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Evaluation of the antibacterial activities of skin mucus from Asian swamp eel (*Monopterusalbus)*

Ayah Rebhi Hilles[1,2*], Syed Mahmood[3,4*], &Ridzwan Hashim[2]

[1]Faculty of Health Sciences, Department of Medical Science and Technology, PICOMS International University College of Medical Sciences, 68100 Kuala Lumpur, Malaysia.

[2]Department of Biomedical Sciences, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia.

[3]Department of Pharmaceutical Engineering, Faculty of Chemical and Process Engineering Technology, University Malaysia Pahang, Kuantan 26300, Pahang, Malaysia.

[4]Centre for Excellence for Advanced Research in Fluid flow (CARIFF), University Malaysia Pahang, Kuantan 26300, Pahang, Malaysia.

*[E-mail: syedmahmood@ump.edu.my]

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The water covers more than two-thirds of the Earth's surface, and almost 90 % of the world's species are found in the water environment. Therefore, it is necessary to explore drugs from marine and freshwater organisms. The skin of marine and freshwater animals are covered with a mucus layer, which acts as a biochemical and mechanical barrier for their skin. This study aimed to investigate the potential antibacterial activity of Asian swamp eel(*Monopterusalbus)* skin mucus. Aqueous and methanol extracts were prepared to detect the antimicrobial activities with different extract concentrations from 0.49 to 1000 g/mL against various pathogens, i.e. *Staphylococcus aureus and Escherichia coli.* The antibacterial activities were determined by measuring the diameter of inhibition zone, minimal inhibitory concentration, minimal bactericidal concentration, inhibition percentage, and survival percentage. The results showed inhibition in bacterial growth, which was treated with both methanol extract and the aqueous extract. However, methanol extract has significantly higher antibacterial activities than the aqueous one. At 100 µl/disc, the inhibition zone of methanol extract against *E.* coli was10.7 ± 0.17 mm while 9.9 ± 0.06 mm against *S. aureus*. The percentage of bacterial inhibition for eel skin mucus (ESM) methanol extract against *E. coli* showed higher inhibition (72.46 %) than against *S. aureus*(68.45 %) at 1000 µl/mL. ESM aqueous extract showed the highest bacterial survival rate against *S. aureus* at 7.81 µg/mL, which was 71.11 %, whereas the methanol extract was 58.25 %. The results were statistically significant, with $p < 0.001$. In conclusion, the current study revealed that eel skin mucus might be considered as a promising source for antibacterial activities.

[Keywords: Antimicrobial;Inhibition zone; Minimal bactericidal concentration; Minimal inhibitory concentration; *Monopterusalbus*].

Introduction

The Asian swamp eel (*Monopterusalbus*) taxonomically belongs to the Synbranchidae family under Synbranchiformes order[1]. It is a freshwater fish that is widely distributed across East India, mainly the Greater Sunda Islands, Indochinese Peninsula, and the Malay Peninsula. They are also widely distributed in the southern part of East Asia, including, the western Japanese Archipelago, southeastern China, and the Korean Peninsula[2]. They are barely found in the United States, as this species is mostly distributed in Asia[3]. Asian swamp eel mucus is secreted from the epidermis by epidermal goblet cells, which contain from immunoglobulins, lipids, and gel-forming molecules like mucins and other glycoproteins suspended in water[4], which gives the mucous lubricating properties[5]. The mucus layer is continuously replaced which protects the eel skin from bacterial and fungal colonisation[6] and produces antimicrobial molecules that serve as the first line of a host's defense against microbial invasion[7]. It has been recorded that skin mucus from*M. albus* can be considered a promising antibacterial agent against oral pathogens[8]. Different extracts have been used to examine the antibacterial properties of *M. albus,* and it showed a significant bacteriostatic effect[9].

Materials and Methods

Extract Material

Asian swamp eels were purchased from the eel farm at Pahang, Malaysia.

Preparation of eel skin mucus (ESM) extract

Eel skin mucus was collected by slightly scraping the surface of the eel skin, then it was homogenised with two volumes of distilled water, after that, it was centrifuged for 30 minutes at 13,000 rpm, the supernatant was freeze-dried for five days. The dried substance was weighed and dissolved in distilled water to generate aqueous extract and in methanol to produce a methanolic extract, then, the dissolved material was filtered using a 0.22 µm syringe filter and kept in -20 ºC until use[10].

Determination of antimicrobial activities

Microbial strains

The bacterial strains used in this study; Gram-positive bacteria which were *Staphylococcus aureus* (ATCC 25923), and Gram-negative bacteria which was *Escherichia coli* (ATCC 25922). All strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Strains preparation

For bacterial inoculum adjustment; the strains were incubated in Mueller-Hinton broth (MHB) (Oxoid) under an aerobic condition at 37 ºC for a period of 18-24 h to be used as inoculum. The turbidity of the suspensions were adjusted based on *McFarland standard* (5 x 10^8 CFU/ml), which corresponds to an absorbance of 0.08 – 0.10 at 625 nm wavelength[11].

Disc diffusion assay

The antibacterial susceptibility testing was performed using a disc diffusion assay. Inocula 0.1 ml of the adjusted inoculum were spread on the surface of agar plates. The sterile discs were soaked in added concentrations 3.13, 6.25, 12.5, 25, 50 and 100 µl/disc of aqueous and methanol ESM extracts, impregnated with solvent followed by drying. Standard antibiotic (penicillin) was used as a positive control. The agar plates were incubated for 24 h at 37 °C. Then plates were observed for clear zone around the discs, and the size of inhibition zones was measured and expressed in millimetres. The test was achieved in triplicate[12].

Growth of inhibition method

The growth of the inhibition method was carried out using a 96-well plate, and all wells were filled with 100 L of MHB. After this 100 L volume of the extract with 1000 g/ l concentration was added to the first wells of the first row. Then, serial dilution was applied to create a concentration of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.90, 1.95, 0.98 and 0.49 g/mL. After this 50 l of adjusted inoculum was seeded into each well. MHB and bacterial suspension represented the negative control and MHB with an antibiotic (penicillin) represented positive control. The agar plates were incubated at 37 °C for 24 h, and then the turbidity was measured using ELISA microplate reader (Tecan: Infinite M200 PRO, Switzerland) at 630 nm wavelength. The growth of inhibition for the test wells at each dilution was determined using the formula: The percentage of inhibition =1- (Absorbance of test well/Absorbance of corresponding control well) × 100 for each row of the 96-well plate[13].

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and IC_{50}

MIC is defined as the lowest concentration required to prevent the visible growth of bacteria. The final dilution of the extracts that maintained its inhibitory effect resulting in 85 % growth of bacteria was recorded as the MIC value of the extract. MBC is the lowest concentration of the extract, which is needed to kill the bacteria. The MBC of the extract was determined by subculturing each inoculated well and further incubating for 24 h at 37 °C. The highest diluted well that generated with 95 % growth on the agar plates was considered as MBC. The experiment was carried out in triplicate for each concentration[14]. IC_{50} values have been determined by calculating the concentration needed for 50 % inhibition of bacterial proliferation after intervention with the extract[15].

Bacterial viability assay

Measurement of viable bacteria was determinedusing MTT method which have done by preparing [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) in PBS (pH 7.2) to get a concentration of 5 mg/mL MTT. The bacteria were cultured and incubated for 9 h at room temperature in 96-plate well, then 20 l of MTT solution was added to every single well then, the 96-plate well was incubated 30 min at room temperature in a dark place.After 30 min incubation with MTT solution, the absorbance was measured at 540 nm wavelength. Bacterial viability was detected using the formula: (Absorbance of the sample – Absorbance of the control); where the control contains the culture medium and bacteria without treatment[16].

Statistical analysis

All results are represented as a mean ± standard deviation. The data were analysed by one-way

ANOVA. The *p*value< 0.05 was considered as significant. The software SPSS (version 21.0) was employed for statistical analysis.

Results:

Determination of antibacterial activity

Disc Diffusion assay

The disc diffusion method was conducted to determine the antibacterial activity of ESM extract against *E. coli* and *S. aureus*. The results showed that 100 µl/disc of ESM methanol extract has the largest inhibition zone against *E. coli* with 10.7 ± 0.17 mm, while the inhibition zone for ESM aqueous extract with the same concentration was (8.1 ± 0.33) mm. Whereas, the inhibition zone of 100 µl/disc of ESM methanol and aqueous extract against *S. aureus* was (9.9 ± 0.06) mm and (7.3 ± 0.33) mm, respectively. The diameter of clear inhibition zone was measured for the positive control (penicillin) against *E. coli* and *S. aureus* which was (23.5 ± 0.21) mm and (34.1 ± 0.01) mm, respectively for all concentrations of penicillin from 100 µl/discto 3.13 µl/disc. All values were expressed by (mean ± SD). The results of both *E. coli* and *S. aureus* were highly significant with $p < 0.001$compared with the positive control (Table 1 & 2).

MIC, MBC, and IC$_{50}$determination

MIC was conducted to assess the lowest concentration of ESM needed to inhibit bacterial growth. The concentrations that were used to evaluate the MIC varied from (1000-0.49) g/mL. The methanol extract of ESMwas observed with the lowest value of MIC at 0.98 g/ml against *E. coli* while in the aqueous extract it was 1.95 g/mL. The highest value of MIC was 3.90 µg/ml observed using ESM aqueous extract against *S. aureus* while MIC for the methanol extract was 1.95 µg/mL. The lowest MBC value was 1.95 g/mL obtained by ESM methanol extract against *E. coli* while that for aqueous extract was 3.90 g/mL.In contrast, the highest MBC value was 7.81 g/mL obtained by ESM aqueous extract against *S. aureus,* while for methanol extract it was 3.90 g/mL. IC$_{50}$value was observed by ESM aqueous and methanol extracts against *E. coli* at a concentration of 27.46 and 150.93 g/mL,respectively. Whereas IC$_{50}$ value was observed by ESM aqueous and methanol extracts against *S. aureus* at a concentration of 31.25 and 406.25 g/mL, respectively.

Growth of inhibition method

The highest inhibition (72.46 %) was obtained by ESM methanol extract at 1000 µg/mL against *E. coli* while that using aqueous extract was 70. 05 % whereas *E. coli* growth inhibition occurred at the lowest concentration of ESM 0.49 µg/mL (14.84 %), and by using aqueous extract was 11.25 % (Fig. 1). ESM methanol extract against *S. aureus* showed 68.45 % at inhibition 1000 µg/mL while the aqueous extract showed 57.85 % at the same concentration while 13.62 % *S. aureus* inhibition was observed at the lowest concentration of 0.49 µg/mL using ESM methanol extract and 10.58 % using ESM aqueous extract as shown in Figure 1. The results were highly significant ($p < 0.001$) as compared to the positive control.

Bacterial viability assay by MTT

The bacterial survival rate of *E. coli* after treatment with ESM methanol extract was recorded to be lowest at 1000 µg/mL (17.33 %) while the aqueous extract was 43.51 %. The survival rate of bacteria treated with ESM methanol extract against *S. aureus* at the same concentration was insignificantly lower than *E. coli*(19.76 %) for 1000 µg/ml while for the aqueous extract was 26.58 %. The highest bacterial survival rate was found in ESM aqueous extract against *S. aureus* at a concentration of 7.81 µg/mL, which was 71.11 %, while for the methanol extract it was 58.25 %. Whereas the bacterial survival rate for ESM

Table 1 — Antibacterial activities of ESMagainst *E. coli* using disc diffusion method (mm). Results were expressed as mean ± SD (n=3).

Extract (µl/disc)	100	50	25	12.5	6.25	3.13
Aqueous extract	8.1±0.33	8.3±0.11	7. 4±0.19	7.3±0.45	6.3±0.07	6.0±0.02
Methanol extract	10.7±0.17	10.0±0.09	9.3±0. 34	8.4±0.03	6.6±0.46	5.3±0.45
Penicillin	23.5±0.21	23.5±0.21	23.5±0.21	23.5±0.21	23.5±0.21	23.5±0.21

Table 2 — Antibacterial activities of ESMagainst *S. aureus* using the disc diffusion method (mm). Results were expressed as mean ± SD (n=3).

Extract (µl/disc)	100	50	25	12.5	6.25	3.13
Aqueous extract	7.3±0.21	6.1±0.15	5.9±0.43	5.4±0. 01	4.7±0.52	4.2±0.13
Methanol extract	9.9±0.06	8.6±0.10	7.3±0.23	6.2±0.13	5.0±0.07	4.8±0.26
Penicillin	34.1±0.01	34.1±0.01	34.1±0.01	34.1±0.01	34.1±0.01	34.1±0.01

aqueous extract against *E. coli* was 73.30 % while the aqueous extract was 81.88 % at a concentration of 7.81 µg/mL. It shows that the values were statistically significant, with $p < 0.001$ compared with the positive control (penicillin) as shown in Figure 2.

Discussion

ESM methanol extract exhibited a higher degree of antibacterial activities as compared with ESM aqueous extract, which shows that the active antimicrobial components were higher in the methanol extract, which might be a reason for this. ESM extracts showed higher inhibition properties against *E. coli* than *S. aureus*, and this might be related to their strain as *E. coli* are gram-negative bacteria, while *S. aureus* gram-positive bacteria. A gram-positive bacteria have thick peptidoglycan cell wall with teichoic acids while gram-negative bacteria have thin peptidoglycan cell wall with the outer plasma membrane. It might indicate that ESM can penetrate the thin peptidoglycan cell wall, not the thick one. At 100 µl/disc,ESM methanol extract showed the highest inhibition zone against *E.* coli, which was(10.7 ± 0.17) mm while (9.9 ± 0.06) mm against *S. aureus*. Penicillin was used as a positive control, it showed (23.5 ± 0.21) mm inhibition zone against *E. coli* and (34.1 ± 0.01) mm against *S. aureus.* Penicillin inhibition was more significant in *S. aureus* as penicillin is most effective against gram-positive bacteria. The lowest concentration of aqueous and methanol ESM extracts required to inhibit *E. coli* growth was 0.98 g/ml and 1.95 g/mL, respectively while *S. aureus* was 3.90 µg/ml and 1.95µg/mL respectively. A small amount of aqueous and methanol ESM extracts would be enough to inhibit bacterial growth.

The percentage of bacterial inhibition for penicillin at 1000 µg/mL was the highest (90.45 %), ESM methanol extract against *E. coli* showed higher inhibition (72.46 %) than against *S. aureus*(68.45 %) which might be because of the strain differences between the two bacteria. The inhibition of ESM methanol extract against *E. coli* at 1000 µg/mL was

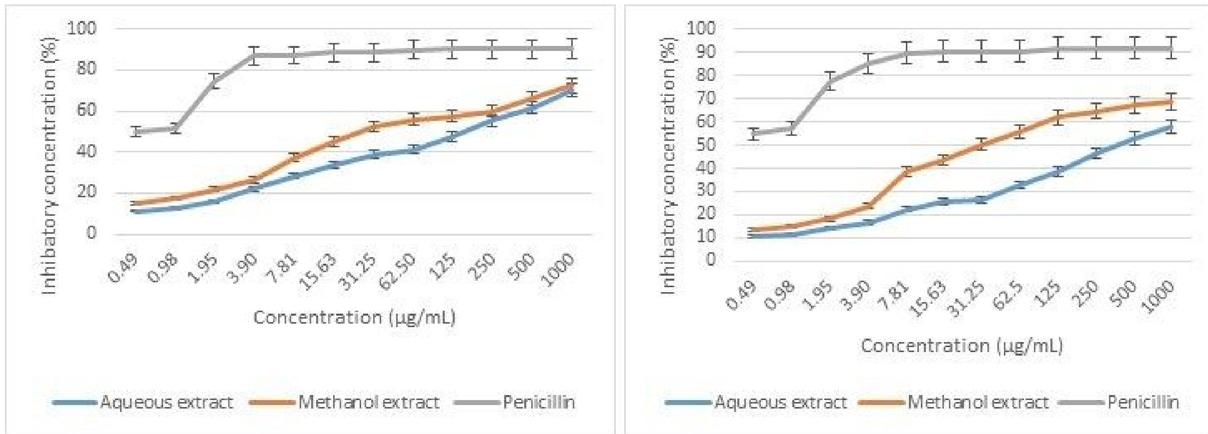

Fig.1 — Percentage of inhibition using ESM extract against *E. coli* and *S. aureus.* Data presented as means ± SD (n=3).

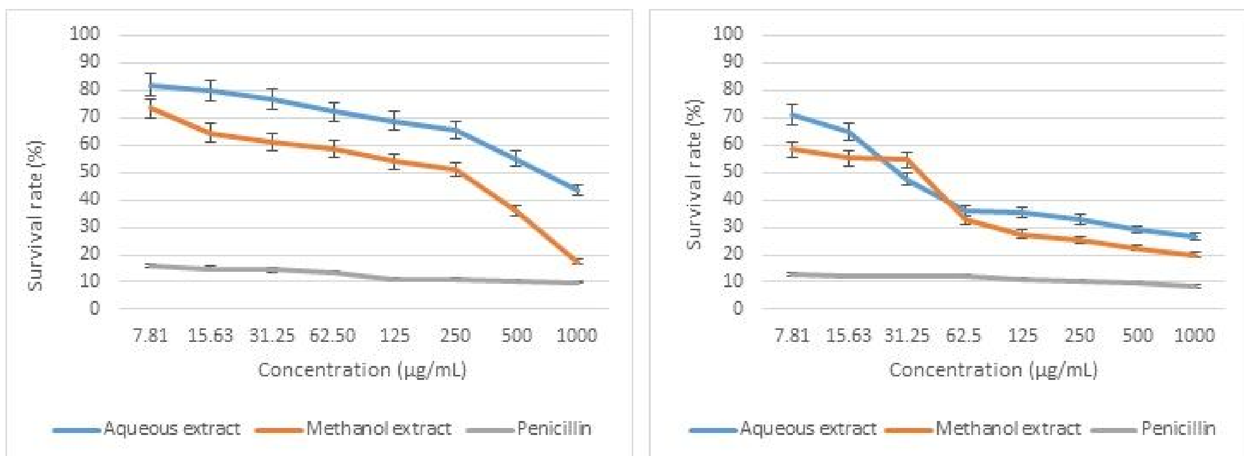

Fig. 2 — Survival rate of ESM extract against *E. coli* and *S. aureus*. Data presented as means ± SD (n=3).

more likely to be close to the standard antibiotic (penicillin), which was 90.45 %.Even though, at a low concentration of penicillin; the inhibition percentage of penicillin was still high while aqueous and methanol ESM extracts dramatically decreased. For example, at the lowest concentration, which was 0.49 µg/mL, penicillin inhibits 50.09 % while aqueous ESM extract inhibits 11.25 %, and the methanol extract inhibits 14.84 % *E. coli*. In contrast, penicillin inhibits 54.79 % at a concentration of 0.49 µg/mL against *S. aureus* while aqueous ESM extract inhibits 10.58 % and the methanol extract inhibits 13.62 %. At 1000 µg/mL, the lowest survival rate of ESM methanol extract was recorded against *E. coli* with 17.33 % while against *S. aureus* with 19.76 %, which shows the effectiveness of the extract to inhibit the bacteria, as at 1000 µg/mL, less than 20 % of the bacteria were survived.

Conclusion

ESM methanol and aqueous extracts revealed varying degrees of antibacterial properties against the microorganisms tested. The chance to find the antibacterial activities were more apparent in methanol than aqueous extracts of ESM. The present study suggests that ESM could be an alternative source of a new antibiotic to treat some of the infectious diseases. Further work is required to isolate and identify the antibacterial bioactive compounds in ESM as well as investigate the exact mechanism of action.

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