GSK3 BETA-INHIBITORY ACTIVITY IN MICROFUNGAL ISOLATED FROM SABAH RAINFOREST SOILS

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2017

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UNIVERSITI MALAYSIA SABAH

THESIS SUBMITTED IN PARTIAL FULFILLMENT
FOR THE DEGREE OF MASTER
OF SCIENCE

FACULTY OF SCIENCE AND NATURAL RESOURCES
UNIVERSITI MALAYSIA SABAH
2017

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries, and references which have been duly acknowledged.

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DEGREE	:	**MASTER OF SCIENCE (BIOTECHNOLOGY)**
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ACKNOWLEDGEMENT

Alhamdulillah to Almighty Allah s.w.t. for giving me strength and patience to complete this research works. My great appreciation goes to my main supervisor Associate Professor Dr. Lee Ping Chin. Her guidance in scientific knowledges, motivations and warm kindness has made my research become meaningful. Great thanks also to my co-supervisor Associate Professor Dr. How Siew Eng that encourage me to learn more about organic chemistry which ultimately improved my chemistry knowledge. Thank you also to Associate Professor Dr. Jualang Azlan Gansau, Associate Professor Dr. Zaleha Aziz and Miss Hartinie Marbawi for giving me valuable motivation and spirits. Thanks to Dr. Ling Yee Soon and Yoong Soon Yong for their guidance, assistance and warm kindness which I will never forget. Sincere thanks to MOSTI for the grant and scholarship scheme.

I dedicated this study to my parents Haji Abdul Aziz Ahmad and Samarah@Hasrah Nurajim which never give up on me, understand my works and very supportive in every matters. Thank you and I love you so much. My warm gratitude also goes to my siblings, cousins and close relative for their motivations, spirits and financial supports. Endless thankful goes to Aidy Michael for his strength and patience on me in every best and worst situation throughout the years.

My gratefulness also goes to my seniors and close friend in the laboratory; Fauze Mahmud, Norhanizah Johansah, Aizuddin Abdullah, Eric Tzyy Jiann Chong, Lucky Poh Wah Goh, Nur Ashikin Gandah, Cherry Prissie Philip Jukim, Pan Shing Yi, Leong Yeh Chwan and Sakinah Yusof. Thank you so much for every act of kindness, inspiration and motivation during my study. Not to forget to thank the kindness from Puan Nadirah Yusof, Encik Asram Rasmun and Puan Nur Hidayah Thaddeus Abdullah for the chemical/reagents, instruments and laboratory that I used during my master study.

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ABSTRACT

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase that has been implicated in several diseases such as diabetes, cancer, inflammation, Alzheimer and bipolar disorder. Therefore, GSK-3β has become a prior target in drug discovery. This study was aimed to search for potential GSK-3β inhibitors in soil microfungi isolated from Sabah rainforests. In this study, a total of 122 soil samples were collected from west coast and interior division of Sabah whereby 165 microfungi strains were successfully isolated on potato dextrose agar and malt extract agar. All strain was cultured aerobically and the prepared extracts were tested using a yeast-based screening assay for their inhibitory activity on GSK-3β. The homologous genes of GSK-3 in the yeast (*MCK1, MDS1, MRK1*, and *YOL128C*) were knocked out and inserted with mammalian GSK-3β to overcome temperature-sensitive phenotype of the mutant at 37°C thus created a yeast strain that is capable to grow at both 25°C and 37°C. Positive result was scored when there is a large inhibition zone on the grown yeast at 37°C. Furthermore, targeting Cys199 residue on GSK-3β is a possible mechanism of inhibition in this assay and this residue lead to a selective inhibitor. Fourteen out of 165 strains gave detectable inhibition zones in the screening assay but only one strain namely MAN15558 isolated from Mantanani's island gave consistent inhibitory activities at 37°C and 25°C which were 17.75 mm ± 0.35 (clear inhibition) and 11.5 mm ± 0.53 (partial inhibition), respectively when tested at 5 mg/disk of acetone crude extracts. The MAN15558 strain is classified as *Aspergillus* sp. based on morphology and molecular techniques using 18sRNA. This strain is also a non-virulence strain based on biochemical assay. Crude extracts of MAN15558 strain was separated into polar and non-polar layer using rapid extraction method. Non-polar layer showed potential inhibitory activity at 100 µg/disk thus further fractionation of this layer was performed using automated semi preparative HPLC. Impure fraction 2 (F2) was obtained and the inhibitory activity on GSK-3β was confirmed using screening assay (100 µg/disk) and Kinase-Glo luminescent assay (10 µg). Active F2 was analysed using LC-MS/Qtof and 639.1664 m/z detected as the potential precursor ion. Identification of compounds performed using MS/MS fragmentation data of 639.1664 m/z in MassBank programme. Five hits were obtained and two of it was predicted as the potential compound based on their chemical substructure and peak relationship. The compounds were Isoscoparin 2''-O-ferulate and Okanin 4'-(4''-acetyl-6''-p-coumarylglucoside). These compounds were predicted as anti GSK-3β agent produced by MAN15558 strain. They are flavonoid compound and contain sub-structure like ferulic acid and coumaric acid which confers anti-diabetic and anti-oxidant activity as reported extensively. Further purification and structure are required to confirm these compounds. This present study concluded that MAN15558 strain is the first soil microfungi isolated from Mantanani's island

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identified as *Aspergillus* sp. that has produced anti GSK-3β agents targeting on Cys199 and predicted as Isoscoparin 2''-O-ferulate and Okanin 4'-(4''-acetyl-6''-p-coumarylglucoside).

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ABSTRAK

GSK3 BETA-INHIBITORY ACTIVITY IN MICROFUNGAL ISOLATED FROM SABAH RAINFOREST SOILS

Glikogen sintase kinase-3β (GSK-3β) adalah serin/threonin kinase yang terlibat dalam pelbagai penyakit seperti diabetes, kanser, inflamasi, Alzheimer dan gangguan bipolar. Maka, GSK-3β telah menjadi sasaran utama dalam pencarian ubat-ubatan. Kajian ini mensasarkan pencarian perencat GSK-3β dalam mikrofungi tanah yang dipencilkan dari hutan hujan di Sabah. Dalam kajian ini, sebanyak 122 sampel tanah dikumpulkan dari pantai barat dan kawasan pedalaman Sabah yang mana 165 strain mikrofungi telah dipencilkan di atas agar dektros kentang dan agar ekstrak gandum. Semua strain dikulturkan secara aerobik dan ekstrak yang disediakan diuji dengan kaedah penyaringan menggunakan yis untuk penyaringan aktiviti perencatan ke atas GSK-3β. Gen homolog GSK-3 di dalam yis (MCK1, MDS1, MRK1, dan YOL128C) dikeluarkan dan digantikan dengan GSK-3β mamalia untuk mengatasi fenotip mutan yang sensitif-suhu pada 37°C seterusnya mencipta strain yis yang dapat tumbuh pada 25°C dan 37°C. Keputusan yang positif akan diskorkan apabila terdapat perencatan yang besar pada pertumbuhan yis di suhu 37°C. Selain itu, pensasaran Cys199 di GSK-3β merupakan mekanisma perencatan yang berkemungkinan untuk kaedah ini dan sasaran ini akan membawa kepada perencat yang selektif. Empat belas daripada 165 strain yang diuji menunjukkan kehadiran zon perencatan dalam kaedah penyaringan tetapi hanya satu strain dengan nama MAN15558 yang dipencilkan dari Pulau Mantanani memberikan aktiviti perencatan yang konsisten pada suhu 37°C dan 25°C iaitu 17.75 mm ± 0.35 (perencatan jelas) dan 11.5 mm ± 0.53 (perencatan separa) masing-masing pada 5 mg/disk aseton ekstrak yg diuji. Strain MAN15558 diklasifikasikan sebagai Aspergillus sp. berdasarkan morfologi dan teknik molekular menggunakan 18sRNA. Strain ini juga tidak virulen berdasarkan ujian biokimia. Ekstrak daripada strain MAN15558 dipisahkan kepada bahagian polar dan tidak polar menggunakan kaedah ekstraksi cepat. Bahagian tidak polar menunjukkan aktiviti perencatan yang berpotensi pada 100 µg/disk seterusnya bahagian ini menjalani pemeringkatan lanjutan menggunakan semi preparative HPLC yang automatik. Pecahan aktif separa asli 2 (F2) telah diperoleh dan aktiviti perencatannya ke atas GSK-3β disahkan menggunakan kaedah penyaringn (100 µg/disk) dan kaedah luminesen Kinase-Glo (10 µg). Aktif F2 dianalisis menggunakan LC-MS/Qtof dan 639.1664 m/z dikesan sebagai ion prekursor yang berpotensi. Pengenalpastian sebatian dilaksanakan menggunakan data fragmentasi MS/MS 639.1664 m/z dalam program MassBank. Lima hit diperoleh dan dua daripadanya diramalkan sebagai sebatian yang berpotensi berdasarkan hubungan sub-struktur kimia dan fragmen. Sebatian itu adalah Isoscoparin 2''-O-ferulate dan Okanin 4'-(4''-acetyl-6''-p-coumarylglucoside). Sebatian ini diramalkan sebagai agen anti GSK-3β dihasilkan oleh MAN15558 strain.

Sebatian ini merupakan sebatian flavonoid dan mempunyai sub-struktur seperti asid ferulik dan asid komarin yang memberikan anti-diabetik dan anti-oksidan aktiviti seperti yang dilaporkan secara meluas. Purifikasi dan struktur lanjutan adalah diperlukan untuk mengesahkan sebatian-sebatian ini. Kajian ini menyimpulkan bahawa strain MAN15558 merupakan mikrofungi tanah yang pertama dipencilkan dari Pulau Mantanani dikenalpasti sebagai Aspergillus sp. yang menghasilkan agen anti GSK-3β yang mensasarkan Cys199 dan diramalkan sebagai Isoscoparin 2''-O-ferulate dan Okanin 4'-(4''-acetyl-6''-p-coumarylglucoside).

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LIST OF ABBREVIATIONS

ATP	-	Adenosine triphosphate
Arg	-	Arginine
Asn	-	Asparagine
Asp	-	Aspartic acid
C18	-	Carbon 18
C-terminal	-	Carboxyl-terminal
CDK	-	Cyclin dependent kinase
CK	-	Casein kinase
CNS	-	Central nervous system
CREB	-	cAMP response element binding protein
CRMP	-	Collapsin response mediator protein
Cys	-	Cysteine
DNA	-	Deoxyribonucleic acid
DYRK	-	Dual-specificity tyrosine(Y)-phosphorylation-regulated kinase
EDTA	-	Ethylenediaminetetraacetic acid
EGFR	-	Epidermal growth factor receptor
EGTA	-	Ethylene glycol tetraacetic acid
ERK	-	Extracellular signal regulated kinase
FRAT	-	Frequently rearranged in advanced T-cell lymphoma
GID	-	GSK-3 interacting domain
Gln	-	Glutamine
Glu	-	Glutamic acid
GSK-3	-	Glycogen synthase kinase-3
Ile	-	Isoleucine
Leu	-	Leucine
LC-MS	-	Liquid chromatography-mass spectrometry
LRP	-	Lipoprotein receptor related protein
LTD	-	Long term depression
Lys	-	Lysine
MAPK	-	Mitogen activated protein kinase
MLCK	-	Myosin light chain kinase
N-terminal	-	Amino-terminal
NFAT	-	Nuclear factors of activated T
NYL	-	Nylon
PCR	-	Polymerase chain reaction
Phe	-	Phenylalanine
PKA	-	Protein kinase A

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PKB	-	Protein kinase B
PKC	-	Protein kinase C
Pro	-	Proline
RNA	-	Ribonucleic acid
Ser	-	Serine
TCF/LEFs	-	T cell factor/lymphoid enhancing factors
TE	-	Tris-HCL EDTA
Thr	-	Threonine
Tyr	-	Tyrosine
Val	-	Valine

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LIST OF SYMBOLS

A	-	Alpha
B	-	Beta
Z	-	Zeta
H	-	Eta
Δ	-	Delta
Γ	-	Gamma
E	-	Epsilon
%	-	Percentage
^{32}P	-	Phosphorus-32 (radioactive isotope of phosphorus)
^{o}C	-	Degree Celsius
Bp	-	Base pair
IC_{50}	-	Half maximal inhibitory concentration
Kb	-	Kilo base
kDA	-	Kilo Dalton
K_m	-	Michaelis-Menten constant
mM	-	Mili molar
Mm	-	Millimetre
Ml	-	Millilitre
Mg	-	Milligram
Mg^{2+}	-	Magnesium ion
MW	-	Molecular weight
nM	-	Nano molar
Nm	-	Nanometre
Ng	-	Nano gram
µM	-	Micro molar
µg	-	Microgram
OD	-	Optical density
RLU	-	Relative luminescence unit
Rpm	-	Rotary per minute
S. D.	-	Standard deviation
™	-	Trademark
w/v	-	Weight over volume
V_{max}	-	Maximum velocity
v/v	-	Volume over volume
V	-	Voltan
m/z	-	Mass over charge ratio

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CHAPTER 1

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is first identified in rabbit skeletal muscle which had phosphorylation activity against glycogen synthase (Embi *et al.*, 1980) and later implicated in diabetes disease (Frame and Cohen, 2001). Active GSK-3 was found in brain which leads to Alzheimer's disease (Bhat *et al.*, 2004). Other studies reported that GSK-3 is also involved in multiple cellular pathway like Wnt signalling (Woodgett, 2001), cell fates and protein synthesis (Jacobs *et al.*, 2012) due to its numerous substrate (Sutherland, 2011). Therefore, GSK-3 activity is not limited to diabetes and Alzheimer's disease but also responsible for regulating most of the central nervous system disorder (Bhat *et al.*, 2004; Avrahami *et al.*, 2013), inflammation and cancers (Mishra, 2010).

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Scientific studies of GSK-3 activity in several diseases have leads to the development of inhibitor for GSK-3. At present, many GSK-3 inhibitors have been found and reported worldwide by researchers and scholars ranging from chemical element, natural resources, synthetic molecules and peptides (Finkelman and Martinez, 2011). Unfortunately not all of them act specifically on GSK-3 and pass in the trials using model animal even though their inhibitory activity is good in the cell free assay (Kramer *et al.*, 2012). Therefore, searching for GSK-3 inhibitor is still needed and the biggest challenge is to find the inhibitor with better selectivity, confers favourable outcome plus the inhibition of GSK-3 did not prevents the cell to function in a normal ways (Avrahami *et al.*, 2013).