

EVALUATION OF THE ANTIMICROBIAL PROPERTIES OF EEL SKIN MUCUS FROM *MONOPTERUS ALBUS* AGAINST SELECTED ORAL PATHOGENS AND IDENTIFICATION OF THE ANTI-ORAL BIOACTIVE COMPOUNDS USING LC-QTOF-MS

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ABSTRACT

Despite great achievements in the oral health of populations globally, oral health problems remain in many communities all over the world, thus our study aimed to evaluate the antimicrobial activity of *Monopterus albus* skin mucus against selected oral pathogens. *Monopterus albus* is Asian swamp eel with elongated body like snakes. Their skin covered with a thick layer of mucus. They are usually found as a sluggish in the stagnant waters as it has a weak swimming behavior. Although eels are capable of quick movements, but they tend to be lethargic and they rely on stealth swimming movements to capture their prey such as shrimp, frogs and other small fishes. With regard to this matter, aqueous and methanol extracts were prepared to test antimicrobial activities against selected oral pathogens; Gram-positive bacteria i.e. *Enterococcus faecalis*, *Streptococcus pyogenes*, *Streptococcus mutans*, Gram-negative bacteria which are *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and fungus pathogens i.e. *Candida albicans*. The antimicrobial activities were determined by inhibition percentage. The results showed a dramatic decrease in the oral pathogens treated with eel skin mucus methanol extract higher than the aqueous extract. *Enterococcus faecalis* showed the highest activity while *Candida albicans* showed the lowest activity. After *in-vitro* evaluation for eel skin mucus activities, identification study using liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) was performed to investigate the compound responsible for the anti-oral pathogens activities, the results showed the presence of salvianolic acid G which strongly correlated with the antimicrobial activity against selected oral pathogens. Results were statistically significant with $p < 0.001$. In conclusion, the present study revealed that eel skin mucus can be considered as promising source for anti-oral pathogens activities.

Keywords: *Monopterus albus*, oral pathogens, liquid chromatography-quadrupole-time-of-flight mass spectrometry, salvianolic acid G

INTRODUCTION

Asian swamp eel, *Monopterus albus* (*M. albus*) belongs to synbranchidae family under the order of synbranchiformes (Rossen, & Greenwood, 1976). It is native to the tropical and subtropical areas of northern India, China, Malaysia, Thailand, Indonesia, Philippines and possibly North-Eastern Australia (Collins et al., 2002). It has elongated shape of the body which covered by a thick protective mucous layer (Liem, 1967).

Oral diseases remain a major public health problem worldwide as the oral cavity exposed to many pathogens. A global review on oral health that has been published by WHO emphasized that oral health is still considered as a global problem even though some countries have achieved great improvements in the oral health management (Petersen, 2003).

Enterococcus faecalis is known to be the most frequent species in root canals with failed endodontic therapy (Wang et al., 2011). *Streptococcus pyogenes* can cause a systemic infection through oral infection (Inagaki et al., 2017). *Streptococcus mutans* is a major risk cause for childhood and future dental caries (Berkowitz, 2003). Lung destruction caused by the chronic colonization of *Pseudomonas aeruginosa* (Caldas et al., 2015). Oral candidiasis is the most common fungal infection (Abu-Elteen & Abu-Alteen, 1998) which caused by overgrowth of *Candida* species in the oral cavity (Akan & Morgan, 2002)

Liquid chromatography quadrupole time-of-flight mass spectrometry performed very well, with high sensitivities and specificities reach up to 95 %. It is a powerful analytical method which has high-resolution mass (Kronstrand et al., 2014). The importance of liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) has appeared as a useful technique for identification of the compounds (Kosjek & Heath, 2008), due to the special combination of high selectivity and structural information originated from accurate-mass MS and MS/MS spectra (Gómez et al., 2010). Thereby, the

potential of LC-QTOF-MS evaluated both quantitative and qualitative abilities (González-Mariño et al., 2011).

MATERIALS AND METHODS

Preparation of eel skin mucus (ESM) extract

Healthy Asian swamp eels (*Monopterus albus*) were collected from eel farm in Pekan, Pahang, Malaysia. Eel skin mucus was homogenized with 2 volumes of distilled water, then centrifuged at 13,000 rpm for 30 min, the supernatant lyophilized. Dried substance weighed and dissolved in distilled water to form aqueous extract and in methanol to form organic extract, after that, the dissolved substance filtered using a syringe filter and kept until use (Sadakane et al., 2007).

Determination of antimicrobial activities

Microbial strains

The microorganisms used in this study; Gram-positive bacteria which are; *Enterococcus faecalis* (ATCC 29212), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus mutans* (ATCC 25175), Gram-negative bacteria which are *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and fungi which is *Candida albicans* (ATCC MYA 4901) as oral pathogens. All strains have been procured from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Preparation of Inoculums

All the oral bacterial strains were incubated at 37 °C for 18 -24 hrs whereas 24 hrs at 30 °C for fungi. The turbidity of the suspensions adjusted according to *McFarland standard* (approximately 5×10^8 CFU/mL) which represent the absorbance of 0.08 – 0.10 at 625 nm for bacteria, while for fungi, the suspension was adjusted at 600 nm to match the turbidity of 0.5 of *McFarland standard* ($0.5-2.5 \times 10^5$ CFU/mL) (Leite et al., 2014)

Determination of anti-oral pathogens activities by growth of inhibition method and IC₅₀ determination

The growth of inhibition method was conducted using sterile 96-well plate, all wells were filled with 100 µL of Mueller-Hinton agar. Then 100 µL volume of serial dilution from the extracts were added from the concentration 1000 to 7.81 µg/mL. After this 50 µl of adjusted inoculum was seeded into each well. The plates were incubated at 37°C for 24 hrs. The turbidity of the medium was measured by ELISA microplate reader at 630 nm. The percentage of inhibition calculated from the formula: $1 - (\text{Absorbance of test well} / \text{Absorbance of corresponding control well}) \times 100$ (Patton et al., 2006). IC₅₀ values were calculated by determining the concentration required for 50% inhibition of bacterial growth after adding the extracts (Miyoshi et al., 2003).

Identification the bioactive compounds against oral pathogens using LC-QTOF-MS

The solvents used for the mobile phase were as follows: solvent name A (water +0.1%Formic Acid), solvent B: (acetonitrile) for LC–MS in gradient grade solvents as shown in table 1 and ammonium acetate was used for HPLC grade Column: C-18. All measurements were performed with a Q-TOF LC–MS instrument (Waters VION Ion Mobility QTOF MS). The QTOF-MS was operated with an electrospray positive and negative ionization mode, mass resolution of (100-1000) m/z measure the frequency of 10,000 transients s⁻¹, the low collision energy was 4.00 eV while the high collision energy ramp started with 10.00 eV and ended with 45.00 eV.

Table 1 The Gradient grade of solvents in the mobile phase.

Time (min)	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)	curve
0.00	0.600	99.0	1.0	Initial
0.50	0.600	99.0	1.0	6
10.00	0.600	65.0	35.0	6
13.00	0.600	0.0	100.0	1
15.00	0.600	99.0	1.0	1

RESULTS

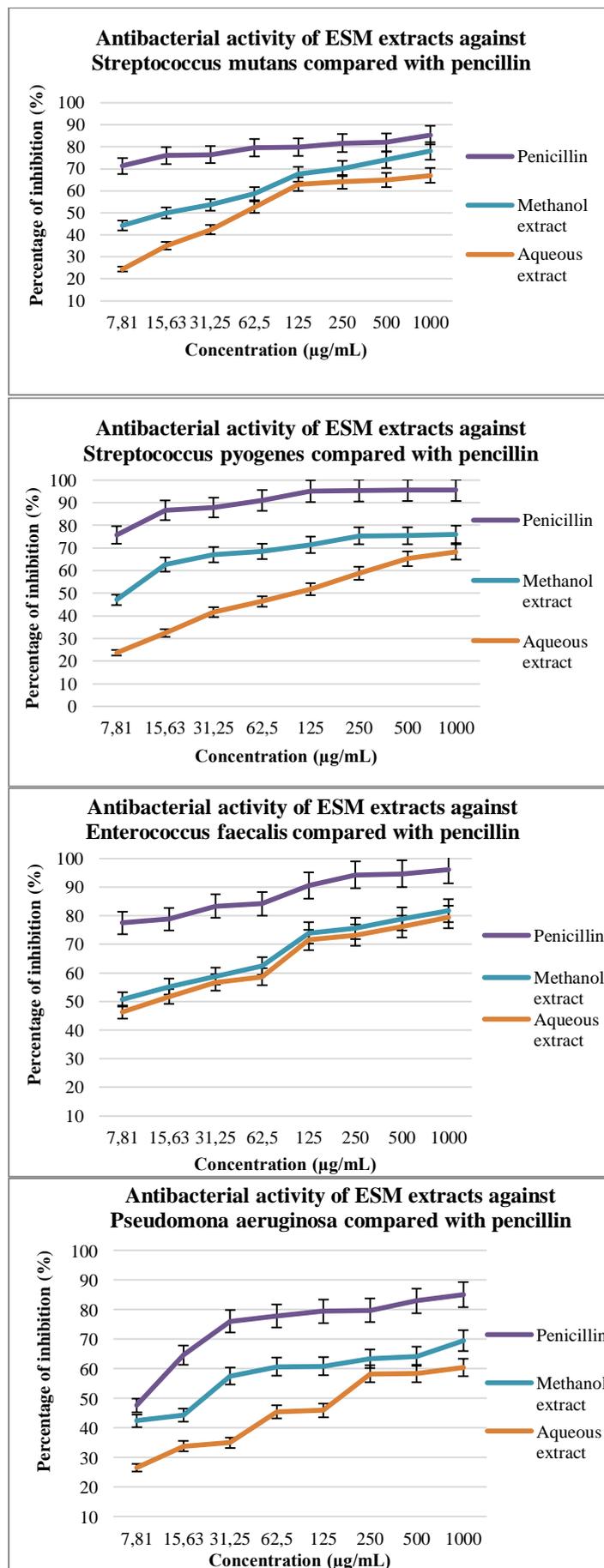
Percentage of inhibition and IC₅₀

The results in figure 1 showed that methanol eel skin mucus extract has higher antimicrobial activities than the aqueous extract, the highest inhibition activity of methanol extract was against *Enterococcus faecalis* which was 81.74 ± 0.43 % and 50.74 ± 0.19 % at the concentration of 1000 µg/mL and 7.81 µg/mL respectively. The extracts were exhibited inhibition activity against *Streptococcus mutans* with 78.13 ± 0.61 % and 44.30 ± 0.44 % at 1000 µg/mL of methanol and aqueous extracts respectively. Whereas the percentage of inhibition against *Streptococcus pyogenes* was 76.01 ± 0.23 % in the methanol extract and 68.24 ± 0.77 % and in the aqueous extract at 1000 µg/mL respectively. *Klebsiella pneumoniae* was inhibited with 72.84 ± 0.11 % in the methanol extract and 68.21 ± 0.34 % in the aqueous extract at 1000 µg/mL. *Pseudomonas aeruginosa* was inhibited in the methanol extract with 69.5 ± 0.79 % while 51.44 ± 0.61 % in the aqueous extract at 1000 µg/mL respectively. The lowest inhibition was against *Candida albicans* which was 59.37 ± 0.42 % in the methanol extract and 50.78 ± 0.18 % in the aqueous extract at 1000 µg/mL. The results showed variation in the IC₅₀ in different extracts and different pathogens as shown in table 2.

Table 2 Determination of IC₅₀ (50% inhibition concentration) of ESM extracts.

Oral pathogen	Methanol extract	Aqueous extract
<i>Enterococcus faecalis</i>	7.15±0.73**	15.93±0.52**
<i>Streptococcus mutans</i>	12.89±0.15**	54.68±0.81**
<i>Streptococcus pyogenes</i>	9.75±0.91**	97.21±0.74**
<i>Klebsiella pneumoniae</i>	13.56±0.28**	124.82±0.01**
<i>Pseudomonas aeruginosa</i>	23.44±0.43**	187.47±0.6**
<i>Candida albicans</i>	379.91±0.5**	761.38±0.29**

Data expressed in µg/mL, mean ± SD (n = 3). ** Significant difference at $p < 0.001$ (one-way ANOVA).



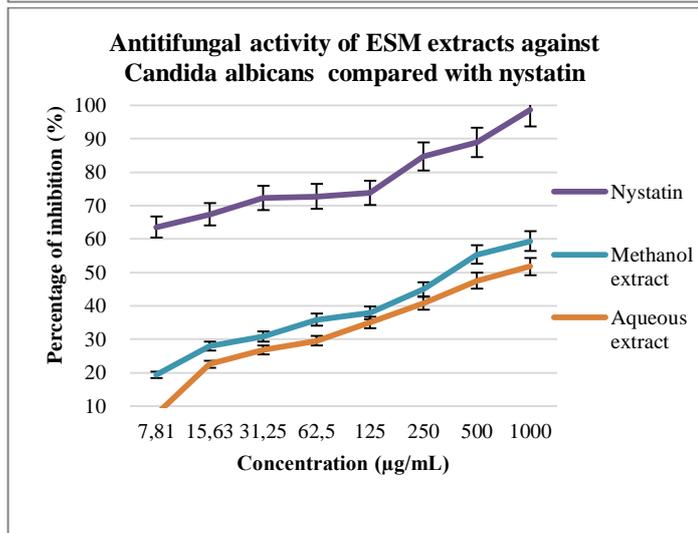
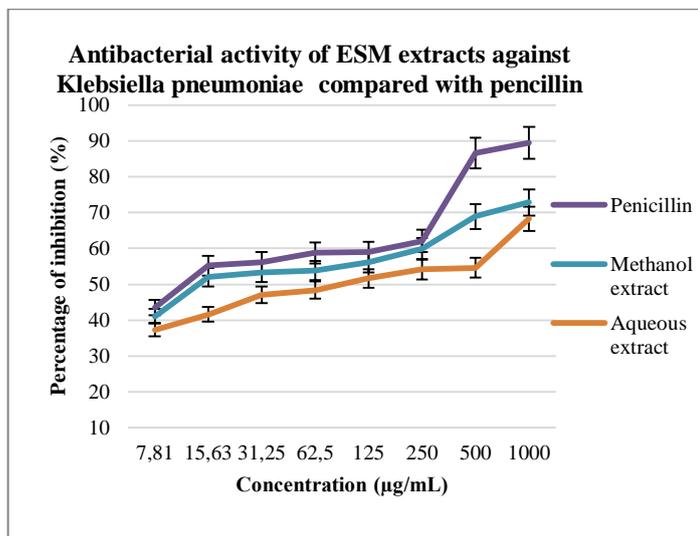


Figure 1 Percentage of inhibition antimicrobial activities of eel skin mucus aqueous and methanol extracts against selected oral pathogens compared with positive control. Data were expressed mean. Data presented as means ± SD (n=3).

Compound identification using LC-QTOF-MS

LC-QTOF-MS data showed the presence of salvianolic acid G (figure 2), which identified and characterised as shown in table 2. It has been invented that salvianolic acid effective to kill viruses and bacteria in the mouth to prevent dental caries and periodontitis, and the treatment of other oral and throat diseases (Chinese Patent number CN 102743436 A).

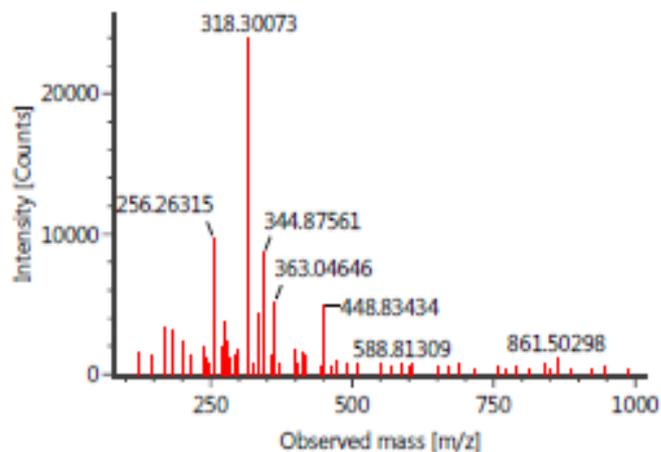
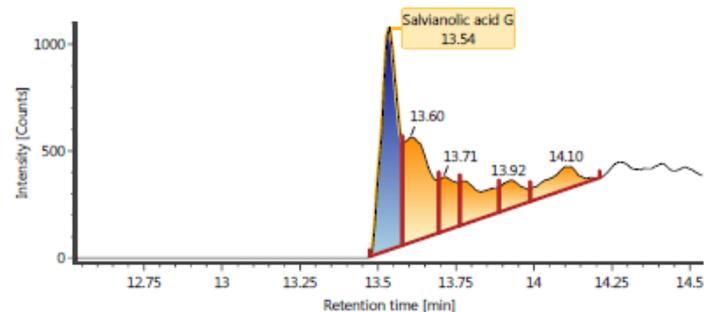


Figure 2 (A) Chromatogram measured single-stage mass spectrum of isotope peak of Salvianolic acid G in ESM methanol extract at 13.54 min with the peaks of [M+H]. (B) Auto-MS-MS mode shows the ionization of the compound.

Table 3.2 Identification and characterization of Salvianolic acid G

	Formula	Identification status	Observed neutral mass (Da)	Observed m/z	Ma err (m)
acid G	C18H12O7	Identified	340.0570	363.0463	-1.

CONCLUSION

Development of new anti-oral pathogens treatment remains one of the most challenging exploration in the biomedical researches. Thus, our research focused on investigating the antimicrobial activities of eel skin mucus (*Monopterus albus*) against selected oral pathogens and determination of the bioactive compounds lead to explore the reason of these activities which was salvianolic acid G, as it has proven that salvianolic acid has anti-oral pathogens activities.

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