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IN-VITRO AND *IN-VIVO* EVALUATION OF THERAPEUTIC PROPERTIES OF SKIN MUCUS FROM ASIAN SWAMP EEL (*Monopterus albus*)

BY

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ABSTRACT

Swamp eels have been widely accepted as sources of food, especially among various Asian cultures. However, their potential values as novel sources of therapeutic agents have not been widely appreciated. Like most other tropical fishes and amphibians, the outer integumentary system of *Monopterus albus* is covered with mucus layers, which may act as mechanical and biochemical barrier for their skin. The biochemical components of these mucus layers may have certain compounds that may be medically beneficial to human. The current study was interested to screen selected biological activities of skin mucus from the tropical eel *in-vitro* and *in-vivo*. The first stage of the study was the screening of the eel skin mucus extracts for bioactive compounds using gas chromatography-mass spectrometry and liquid chromatography quadrupole-time-of-flight mass spectrometry. Several compounds, which had been previously identified to have different biological activities mainly cytotoxic, antioxidant and antimicrobial activities were discovered. The second stage of the study aimed to demonstrate and investigate the anti-oxidative, anti-cancer and antimicrobial activities *in-vitro* models. The antioxidant results revealed that methanolic extract showed higher activity than aqueous extract with higher phenolic and flavonoid contents as well as higher antioxidant assays which including DPPH radical scavenging and β-carotene bleaching. Preliminary cytotoxicity study was demonstrated against human lung carcinoma cell line (A549) using cell viability assay which revealed that methanolic extract is more potent than aqueous extract as IC_{50} values were 621±0.09 µg/mL and 845 ± 0.25 µg/mL respectively. It was then established that the methanolic extract was able to induce apoptosis in A549 cell line by the activation of caspase-3, 8 and 9. Further analyses to investigate the mode of cell death induction and cell cycle arrest pathways by flow cytometry analysis revealed that the methanolic extract was able to induce late apoptosis and arrested the cells in G0/G1 phase. Assessment of antimicrobial activities of the eel mucus extracts against several bacterial and fungal strains was conducted using diffusion method. Methanolic extract at 100 µL/well was found to inhibit the growth of *Microsporum gypseum* and *Aspergillus niger*. Significantly. Furthermore, the methanolic extract at 100 µL/disc significantly inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*. Comprehensive evaluation of antimicrobial activities against selected oral pathogens showed that methanolic extract exhibited high activity against *Enterococcus faecalis, Streptococcus mutans, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Candida albicans*. For *in-vivo* study, a topical gel delivery system used from eel skin mucus formulated gel to apply into the infected rat skins. Sprague Dawley rats were divided into normal, positive control, negative control, and treated groups. The infections were introduced to the rats by intracutaneous injections of pathogenic bacteria and fungi. The development of impetigo, tinea capitis and cutaneous candidiasis in the animal model was confirmed based on the clinical and histological observations. Following that, the infected rats were treated topically with the formulated gel from eel mucus extract. The histological analysis of the skin tissues which treated with the formulated gel was shown a complete recovery in the skin tissues at a similar rate as the control antibiotic groups. In conclusion, the present study revealed that eel skin mucus formulated gel efficient therapeutic candidate in treating skin infections which can be considered as a novel discovery as a natural alternative treatment for certain skin diseases.

خلاصة البحث

يعد اكتشاف وتطوير الأدوية البحرية الجديدة واحد من الأشياء الأكثر تحديا في مجال العلوم البحرية مؤخرا. تغطي المحيطات أكثر من ثلثي سطح الأرض، وبالتالي، فمن الضروري استكشاف الأدوية من الكائنات البحرية. تغطي جلد الحيوانات البحرية والبرمائية بطبقة من المخاط، الذي يعمل بمثابة حاجز كيميائي وميكانيكي لجلدهم. هذه الدراسة تهدف إلى تحديد الأنشطة البيولوجية لمخاط الجلد من ثعبان المستنقع الآسيوي (*Monopterus albus*) في المختبر وفي الجسم الحي. وكانت المرحلة الأولى من الدراسة هي فحص المركبات النشطة بيولوجيا باستخدام الغاز اللوني للطيف الكتلي والسائل اللوني –رباعي– المطياف لزمن الطيران، من خلال تحديد المركبات تبين أنها تمتلك أنشطة مختلفة خاصة بسمية الخلايا، ومضادات الأكسدة ومضادات الميكروبات. لذلك ، تهدف المرحلة الثانية من الدراسة إلى فحص هذه الأنشطة في المختبر. بشكل عام، أظهر مستخلص الميثانول أنشطة أعلى من المستخلص المائي. كشفت النتائج أن مضادات الأكسدة لمستخلص الميثانول متلك نشاطا عاليا، حيث بلغ إجمالي قيمة IC_{50} للمحتوى الفينولي 120.29±0.11 mg(GAE)/g ، وبلغ إجمالي محتوى الفلافونويد 260.28±0.19mg (QE)/g ، والفحص الجذري الكاسح 0.1±0.08 mg/mL وفحص بيتا كاروتين المبيض 23.2±0.44%. قد أشارت الدراسة الأولية للسمية الخلوية ضد الخلايا البشرية لسرطان الرئة (A549) باستخدام فحص بقاء الخلايا، والذي أظهر أن مستخلص الميثانول أكثر قوة (IC_{50} 621±0.09 µg/mL) .بعد ذلك ، تم تحديد آلية موت الخلايا بعد إجراء اختبارت كيميائية مختلفة، وثبت أن مستخلص الميثانول مسؤول عن موت الخلايا من خلال موت الخلايا المبرمج عن طريق تفعيل كاسباس 3 و 8 و 9. مستخلص الميثانول استحث موت الخلايا المبرمج المتأخر واعتقال الخلايا في مرحلة G0/G1 .أجريت الخصائص المضادة للميكروبات باستخدام طريقة النشر، حيث كانت منطقة تثبيط مستخلص الميثانول ضد البويغاء الجبسية لتركيز 100 µL/well بقيمة 25.72±0.75mm بينما ضد رشاشيات النيجر كانت 11.21±0.59mm. وفي الوقت نفسه، كانت منطقة تثبيط مستخلص الميثانول لتركيز 100 µL/disc ضد المكورات العنقودية الذهبية بقيمة 9.91±0.06mm بينما ضد القولونية كانت 10.79±0.17mm. أظهر تقييم شامل للأنشطة مضادة الميكروبات ضد مسببات الأمراض عن طريق الفم بأن مستخلص الميثانول أكثر فعالية حيث كانت فعاليته ضد المكورات المعوية البرازية(IC_{50} 7.15±0.73 µg/mL) ، ثم العقدية الطافرة (IC_{50} 12.89±0.15 µg/mL) ، تليها العقدية المقيحة (IC_{50} 9.75±0.91µg/mL) ، ثم الكلبسيلة الرئوية (IC_{50} 13.56±0.28 µg/mL)، ثم الزائفة الزنجارية (IC_{50} 23.44±0.43 µg/mL) وأدنى نشاط كان ضد المبيضات للبيض (IC_{50} 379.91±0.5 µg/mL) .تمت دراسة في الجسم الحي لنظام العلاج الموضعي يستخدم من هلام المخاط لجلد ثعبان المستنقع حيث استخدمت لتطبق على جلود الفئران المصابة. تم تقسيم فئران سبراغ داولي إلى التحكم الطبيعي والإيجابي ، والتحكم السلبي والمجموعات المعالجة. تم إدخال العدوى إلى الفئران عن طريق الحقن تحت الجلد بالبكتيريا والفطريات المسببة للأمراض، تم تطوير نماذج حيوانية من القوباء، سعفة الرأس والأمراض الجلدية المبيضة من خلال التحقق من الملاحظات السريرية والنسيجية، تم علاج الفئران المصابة موضعيا بالهلام المحضر. ويوضح التحليل النسيجي لجلود الفئران المعالجة بالهلام ، الانتعاش الكامل في أنسجة الجلد تماما كما هو الحال في المضادات الحيوية. وفي الختام، كشفت الدراسة أن الهلام المحضر من مخاط جلد ثعبان المستنقع مرشح لعلاجات فعالة لبعض لإلتهابات الجلدية التي تنظر في اكتشاف جديد كعلاج طبيعي بديل لأمراض الجلد.

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Ayah Rebhi Mohammad Hilles

Signature ..... Date .....

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The real of infinite and grateful praises, thanks to ALLAH for all this gift thus this text would not have been possible without the help of ALLAH under whose blessing and guidance I have completed this thesis as a requirement for my PhD of Biomedical Sciences and may His peace and blessings be upon our beloved Prophet Mohammad. Almighty ALLAH was the most merciful in providing me the opportunity to have worked with great people who have contributed in various ways to my research and thesis that deserve special mention.

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LIST OF ABBREVIATIONS

ESM	Eel skin mucus
SSTIs	Skin and soft tissue infections
GC-MS	Gas Chromatography- Mass Spectrometery
LC-QTOF-MS	Liquid chromatography quadrupole time flight mass spectrometry
µg/mL	Microgram per millilitre
TPC	Total Phenol Content
TFC	Total Flavonoids Content
DPPH	2,2-diphenyl-1-picrylhydrazyl
IC_{50}	Inhibition concentration (reduces the effect by 50%)
SD	Standard Deviation
PBS	Phosphate Buffered Saline
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RPM	Revolutions per minute
NA	Nutrient Agar
NB	Nutrient Broth
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
TEA	Triethanolamine
ANOVA	One-way analysis of variance
H&E	Haematoxylin and eosin stain

LIST OF SYMBOLES

β	Beta
g	Gram
M	Mitosis
S	Synthesis
µg/mL	Microgram per millilitre
mg GAE/g	Milligrams of gallic acid equivalent per gram of dry weight
mg QE/g	Milligrams of quercetin equivalent per gram of dry weight
°C	Degree Celsius
%	Percent
<	Less than
±	Plus-minus
=	Equal to
*	Statistical significance denotation

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Development and discovery of new marine drugs consider one of the most challenging fields in the recent marine sciences area. The water covers more than two-thirds of the Earth's surface and there are almost 90% of the world's species found in the marine environment (Napolitano et al., 2009), therefore, it is necessary to explore new drugs from marine organisms. Finding new, anti-inflammatory agents with fewer side effects is highly needed. Therefore, many types of research have been proven the anti-inflammatory activity of marine organisms, overall the fish has long-chain omega-3 fatty acids which is potent anti-inflammatory (Wall et al., 2010).

Marine natural products have a novel antioxidant prototype (Takamastu et al., 2003). Fish and marine invertebrates stimulate the induction of antioxidant defense systems (Abele and Puntarulo, 2004). Marine plants play a critical role to fulfill the requirement of food and nutrition, as it has high dietary fibres, low energy density, non-starchy vegetables, and fruits, which might protect against some cancers (Marmot et al., 2007). The marine environment provides novel leads against fungal, parasitic, bacterial, and viral diseases. Many marine natural products have successfully advanced into the late stages of clinical trials (Donia and Hamann, 2003).

Asian swamp eel (*Monopterus albus*) belongs to the family of Synbranchidae under Synbranchiformes order (Cheng et al., 2003). It is a freshwater fish which distributed widely in the East of India mainly across Greater Sunda Islands, Malay Peninsula and Indochinese Peninsula, it is also broadly distributed in the Southern areas of East Asia including, southeastern China, Western Japanese Archipelago, and Korean Peninsula (Banarescu, 1990). However, the eel is rarely found in the United States, as this species mainly distributed in Asia (Collins et al., 2002). It lives in muddy places, rice paddies, and slow-flowing currents areas. Asian swamp eel has a unique morphological elongated body which is similar to snake and covered with a thick layer of the mucus with no scales or fins. It can breathe air through the buccal mucosa (Chan and Phillips, 1967).

The skin of marine animals and amphibians are covered with a mucus layer, which acts as a biochemical and mechanical barrier for their skin. Several mucus sources have been isolated and studied for their biochemical and immunological functions, but the precise mechanism of action still not fully understood. Our study aimed to investigate the antimicrobial potential activity of Malaysian local swamp eel (*Monopterus albus)* skin mucus. Asian swamp eel mucus is secreted by the epidermal goblet cells in the epidermis which composed from inorganic salts, immunoglobulins, lipids and gel-forming macromolecules such as mucins, and other glycoproteins suspended in water (Bragadeeswaran and Thangaraj, 2011), which gives the mucus lubricating properties (Pearson and Brownlee, 2005).

The mucus layer is continuously replaced which protect the eel from stable colonization by bacteria, parasites, and fungi (Villarroel et al., 2007). The functional properties of the mucus depend on its ability to form a gel on the epithelial surface (Martínez-Antón et al., 2006), which produce antimicrobial molecules serve as the first line of a host's defense against microbial invasion (Manivasagan et al., 2009). The mucopolysaccharides in the mucus have a high value for immunoprotection and defense mechanism (Watanabe et al., 2012; Dirks et., 2014). It has been proven that mucin has the potential of antimicrobial and noxious properties (Knouft et al., 2003) as it plays an important role to protect the eel from pathogens (Yan et al., 2010). Overall mucus layer on the surface of eel functions as a physical and biochemical barrier between the eel and its environment (Palaksha et al., 2008).

Human skin infections can be caused due to many factors, such as low socioeconomic status, poor skin health, low level of hygiene and lack of awareness (Goonmatee and Rajesh, 2013; Kingman, 2005; Balai, 2012; Puri and Puri, 2013). It has been reported in Bari and Sierra Leone that skin infection was 42 % in African population because of social and environmental factors. (Ul Bari, 2007). The infection in Tunisia has been demonstrated as 16.9% fungal infection and 11.9% eczema were associated with their climatic conditions (Souissi, et al., 2006). In Nigeria, the most common fungal skin infection is tinea versicolor which correlates with several factors such as humid environment, heavy sweating, malnutrition, and genetics. Furthermore, the treatment from this skin fungal infection is found to be quite expensive such as Clotrimazole and due to the poor socioeconomic status in the infected population thus the number of infected patients with skin infections are very high (Oladele, et al., 2010).

It has also been reported that one-third of Mauritian population are infected with skin diseases with acne which is the most prevalent skin infection, followed by eczema, athlete foot and versicolor infection due to gender, age, personal hygiene, climatic conditions and level of awareness (Goonmatee and Rajesh, 2013). Therefore, the knowledge is an essential prevention factor to prevent skin infection (Schofield et al., 2009). The health knowledge in skin infections is necessary with the prevention, and it is important to seek primary health care to prevent complication and serious infection. knowledge and education about skin infections can play a critical role in prevention and management of skin infections (White et al., 2013).

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF THE STUDY

In the recent decades, the interest in evaluating therapeutic effects of aquatic natural products has been increased, therefore researches exploration of new alternative treatment from natural sources such as eel that possess no/minimal toxic effect is highly needed. According to WHO, 80% of the developing countries in the world's population rely on plant-derived medicines for the health care (Gurib-Fakim, 2006). Marine floras are widely unexplored as potent medicines for the dreadful human disease (Sithranga Boopathy and Kathiresan, 2011). It has been isolated more than 10,000 compounds from marine organisms and there are hundreds of new compounds are still being discovered every year. Around 300 patents were issued on bioactive marine natural products between 1969 and 1999 (Kathiresan et al., 2008). Discovery and development of new aquatic drugs remain one of the most challenging areas in recent marine and freshwater sciences.