

**CHEMICAL COMPOSITION, BIOLOGICAL
ACTIVITIES AND PROTEOMIC PROFILING
OF EPIDERMAL MUCUS IN TWO CATFISH
SPECIES FROM THE GENUS *CLARIAS***

NURUL MARIAM BINTI HUSSIN

**THESIS SUBMITTED IN FULFILLMENT FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY**



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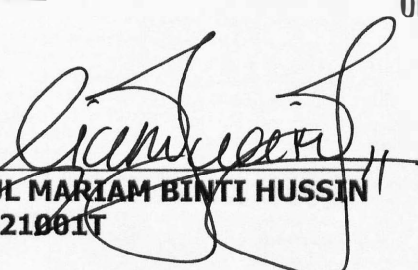
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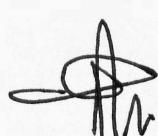
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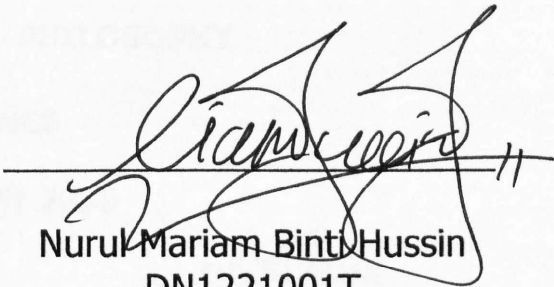

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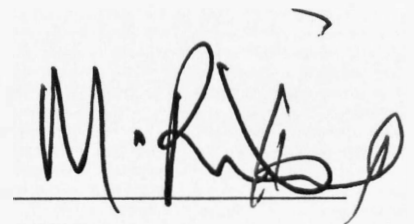
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ABSTRACT

Clarias gariepinus and *Clarias anfractus* (genus *Clarias*) are potentially catfish to secrete epidermal mucus. The mucus serves as a first physical barrier of defence against pathogens, external stressors and chemical attacks. Here, we study the chemical compositions, biological activities and protein profile of epidermal mucus from both catfish species. Proximate analysis (moisture, ash, protein, lipid and carbohydrate), mineral content determined using atomic absorption spectroscopy (AAS) and inductive coupled plasma mass spectrometry (ICP-MS), amino acid determination using high-performance liquid chromatography (HPLC) and fatty acid profile analyzed using gas chromatographic with flame ionization detector (GC-FID) were the evaluation of chemical compositions for the two catfish species epidermal mucus. The moisture content was a major component in the epidermal mucus (*C.gariepinus*: 90.01 ± 3.33 %; *C.anfractus*: 88.15 ± 0.32 %). The level of ash was significant ($p < 0.05$) higher in *C.gariepinus* epidermal mucus (7.65 ± 1.10 %) while the level of carbohydrate was significant ($p < 0.05$) higher in epidermal mucus of *C.anfractus* (5.70 ± 0.37 %). Although the ash content of *C.anfractus* epidermal mucus lowers than *C.gariepinus*, this study discovered its contained high levels of macro-elements minerals (K, Na, Mg, Ca and P). Both Na/K and Ca/P ratios in both catfish species epidermal mucus were less than one, agreement with the requirement of FAO/WHO. High amounts of amino acids (8.998 ± 0.014 %) and polyunsaturated fatty acids (PUFAs; 48 %) as compared to saturated fatty acids (SFAs; 39 %) and monounsaturated fatty acids (MUFAs; 13 %) also found in *C.anfractus* epidermal mucus. These can contribute positively to human nutritional requirements and consumptions. The antioxidant and antimicrobial activities were evaluated for the biological activities of the catfish species epidermal mucus. All the mucus were extracted using acidic, organic (dichloromethane (DCM) and aqueous phase) and water solvents to determine the DPPH radical scavenging, reducing power, ferric reducing power (FRAP), antibacterial and antifungal activities. The antimicrobial activities were based on the disc diffusion method. Overall, the DPPH scavenging effect in epidermal mucus of both catfish extracts ranges from 35 % to 55 %. Interestingly, the acidic extract of *C.gariepinus* epidermal mucus showed significantly ($p < 0.05$) higher of reducing power activity compared to BHT and ascorbic acid at a concentration of 80 $\mu\text{g/mL}$; ascorbic acid at 100 $\mu\text{g/mL}$. All epidermal mucus extracts showed low of FRAP activity compared to BHT and ascorbic acid. Although the antioxidant activities of both catfish epidermal mucus extracts showed low activities ($p < 0.05$) compared to the BHT and ascorbic acid, it revealed these catfish epidermal mucus could be potentially rich sources of natural antioxidants. These findings can be used as a basis for more advanced research on catfish epidermal mucus antioxidant capability. Of the screened epidermal mucus extracts, the acidic epidermal mucus extract of *C.anfractus* exhibited significant ($p < 0.05$) high levels of inhibitory activity (12.2 ± 2.54 mm) against Gram-positive bacteria, *Bacillus cereus*. All epidermal mucus extracts also showed inhibition zone against Gram-negative bacteria, *Escherichia coli* (range 6.77 to 9.44 mm) and *Pseudomonas aeruginosa* (range 6.16 to 8.60 mm). None of the acidic extracts was active against *Candida albicans*. From the biological activities experiments, its demonstrate that *C.gariepinus* and *C.anfractus* epidermal mucus were able to give average 22.52 ± 0.40 % of acidic extract yield, 9.06 ± 1.00 % of DCM phase extract yield, 13.36 ± 1.21 % of aqueous phase extract and 35.43 ± 3.15 % of

water extract yield. The yields expressed as grams of dry extract per 100 g of catfish epidermal mucus. Furthermore, the study also was done to find one the most efficient extraction method for proteomic analysis of the catfish epidermal mucus. It found that precipitation with trichloroacetic acid (TCA) and acetone result was an efficient sample concentration and desalting for proteomic analysis, compared to precipitation with ammonium sulfate. Therefore, this protein extraction method was chosen for a routine procedure for the proteomic analysis of *C.gariepinus* and *C.anfractus* epidermal mucus. A distinctive protein pattern was observed on the SDS-PAGE gels between two different catfish epidermal mucus and managed to get 2D-PAGE proteome map. The image analysis of 2D-PAGE gel was performed with Progenesis SameSpots version 4.5 and two sets of comparison of the 2D-PAGE gel images were done, Set 1: *C.gariepinus* vs *C.anfractus*, and Set 2: *C.anfractus* vs *C.gariepinus* to detect differentially expressed protein spots. Results of image analysis revealed 277 differentially expressed proteins for experiment set of *C.gariepinus* vs *C.anfractus* and 211 differentially expressed proteins for experiment set of *C.anfractus* vs *C.gariepinus*. Only 20 differentially expressed proteins from all two sets of experiments have successfully identified and characterized by the Shimadzu Prominence nano HPLC system [Shimadzu] coupled to a 5600 TripleTOF mass spectrometer [Sciex] and Mascot sequence matching software [Matrix Science]. A reference proteome map of *C.gariepinus* and *C.anfractus* epidermal mucus were obtained for the first time. The proteins identified in *C.gariepinus* and *C.anfractus* epidermal mucus can be categorized (protein biological functions) into three types of the general group of protein-based on the gene ontology (GO) analysis. The 55 % of structural proteins (two proteins of keratin, type II cytoskeletal 8; tropomyosin alpha-1 chain; actin, cytoplasmic 1; intermediate filament protein ON3 and glial fibrillary acidic protein), followed by 36 % of metabolic proteins (alpha-enolase, transcription factor AP-2-delta; Cyclin-dependent kinase 8 and MICOS complex subunit mic25a) and 9 % of protection-related protein (heat shock cognate 71 kDa protein) were successfully identified in *C.gariepinus* epidermal mucus. In *C.anfractus* epidermal mucus, the majority of the proteins were identified as metabolic proteins (67 %: glyceraldehyde-3-phosphate dehydrogenase; alpha-enolase; nascent polypeptide-associated complex subunit alpha; protein PAT1 homolog 1; kynurenine--oxoglutarate transaminase and triosephosphate isomerase B), followed by protection-related proteins (22 %: heat shock cognate 71 kDa protein and ferritin, middle subunit) and structural proteins (11 %: keratin, type I cytoskeletal 18). We can summarize from all the three main functions of protein, the epidermal mucus of catfish genus of *Clarias* consist of 51 % of metabolic proteins, 33 % of structural proteins and 16 % of protective-related proteins. The protein findings from epidermal mucus of *C.gariepinus* and *C.anfractus* may also be related to the biological activity screening evaluation that showed both species able to give some antioxidant, antibacterial and antifungal activities. Furthermore, the proteomic data provide systematic protein information together with the other chemical compositions data to comprehensively understand the biological function of *C.gariepinus* and *C.anfractus* epidermal mucus. In conclusion, the wild catfish, *C.anfractus* epidermal mucus contain various functional proteins and other beneficial chemical compounds compared to cultured catfish, *C.gariepinus* epidermal mucus. Therefore, mucus of this species has the potential too to be used in the nutraceutical and pharmaceutical industries. These were as indicated through the biological activities, bioactive compounds and identified functional proteins found in *C.anfractus* epidermal mucus.

ABSTRAK

KOMPOSISI KIMIA, AKTIVITI BIOLOGI DAN PROFIL PROTEOMIK TERHADAP MUKUS EPIDERMAL DUA JENIS SPESIES IKAN KELI DARI GENUS CLARIAS

Clarias gariepinus dan *Clarias anfractus* (genus *Clarias*) adalah merupakan ikan keli yang berpotensi untuk menghasilkan mukus epidermal. Mukus tersebut berfungsi sebagai penghalang pertahanan fizikal pertama terhadap patogen, tekanan luar dan serangan kimia. Dalam kajian ini, kami telah menjalankan ujikaji bagi komposisi kimia, aktiviti biologi dan profil proteomik mukus epidermal dari kedua-dua spesies ikan keli tersebut. Analisa proksimat (kelembapan, abu, protein, lipid dan karbohidrat), kandungan mineral yang ditentukan oleh spektrometri penyerapan atom (AAS) dan spektrometri jisim plasma gandingan induktif (ICP-MS), penentuan kandungan asid amino dengan menggunakan kromatografi cecair berprestasi tinggi (HPLC) dan penentuan profil asid lemak yang dianalisa dengan menggunakan kromatografi gas dengan api pengesan ionisasi (GC-FID) adalah merupakan penilaian komposisi kimia untuk mukus epidermal bagi kedua-dua spesies ikan keli tersebut. Kandungan kelembapan merupakan komponen utama dalam mukus epidermal (*C.gariepinus*: $90.01 \pm 3.33\%$; *C.anfractus*: $88.15 \pm 0.32\%$). Tahap abu adalah signifikan ($p < 0.05$) lebih tinggi pada mukus epidermal *C.gariepinus* ($7.65 \pm 1.10\%$), manakala tahap karbohidrat adalah signifikan ($p < 0.05$) lebih tinggi pada mukus epidermal *C.anfractus* ($5.70 \pm 0.37\%$). Walaupun kandungan abu di dalam mukus epidermal *C.anfractus* didapati rendah daripada *C.gariepinus*, kajian ini menunjukkan ianya mempunyai kandungan mineral dengan unsur makro yang tinggi (K, Na, Mg, Ca dan P). Kedua-dua nisbah Na/K dan Ca/P pada semua mukus epidermal spesies ikan keli adalah kurang dari nilai satu, iaitu bersesuaian dengan keperluan oleh FAO/WHO. Jumlah kandungan asid amino yang tinggi ($8.998 \pm 0.014\%$) dan asid lemak tidak tepu jenis 'poly' (PUFA: 48 %) berbanding dengan asid lemak tepu (SFA: 39 %) dan asid lemak tidak tepu jenis 'moly' (MUFA: 13 %) juga dijumpai di dalam mukus epidermal *C.anfractus*. Perkara ini seterusnya mampu memberi sumbangan yang positif kepada keperluan serta penggunaan pemakanan manusia. Aktiviti antioksidan dan antimikrob dijalankan bagi menentukan tahap aktiviti biologi di dalam mukus epidermal ikan keli. Semua mukus diekstrak menggunakan tiga jenis larutan pelarut, iaitu larutan asidik, larutan organik (terbahagi kepada fasa diklorometana (DCM) dan fasa 'aqueous') dan larutan air bagi tujuan menentukan tahap aktiviti antioksidan berdasarkan kepada 'DPPH radical scavenging', 'reducing power', 'FRAP reducing power', serta aktiviti antibakteria dan antifungus. Aktiviti antimikrob ditentukan dengan menggunakan kaedah 'disc diffusion'. Secara keseluruhannya, kesan terhadap 'DPPH scavenging' dalam ekstrak mukus epidermal kedua-dua spesies ikan keli ini adalah di dalam julat antara 35 % hingga 55 %. Menariknya, ekstrak asidik bagi mukus epidermal *C.gariepinus* menunjukkan aktiviti 'reducing power' yang tinggi (signifikan, $p < 0.05$) berbanding dengan BHT dan asid askorbik pada kepekatan 80 $\mu\text{g/mL}$; dan asid askorbik pada kepekatan 100 $\mu\text{g/mL}$. Kesemua ekstrak mukus epidermal menunjukkan tahap aktiviti 'FRAP' yang amat rendah berbanding dengan BHT dan asid askorbik. Walaupun aktiviti antioksidan dari kedua-dua ekstrak mukus epidermal ikan keli menunjukkan aktiviti yang rendah ($p < 0.05$) dibandingkan

dengan BHT dan asid askorbik, ia menunjukkan bahawa mukus epidermal ikan keli ini turut berpotensi dijadikan sebagai sumber antioksidan semula jadi. Penemuan aktiviti antioksidan ini boleh dijadikan sebagai asas untuk lebih banyak kajian secara lebih maju mengenai kemampuan antioksidan mukus epidermal *C.gariepinus* dan *C.anfractus*. Daripada ekstrak mukus epidermal yang disaring, ekstrak asidik bagi *C.anfractus* menunjukkan tahap aktiviti 'inhibitory' yang tinggi ($p < 0.05$) (12.2 ± 2.54 mm) terhadap bakteria Gram-positif, *Bacillus cereus*. Semua ekstrak mukus epidermal juga menunjukkan 'inhibition zone' terhadap bakteria Gram-negatif, *Escherichia coli* (julat antara 6.77 to 9.44 mm) dan *Pseudomonas aeruginosa* (julat antara 6.16 to 8.60 mm). Tiada sebarang aktiviti aktif yang ditunjukkan oleh semua ekstrak asidik terhadap fungus *Candida albicans*. Dari eksperimen aktiviti biologi yang telah dijalankan, ia menunjukkan bahawa mukus epidermal *C.gariepinus* dan *C.anfractus* dapat memberikan nilai purata hasil ekstrak asidik sebanyak 22.52 ± 0.40 %, ekstrak fasa DCM sebanyak 9.06 ± 1.00 %, ekstrak fasa 'aqueous' sebanyak 13.36 ± 1.21 % dan ekstrak air sebanyak 35.43 ± 3.15 %. Nilai hasil tersebut adalah bersamaan dengan gram ekstrak kering per 100 g mukus epidermal ikan keli. Objektif kajian semasa juga adalah mendalami apakah kaedah yang paling sesuai untuk pengekstrakan protein mukus ikan keli ini. Kaedah pemendakan trikloroasetik asid (TCA) dan aseton adalah kaedah yang paling sesuai bagi menghasilkan kepekatan sampel dan menyah-garam bagi analisa proteomik. Oleh yang demikian, kaedah pemendakan tersebut telah dipilih untuk segala proses analisa proteomik mukus *C.gariepinus* dan *C.anfractus*. Gambaran pemisahan protein yang sangat baik dapat dilihat pada gel SDS-PAGE dan ini berupaya untuk mendapatkan 2D-PAGE 'proteome map'. Analisa imej gel 2D-PAGE telah dilaksanakan dengan menggunakan 'Progenesis SameSpots version 4.5' dan perbandingan antara dua set telah dijalankan, Set 1: *C.gariepinus* vs *C.anfractus*; dan Set 2: *C.anfractus* vs *C.gariepinus* untuk penemuan tompok protein yang berbeza. Keputusan analisa imej menunjukkan sebanyak 277 'expressed protein' bagi set eksperimen *C.gariepinus* vs *C.anfractus* dan sebanyak 211 'expressed protein' bagi set *C.anfractus* vs *C.gariepinus*. Hanya sebanyak 20 'expressed protein' daripada dua set eksperimen berjaya dikenalpasti dan pencirian dengan menggunakan 'Shimadzu Prominence nano HPLC system [Shimadzu] coupled to a 5600 TripleTOF mass spectrometer [Sciex] dan 'Mascot sequence matching software [Matrix Science]'. Protein yang telah dikenalpasti di dalam mukus epidermal *C.gariepinus* dan *C.anfractus* boleh dikategorikan kepada tiga jenis kumpulan protein berdasarkan daripada analisa 'Gene Ontology (GO)'. 55 % protein struktur (dua protein 'keratin, type II cytoskeletal 8'; 'tropomyosin alpha-1 chain'; 'actin, cytoplasmic 1'; 'intermediate filament protein ON3' dan 'glial fibrillary acidic protein'), diikuti dengan 36 % protein metabolik ('alpha-enolase', 'transcription factor AP-2-delta'; 'Cyclin-dependent kinase 8' and 'MICOS complex subunit mic25a') dan 9 % protein pertahanan ('heat shock cognate 71 kDa protein') telah dikenalpasti dari mukus *C.gariepinus*. Mukus *C.anfractus* pula terdiri daripada protein metabolik (67 %: 'glyceraldehyde-3-phosphate dehydrogenase'; 'alpha-enolase'; 'nascent polypeptide-associated complex subunit alpha'; 'protein PAT1 homolog 1'; 'kynurenine--oxoglutarate transaminase' and 'triosephosphate isomerase B'), di ikuti protein pertahanan (22 %: 'heat shock cognate 71 kDa protein' dan 'ferritin, middle subunit') dan struktur (11 %: 'keratin, type I cytoskeletal 18'). Secara kesimpulannya, mukus epidermal ikan keli dari genus *Clarias* ini terdiri daripada 51 % protein metabolik, 33 % protein struktur dan 16 % protein pertahanan. Penemuan protein dari mukus epidermal *C.gariepinus* dan *C.anfractus* juga boleh dikaitkan dengan hasil penemuan aktiviti biologi yang

menunjukkan kedua-dua spesies ikan keli ini berupaya menunjukkan beberapa aktiviti antioksidan, antibakteria dan antimikrob. Tambahan lagi, data proteomik dari kajian ini mampu memberikan maklumat protein yang sistematik bersama-sama dengan data komposisi kimia yang lain bagi memahami lebih mendalam dan secara menyeluruh fungsi biologi mukus epidermal *C.gariepinus* dan *C.anfractus*. Akhir sekali, dapat disimpulkan bahawa mukus epidermal *C.anfractus* (ikan keli tempatan) didapati turut mengandungi pelbagai protein berfungsi dan sebatian kimia yang bermanfaat berbanding ikan keli kultur, *C.gariepinus*. Oleh yang demikian, mukus epidermal dari spesies tempatan ini juga berpotensi untuk digunakan dalam industri nutraseutikal dan farmaseutikal selain dari menggunakan sumber dari ikan keli kultur, *C.gariepinus*. Perkara ini telah dibuktikan melalui penemuan dalam kajian ini iaitu aktiviti biologi, komposisi kimia dan protein berfungsi.



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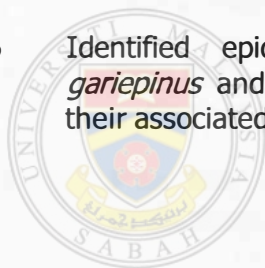


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LIST OF ABBREVIATIONS

A	Absorbance
AA	Amino acid
AAS	Atomic absorption spectrophotometer
AOAC	Association of Official Analytical Chemistry
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CBB	Coomasie Brilliant Blue
CG	<i>Clarias gariepinus</i>
CSP	<i>Clarias anfractus</i>
DHA	Docosahexaenoic
DPPH	1,1-Diphenyl-1-picrylhydrazyl
2D-Page	2 Dimension-polyacrylamide gel electrophoresis
EAA	Essential amino acid
e.g.	For example
et al	And others
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization if the United Nations
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
GC-FID	Gas chromatographic with flame ionisation detector
HDL	High density lipoprotein
HPLC	High performance liquid chromatography

H ₂ O	Distilled water
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
LDL	Low density lipoprotein
MUFA	Monounsaturated fatty acid
NA	Nutrient agar
NB	Nutrient broth
NEAA	Non essential amino acid
NL	Non linear
PUFA	Polyunsaturated fatty acid
PCA	Principal Component Analysis
PDA	Potato dextrose agar
RP	Reducing power
SDS-PAGE	Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
TSP	Total soluble protein
WHO	World Health Organization



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LIST OF SYMBOLS

mg/mL	milligrams per milliliters
mL	milliliters
ppm	parts per million
g	gram
kg	kilogram
cm	centimeter
pH	scale of acidity
%	percentage
°C	degree Celsius
mg	milligram
mM	mill molar
μL	microliter
pmole	pico mole
N (solution)	normality
mm	millimeter
nm	nanometer
μm	micrometer
nm	nanometer
mL/min	milliliter per minute
w/w	weight per weight
x g	gravity at the earth's surface
MΩ.cm	megohm-centimeter
μL/mL	microliter per milliliter
w/v	weight per volume

kDa	kilo Dalton
pI	Isoelectric point
V	Volt
μ A	microampere
PI	Proteomic International
CV	Coefficient of Variation
SD	Standard Deviation
MW	Molecular Weight
ω	omega
α	alpha
β	beta



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