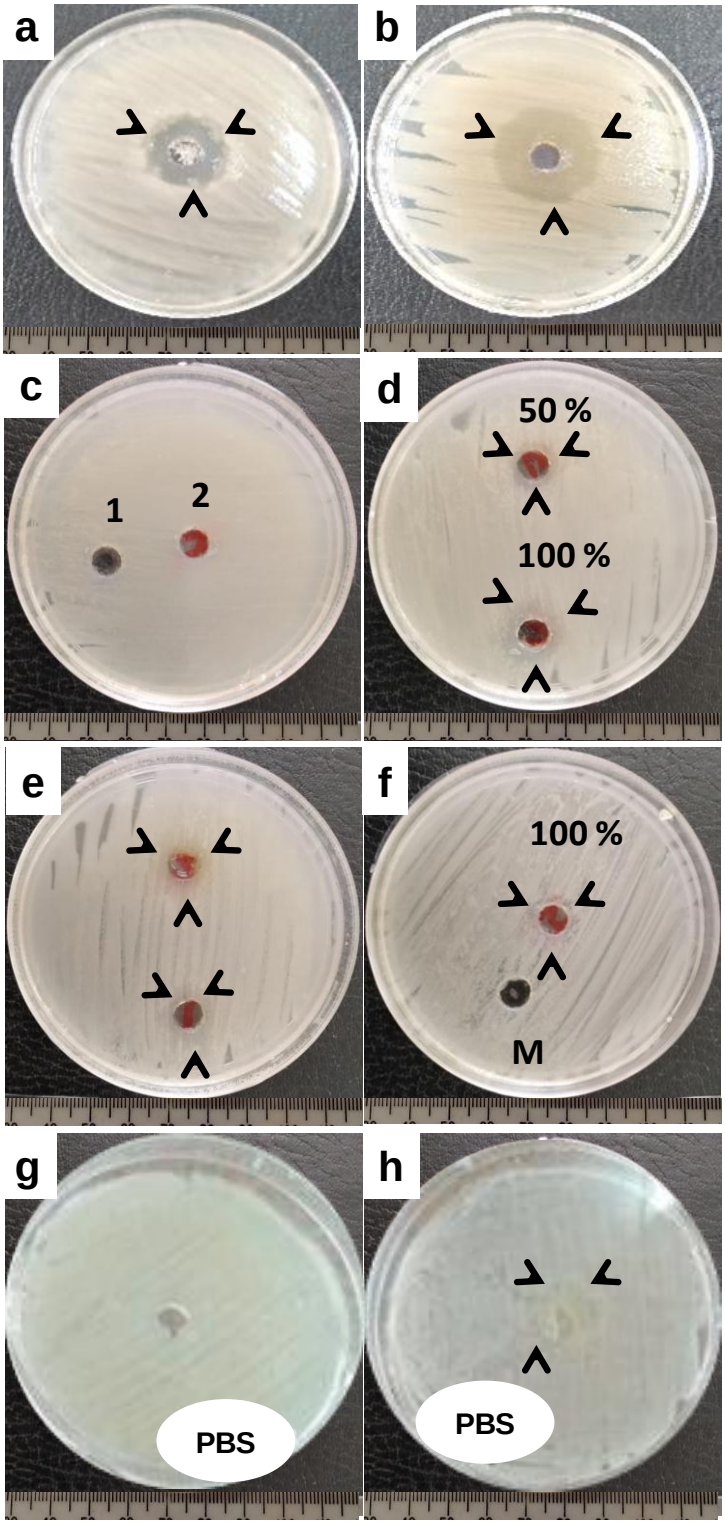

Figure 1: Antibacterial activity of the methanolic and phosphate buffer saline (PBS) viscera extracts of the terrestrial gastropods against *S. aureus* and *E. coli*. **(a)** amoxicillin (1000 µg/mL) antibiotic against *S. aureus*; **(b)** amoxicillin antibiotic against *E. coli*; **(c)** *S. aureus* growth in the absence (1) or presence of the methanol (2); **(d)** *S. aureus* growth in the presence of 50% or 100% methanolic *H. aspersa* viscera extract; **(e)** *S. aureus* growth in the presence of 50% methanolic *E. vermiculata* viscera extract; **(f)** *E. coli* growth in presence of 100% methanolic *H. aspersa* viscera extract, or methanol (M); **(g)** *S. aureus* growth in the presence of PBS; **(h)** *S. aureus* growth in the presence of 100% PBS *H. aspersa* viscera extract. Arrow heads showing the inhibition zone.

The dehydrated pellets were dried and mounted onto stubs using double-sided carbon tape, and then were gold coated. Samples were examined and photographed at the desired magnifications using JSM-IT200 SEM series (JEOL Ltd., Tokyo, Japan), at Electron Microscope Unit, Faculty of Science, Alexandria University, Alexandria, Egypt. All chemicals used in the present study were pure and purchased from Sigma-Aldrich Corp (St. Louis, MO USA).

Statistical analysis

All the tests were run in triplicates or more. Data were expressed as mean ± standard deviation and analysed using Statgraphics

Centurion XVI (Stat-Point Technologies Inc., Warrenton, VA, USA). Statistical analysis was carried out using One-way ANOVA with Fisher's Least Significant Difference (LSD) post-hoc test to set the significant difference ($P \leq 0.05$) between the inhibition zones of the different groups.

RESULTS

The antibacterial activity of the terrestrial gastropod extracts that was detected by using the agar well diffusion method

Amoxicillin antibiotic caused inhibition in the growth of *S. aureus* and *E. coli* by 24 ± 1.0 and 16.3 ± 0.6 mm, respectively (Figures 1a and b). In the 1st trial, PBS extracts of terrestrial gastropods' mucus and haemolymph did not show any antibacterial activity against any of the used bacterial strains at any concentration. However, PBS extract of *H. aspersa* viscera at concentration 100% inhibited the growth of *S. aureus* with a significant inhibition ratio = 17% ($P \leq 0.0001$, Figure 1h and Table 1). In the 2nd trail, the acetone extracts of terrestrial gastropods' mucus, haemolymph, and viscera did not show any antibacterial activity toward any of the used bacterial strains.

In the 3rd trail, the methanolic extracts of terrestrial gastropods' mucus and haemolymph did not show any anti-bacterial activity at any of the investigated concentrations against the used bacterial strains. Meanwhile, the methanolic extracts (50 and 100%) of *H. aspersa* viscera showed a significant ($P \leq 0.05$) antibacterial activity (inhibition zone = 25.7-27.6%) against *S. aureus* when compared with the control group (Figure 1d and Table 1). In addition, the antibacterial activity of the 50% methanolic extract of *H. aspersa* viscera was significantly higher ($P \leq 0.05$, ANOVA) than that of *Eobania vermiculata* viscera methanolic extract at the same concentration (Table 1). All the tested extracts of the terrestrial gastropods did not show any antibacterial activity against *E. coli* in the current study, except the 100% methanolic *H. aspersa* viscera extract, which showed a medium effect on the growth of *E. coli* (Figure 1f).

Table 1: Antibacterial activity of viscera from different terrestrial gastropods extracted with phosphate buffer saline (PBS) or methanol.

Solvents	Gastropods	Bacterial Species	Zone of inhibition, mm and (%)	
			Viscera	
			100%	50%
PBS	*Helix aspersa*	*Staphylococcus Aureus*	12.0 ± 0.6***** (17.0)	–ve
Methanol	*Helix aspersa*	*Staphylococcus aureus*	19.3 ± 0.6***** (27.6)	18.0 ± 0.1***†** (25.7)
	Eobania vermiculata		–ve	12.3 ±0.6***** (17.6)

Data were expressed as mean ± standard deviation ($n \geq 3$). –ve: no antibacterial activity, *****$P \leq 0.05$: compared to the control group, **†**$P \leq 0.05$: compared with the 50% methanolic extract of *Eobania vermiculata* viscera.

The antibacterial activity of the terrestrial gastropod extracts that was detected by using SEM examination

The control *E. coli* appeared as typical rod shape with maintained rigidity and smooth surface (Figure 2a). *E. coli* exposed to PBS or methanol alone showed normal appearance as the control one (Figures 2a-c). The main abnormalities of the *E. coli* exposed to the PBS viscera extract of

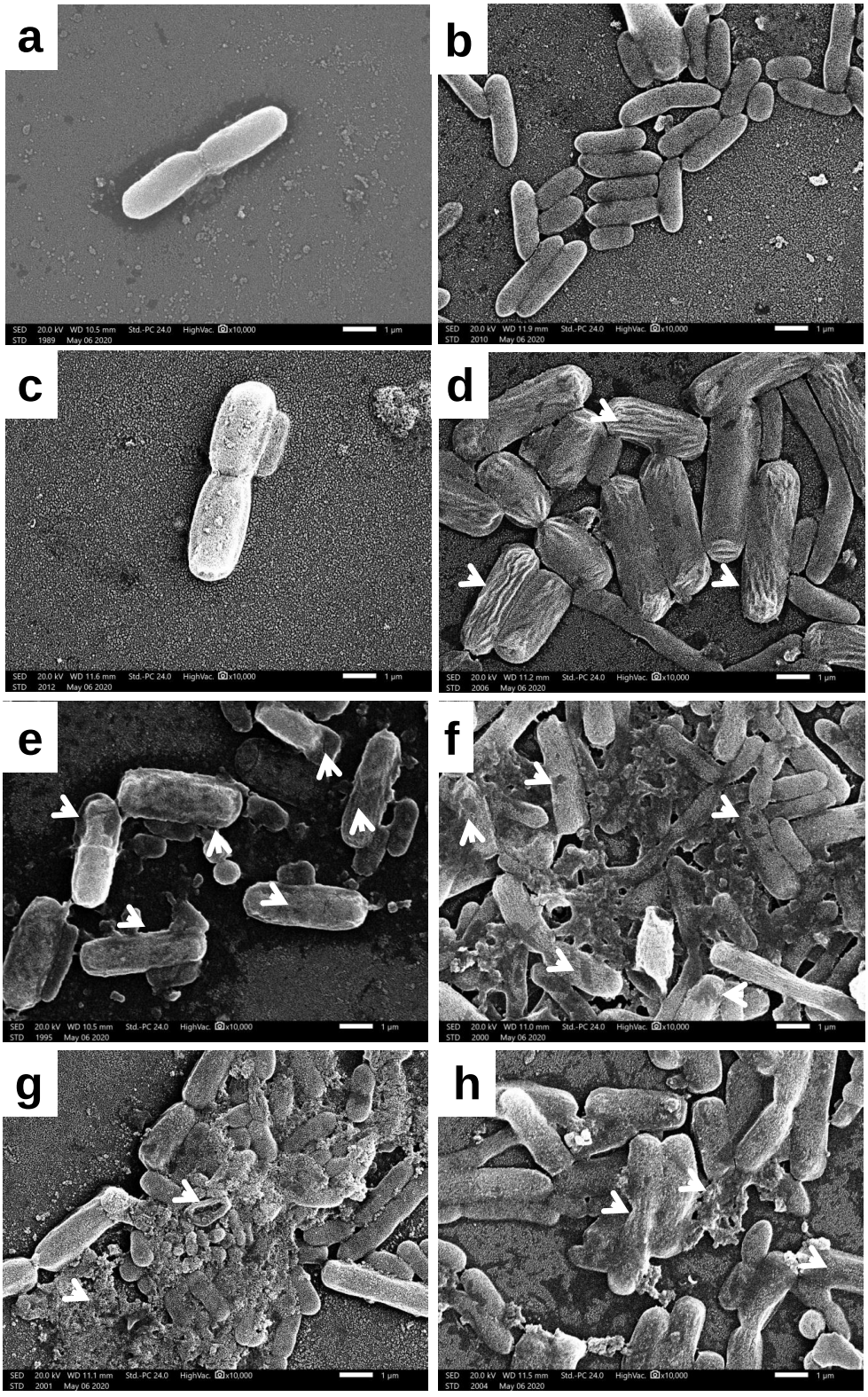

Figure 2: Scanning electron micrographs showing the antibacterial activity of the methanolic and phosphate buffer saline (PBS) viscera extracts of the terrestrial gastropods against *E. coli*. **(a)** control *E. coli*; **(b)** *E. coli* + PBS; **(c)** *E. coli* + methanol; **(d)** *E. coli* + 100% PBS *H. aspersa* viscera extract; **(e)** *E. coli* + 50% methanolic *H. aspersa* viscera extract; **(f)** *E. coli* + 100% methanolic *H. aspersa* viscera extract; **(g, h)** *E. coli* + 50% methanolic *E. vermiculata* viscera extract. Arrow heads showing abnormalities such as curliness, swollen cells, cavities, cell debris, lyses, and cuts.

H. aspersa were wrinkles and curliness of the surface and swollen (Figure 2d). However, exposure of the *E. coli* to the methanolic extracts of *H. aspersa* and *E. vermiculata* viscera at different concentrations induced several morphological alterations. Such alterations were represented in formations of cavities and cell debris between and on the bacterial cells, the cell envelope was eroded and cut abruptly, some cells were lysed, and the surface was completely rough (Figures 2e-h).

The control *S. aureus* appeared as the typical coccoid shape cells, rigid, with smooth round surface (Figure 3a). *S. aureus* exposed to PBS or methanol alone appeared as the control one with normal outer shape (Figures 3b and c). The main abnormalities of *S. aureus* exposed to the PBS viscera extract of *H. aspersa* were the increase in dead cells and debris, as well as changes in the shape, e.g. swelling or flatness (Figure 3d). However, exposure of *S. aureus* to the methanolic extracts of *H. aspersa* viscera at different concentrations induced several morphological alterations, which were represented in cell death, lyses, increased cell debris between and on the bacterial cells, and the rough bacterial surface (Figures 3e-g). In addition, the 50% methanolic extract of *E. vermiculata* viscera caused formations of cavities or pits on the outer wall of *S. aureus*, cell lyses, and change in the cells shape (Figure 3h).

DISCUSSION

In the current work, methanol was the most powerful solvent for extracting the bioactive compounds from the tested gastropods. In addition, the terrestrial snail, *H. aspersa*, viscera methanolic extract recorded the highest antibacterial activity against *S. aureus* at different concentrations, and some of its antibacterial effects were also detected against *Escherichia coli* and were confirmed by SEM examination. The potent inhibitory activity of the snails extract against bacterial growth may due to the presence of proline-rich peptides[18]. Also, some toxic compounds could be present in the extract of internal tissues (hypobranchial body) of the marine gastropod, *Murex trunculus*, named as murexine (choline derivative[19]). In addition, the role of digestive enzymes of the marine snail, *Cypraea errones* against ascidian (Tunicata) antimicrobial molecules was discussed[20]. In addition, the maximum antibacterial activity in the gut tissue of the bivalve, gastropod and crustacean samples were recorded due to the presence of highest content of proteins rather than other body parts[21]. Also, marine invertebrates have bioactive nitrogenous compounds which contain sodium nitrates as an antibacterial chemical (tetrodotoxin, is a potent neurotoxin found in several taxa of terrestrial invertebrates such as the terrestrial flatworms)[22]. Ulagesan and Kim[5] reported potent antibacterial activity in one of the seven studied snails (terrestrial) and attributed this to the different proteins with antimicrobial properties among snails. The antibacterial activity of some snails could be transferred from their diet as biologically active compounds[23]. Poaceae is the natural diet of *H. aspersa*, which has an antimicrobial activity to protect the crop[24].

In the current results, methanolic tissue extracts inhibited the growth of *S. aureus*. Antibacterial activity of the methanolic extract of the whole body marine gastropod "*Hemifusus pugilinus*" against *E. coli*, but not *S. aureus*, was detected by Anand *et al*.[25]. In addition, methanolic extract of molluscs and crustaceans exhibited higher antibacterial activity than water extract[21]. The lipophilic extracts with methanol or chloroform of *Aplysia* eggs showed significant bactericidal effect than water[23]. The highest antibacterial activity of the marine snail, *Babylonia spirata* was extracted by methanolic and ethanolic solvents[13].

In the current work, the agar well diffusion method indicated that tissue extracts of snails viscera inhibited mainly the growth of *S. aureus*, but inhibited the *E. coli* growth with a lesser extent.

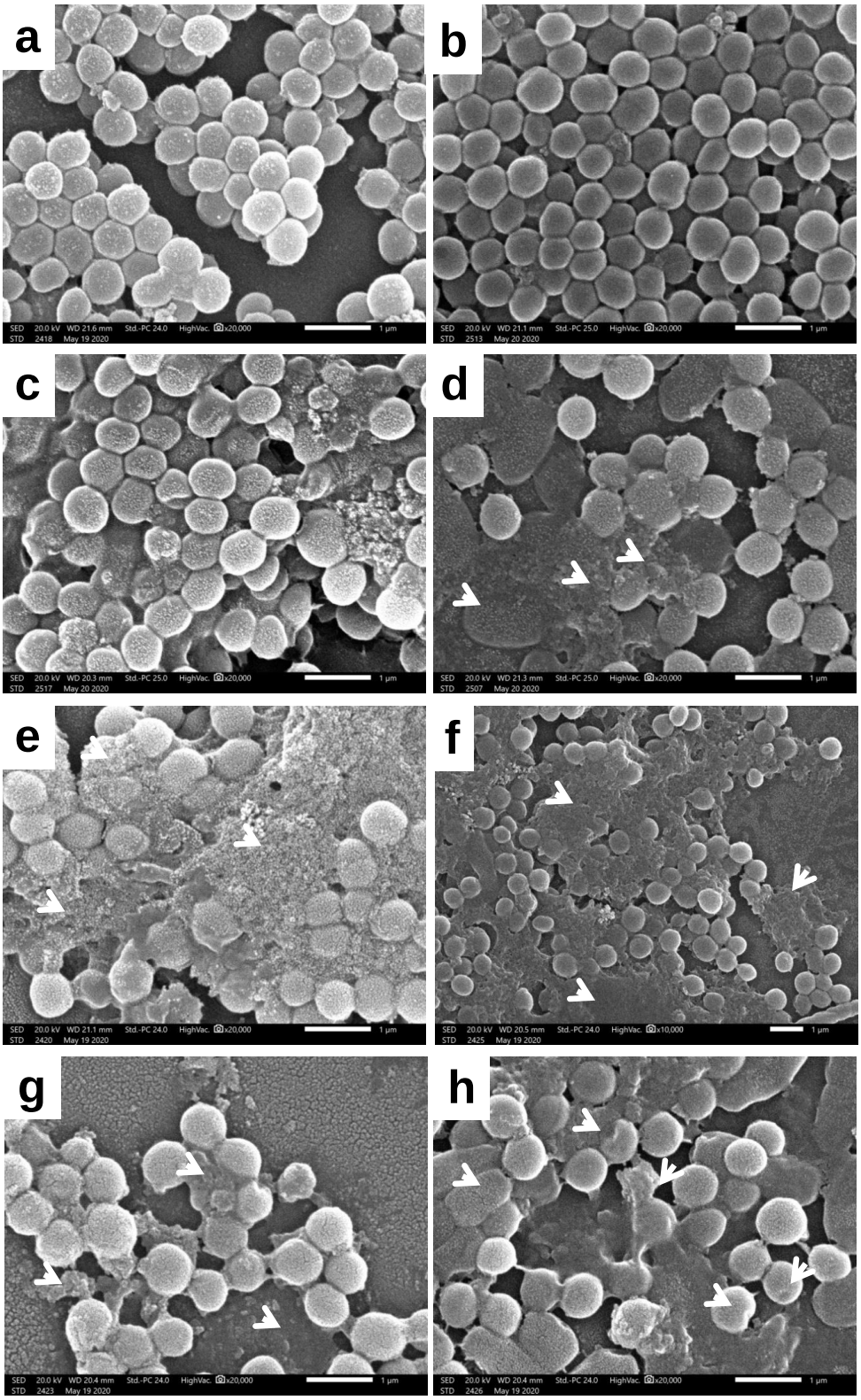

Figure 3: Scanning electron micrographs showing the antibacterial activity of the methanolic and phosphate buffer saline (PBS) viscera extracts of the terrestrial gastropods against *S. aureus.* **(a)** control *S. aureus*; **(b)** *S. aureus* + PBS; **(c)** *S. aureus* + methanol; **(d)** *S. aureus* + 100% PBS *H. aspersa* viscera extract; **(e)** *S. aureus* + 50% methanolic *H. aspersa* viscera extract*;* **(f, g)** *S. aureus* + 100% methanolic *H. aspersa* viscera extract; **(h)** *S. aureus* + 50% methanolic *E. vermiculata* viscera extract. Arrow heads showing abnormalities such as cell debris, lyses, pits on the cell wall, and change in cell shape.

Benkendorff *et al*.[23] found that *S. aureus* as Gram-positive bacteria was more susceptible to inhibition of growth by gastropod extracts than other bacteria species. Also, no antibacterial activity of *Mytilus galloprovincialis* haemolymph against *E. coli* was detected[26]. This could be explained by that the resistance of *E. coli* (Gram-negative) bacteria was due to their complex wall structure (protein-lipopolysaccharides), which can exclude most of the active compounds[27]. Ramli *et al*.[15] and Othman *at al*.[28] discussed the disadvantages of the agar well diffusion methods, as the difficulty of diffusing the hydrophobic compounds (most of the bioactive compounds) through the agar. In addition, Ramli *et al*.[15] recorded more antibacterial effect of the methanolic extract of *Syzygium polyanthum* L. (Salam) leaves against the gram-positive bacteria than the gram-negative bacteria species, as in the present study. However, SEM method approved severe deformities in *E. coli* treated with snails viscera extracts (PBS and methanol). Ibrahim *et al*.[16] recorded similar effects of the ethyl acetate extract of endophytic fungus, *Nigrospora sphaerica*, against the gram-negative bacteria, *Klebsiella pneumonia*. They explained the happened antibacterial effects as follows: (a) the extracts caused damage to the cell envelope (due to their action on the peptidoglycan layer) and consequently loss of cellular contents from cytoplasm of cells, (b) this also could cause influx/efflux of water in/out of the cells (swollen/shrinkage) due to osmotic un-balances. In addition, the active ingredients of the bactericidal material could interfere with the lipid bilayer of the bacterial cell membrane and accumulate between the fatty acids' chains[29].

In the present investigation, haemolymph and mucus extracts of snails did not inhibit the growth of *S. aureus* or *E. coli*. Antibacterial activity of haemolymph and mucus could be modified or degraded during the process of extraction[20] or due to the presence of other micro-organisms (symbiots/pathogens) which already stimulate the mucus production and/or immune activity as these are the first and second lines of defence[23]. No activity of *Achatina fulica* mucus against *Pseudomonas Aeruginosa* bacteria was recorded[30,9]. The *H. aspersa* mucus had a weak antibacterial ability, which may be due its protein content and/or the extraction techniques[9].

In conclusion, the present study confirmed that *H. aspersa*, and to a lesser extent *E. vermiculata*, viscera extracts have bactericidal properties by using the agar well diffusion method and SEM technique. The present study can open a novel avenue in the antibiotics research. Therefore, further studies are required to identify and purify the bioactive compounds of gastropod extracts for manufacturing new antibiotics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

HHA and KSK carried out the experiments, summarised the results, and wrote the manuscript. GYO suggested the research idea and revised the manuscript. SME provided the materials and lab for the microbiology experiments. SKS performed the statistical analysis.

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النشاط المضاد للبكتريا لبعض البطنقدميات الأرضية من مصر ضد بكتريا "*STAPHYLOCOCCUS AUREUS* و *ESCHERICHIA COLI*"

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تتعرض اللافقاريات الأرضية لمجموعة واسعة من الإصابات الميكروبية طوال دورة حياتها. ولذلك فهي مدعمة بعوامل قوية مضادة للميكروبات. وقد تم تصميم الدراسة الحالية لتقييم كفاءة مستخلصات الأنسجة المختلفة لثلاث قواقع أرضية هي "*Helix aspersa* و *Eobania vermiculata* و *Deroceras reticulatum*" ضد نوعين من البكتيريا المسببة للأمراض البشرية هما "*Escherichia coli* و *Staphylococcus aureus*". وقد تم استخلاص كل من الأحشاء، والهيموليمف، والمخاط من القواقع بواسطة المحلول الملحي الفُسفاتي، والأسيتون، والميثانول. وقد وجد أن مستخلصات المحلول الملحي الفُسفاتي للمخاط والهيموليمف للقواقع الأرضية لم تسبب أي تثبيط للنمو البكتري. وأيضا لم تسبب مستخلصات الأسيتون للمخاط والهيموليمف أي تثبيط للنمو البكتري. بينما أحدث المستخلص الميثانولي لأحشاء قوقع "*Eobania vermiculata*" وقوقع "*Helix aspersa*" تثبيطا لنمو البكتيرة "*Staphylococcus aureus*" وخاصة عند التركيزات "50% (0.6 ± 12.3 مليمتر)"، و "50% (0.1 ± 18.0 مليمتر) و 100% (0.6 ± 19.3 مليمتر)" على التوالي. ولم تسبب مستخلصات أنسجة الحلزون "*Deroceras reticulatum*" أي تثبيط للنمو البكتري. وباستخدام الميكروسكوب الإلكتروني الماسح، ظهر تأثيرا واضحا لمستخلصات المحلول الملحي الفُسفاتي والميثانول لكل من القوقع "*Eobania vermiculata*" والقوقع "*Helix aspersa*" علي كل من نوعي البكتيريا. وقد أوضحت الدراسة الحالية الخصائص المضادة للبكتيريا للمستخلصات الميثانولية لأحشاء البطنقدميات الأرضية مما يشجع على اكتشاف مضادات حيوية جديدة وآمنة من أصل حيواني.