SYNTHESIS AND INVESTIGATION OF ANTI-TYROSINASE AND ANTIOXIDANT ACTIVITY OF NEW HALOGENATED XANTHONE DERIVATIVES

By

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Chair : Nadiah Mad Nasir, PhD
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Excess melanin production in skin cells promotes dermatological diseases such as hyperpigmentation, ageing, and other skin-related diseases. Tyrosinase enzyme catalyses the oxidation of L-tyrosine substrate to melanin, via formation of L-3,4-dihydroxyphenylalanine (L-DOPA). Thus, tyrosinase inhibitors can lower melanin production to achieve whitening effect on the skin. Whitening products such as hydroquinone and kojic acid that are commonly used nowadays, might be harmful to human skin. This research aimed to produce 20 non-toxic halogenated tyrosinase inhibitors synthetically using Grover-Shah and Shah (GSS) method and determine their tyrosinase inhibitory activity using tyrosinase mushroom *Agaricus bisporus* assay with kojic acid as positive control. Six compounds were found to inhibit the enzyme with inhibition percentage of more than 50%. Among these six active compounds, three compounds (**15**, **16** and **17**) showed high potency. Their half-maximal inhibitory concentration (IC_{50}), enzyme kinetic analysis and antioxidant properties were determined. The IC_{50} values for compounds **15** (7.8 µg/ml, 75 µg/ml), **16** (9 µg/ml,

118 µg/ml) and **17** (10 µg/ml, 100 µg/ml) in L-tyrosine and L-DOPA substrates respectively, demonstrate their strong action in comparison to kojic acid as control (IC_{50} 4 µg/ml, 7.8 µg/ml). Enzyme kinetic analysis demonstrated that compounds **15** and **17** as uncompetitive inhibitor and compound **16** as mixed type inhibitor respectively. In antioxidant assays, only compound **16** exhibited a high antioxidant activity (IC_{50} 18 µg/ml) in DPPH assay and 2.93 mM/g in FRAP assay with ascorbic acid and quercetin as positive controls. Additionally, compound **16** revealed an IC_{50} value of 43.33 µM against the *in vitro* HaCaT skin cell line, indicating a weak toxicity on human skin cells. Compound **16** also revealed a non-toxic property for screening of toxicity test when tested against the *in vitro* brine shrimp toxicity assay at a dose of 1000 µg/ml. From the *in vivo* zebrafish embryo toxicity assay, results have shown that compound **16** was having a non-toxic property based on the parameters such as hatching rate, survival rate, heartbeat rate and obtained a LC_{50} value of 197.2 µg/mL. Furthermore, the molecular docking analysis showed their binding interactions between mushroom tyrosinase protein structure (PDB ID: 2Y9X) for compound **16** with a binding affinity of (-7.7 kcal/mol) and the compounds as the ligands through in silico approach. These data suggest that potent halogenated xanthone derivatives have the potential to be developed as new candidates for skin whitening agents with antioxidant and non-toxic properties in cosmetic industries and for pigmentation-related diseases.

Keywords: Antioxidant, anti-tyrosinase, synthesis, toxicity, xanthone

SDG: GOAL 3: Good Health and Well-being, GOAL 4: Quality Education, GOAL 9: Industry, Innovation, and Infrastructure

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

SINTESIS DAN PENYIASATAN AKTIVITI ANTI-TIROSINASE DAN ANTIOKSIDAN BAGI TERBITAN XANTON BERHALOGEN BARU

Oleh

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Pengeluaran melanin yang berlebihan dalam sel kulit menggalakkan penyakit dermatologi seperti hiperpigmentasi, penuaan dan penyakit kulit lain yang berkaitan. Enzim tirosinase memangkinkan substrat L-tirosin melalui pengoksidaan, lalu membentuk L-3,4-dihydroksilfenilalanin (L-DOPA) dan bertukar kepada melanin. Oleh itu, mendorong perencat tirosinase boleh mengurangkan pengeluaran melanin untuk mendapat sifat pemutihan pada kulit. Pada masa kini, beberapa produk pemutihan seperti hidrokuinon dan asid kojik mungkin berbahaya kepada kulit manusia. Penyelidikan ini bertujuan untuk menghasilkan 20 perencat tirosinase berhalogen tidak toksik secara sintetik dengan menggunakan kaedah Grover-Shah dan Shah (GSS) dan aktiviti perencatan tirosinasenya menggunakan ujian cendawan *Agaricus bisporus* tirosinase dengan asid kojik sebagai kawalan positif. Enam sebatian didapati merencat enzim tyrosinase dengan peratusan perencatan lebih daripada 50%. Di antara enam sebatian aktif ini, tiga sebatian (**15, 16** dan **17**) menunjukkan potensi

yang lebih tinggi. Kepekatan perencatan separuh maksimum (IC_{50}), analisis kinetik enzim dan sifat antioksidan telah ditentukan. Nilai IC_{50} untuk sebatian **15** (7.8 µg/ml, 75 µg/ml), **16** (9 µg/ml, 118 µg/ml) dan **17** (10 µg/ml, 100 µg/ml) dalam substrat L-tirosin dan L-DOPA secara masing-masing menunjukkan tindakan penghalangan kuatnya dengan asid kojik sebagai kawalan (IC_{50} 4 µg/ml, 7.8 µg/ml). Analisis kinetik enzim menunjukkan bahawa sebatian **15** dan **17** sebagai perencat bukan kompetitif dan sebatian **16** sebagai perencat jenis campuran masing-masing. Dalam ujian antioksidan, hanya sebatian **16** yang menunjukkan aktiviti antioksidan yang tinggi (IC_{50} 18 µg/ml) dalam ujian DPPH dan 2.93 mM/g dalam ujian FRAP dengan asid askorbik dan kuersetin sebagai kawalan positif. Selain itu, sebatian **16** mendedahkan nilai IC_{50} sebanyak 43.33 µM terhadap ujian *in vitro* MTT sel kulit HaCaT yang menunjukkan ketoksikan yang lemah pada sel kulit manusia. Sebatian **16** juga mendedahkan sifat tidak toksik untuk saringan ujian ketoksikan *in vitro* apabila diuji terhadap ujian ketoksikan udang air garam pada dos 1000 µg/ml. Daripada ujian ketoksikan embrio ikan zebra *in vivo* pula, keputusan telah menunjukkan bahawa sebatian **16** mempunyai sifat tidak toksik berdasarkan parameter-parameter seperti kadar penetasan, kadar kelangsungan hidup, kadar degupan jantung dan memperoleh nilai LC_{50} iaitu 197.2 µg/mL. Tambahan pula, analisis dok molekul menunjukkan interaksi pengikatannya antara struktur protein cendawan tirosinase (ID PDB: 2Y9X) untuk sebatian **16** dengan nilai pengikatan (-7.7 kcal/mol) dan sebatian ini sebagai ligan melalui pendekatan kaedah secara dalam siliko. Data-data ini menunjukkan bahawa derivatif xanton berhalogen yang kuat berpotensi untuk dibangunkan sebagai calon baharu untuk agen pemutihan kulit dengan sifat antioksidan dan tidak toksik dalam industri kosmetik dan untuk penyakit berkaitan pigmentasi pada waktu akan datang.

Kata Kunci: Antioksidan, anti-tirosinas, ketoksikan, sintesis, xanton

SDG: GOAL 3: Good Health and Well-being, GOAL 4: Quality Education, GOAL 9: Industry, Innovation, and Infrastructure

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		vi
APPROVAL		x
DECLARATION		xi
LIST OF TABLES		xv
LIST OF FIGURES		xvii
LIST OF SCHEMES		xxii
LIST OF ABBREVIATIONS		xxiii
CHAPTER		
1	**INTRODUCTION**	1
	1.1 General Introduction	1
	1.2 Synthetic Approaches to Xanthone	2
	1.3 Biological Activities of Xanthone	2
	1.4 Problem Statement	8
	1.5 Objectives of Study	10
2	**LITERATURE REVIEW**	11
	2.1 Sources of Xanthone	11
	2.1.1 Biological Activities for Natural Xanthones	11
	2.1.2 Chemical Synthesis of Xanthone	14
	2.2 Xanthone Groups	20
	2.2.1 Halogenated Xanthones	20
	2.2.2 Prenylated Xanthones	23
	2.3 Antityrosinase Activities of Xanthone	24
	2.4 Antioxidant Activities of Xanthone	28
3	**MATERIALS AND METHODS**	31
	3.1 Chemicals, reagents and solvents	31
	3.2 Instrumentation	32
	3.2.1 Melting Point	32
	3.2.2 Nuclear Magnetic Resonance Spectrometer (NMR)	32
	3.2.3 Fourier Transform Infrared Spectroscopy (FTIR)	33
	3.2.4 Gas Chromatography (Direct Injection Mass Spectrometry-DIMS)	33
	3.3 Chromatographic Methods	34
	3.3.1 Thin Layer Chromatography (TLC)	34
	3.3.2 Column Chromatography	34
	3.4 Synthesis	35
	3.4.1 Synthesis of Halogenated Xanthone Derivatives **(5-20)**	35
	3.4.2 Experimental Data for Synthesis of Halogenated Xanthones	37

3.4.3 Synthesis of prenylated halogenated xanthone derivatives **(21-24)** 53
3.4.4 Experimental Data for Prenylated Halogenated Xanthone Derivatives 54
3.5 Tyrosinase Inhibition Assay 58
3.5.1 L-tyrosine and L-DOPA substrates 58
3.6 Enzyme Kinetic Analysis 59
3.7 Antioxidant Assays 60
3.7.1 2,2-diphenylpicrylhydrazyl (DPPH) assay 60
3.7.2 Ferric ion reducing antioxidant power (FRAP) assay 61
3.8 Cytotoxicity 61
3.8.1 HaCaT skin cell Assay 61
3.8.2 Brine Shrimp Lethality Assay (BSLA) 62
3.8.3 Zebrafish (*Danio rerio*) Embryo *in vivo* Toxicity Assay 62
3.9 Computational Analysis 64
3.9.1 Molecular Docking Study 64

4 RESULTS AND DISCUSSION 67
4.1 Synthesis of Halogenated Xanthone Derivatives 67
4.1.1 Characterization of Compounds **15, 16** and **17** 70
4.1.2 Characterisation of 4-chloro-1,8-dihydroxy-9H-xanthen-9-one **(15)** 78
4.1.3 Characterisation of 4-chloro-1,5,6-trihydroxy-9H-xanthen-9-one **(16)** 84
4.1.4 Characterisation of 4-chloro-1,6-dihydroxy-9H-xanthen-9-one **(17)** 92
4.1.5 Synthesis of prenylated halogenated xanthone derivatives **21-24** 99
4.2 Tyrosinase Inhibition Assay 113
4.2.1 Preliminary Screening of Two Substrates 113
4.2.2 Half-Maximal Inhibitory Concentration Mushroom Tyrosinase Inhibition Assay (IC_{50}) 117
4.3 Enzyme Kinetic Analysis on Potent Compounds **15, 16** and **17** 121
4.4 Antioxidant Assays 125
4.4.1 2,2-diphenylpicrylhydrazyl (DPPH) assay and Ferric ion Reducing Antioxidant Power (FRAP) assay 125
4.5 Cytotoxicity Assays 129
4.5.1 Skin HaCaT cell MTT Assay 129
4.5.2 Brine Shrimp Lethality Assay (BSLA) 130
4.5.3 Zebrafish Embryo Toxicity Test 132
4.6 Computational Study 140
4.6.1 Computational Study of Selected Compounds 140

5 CONCLUSION AND RECOMMENDATIONS 156
5.1 Conclusion 156
5.2 Recommendation 157
5.3 Bioactivity Recommendation 159

REFERENCES 160
APPENDICES 167
BIODATA OF STUDENT 216
LIST OF PUBLICATIONS 217

LIST OF TABLES

Table		Page
1.1	Structure of synthesized xanthone derivatives	7
2.1	Previous reported series of 1,3-dihydroxyxanthone derivatives by Menéndez et al., 2017	19
2.2	*In vitro* anti-malarial activity against D6 and W2 strains	21
2.3	1,3-dihydroxyxanthone derivatives with a variety of substituents against tyrosinase enzyme	26
3.1	List of chemicals used in synthesis process	31
3.2	List of chemicals & reagents used in biological activities	32
3.3	List of halogenated xanthones **5-20**	35
3.4	List of prenylated halogenated xanthones **21-24**	53
4.1	Comparison of ^{1}H and ^{13}C NMR values for compound **15, 16** and **17**	76
4.2	Comparison of FTIR spectrum, GC (DI-MS) spectroscopy and yields for compound **15, 16** and **17**	77
4.3	The ^{1}H-NMR and ^{13}C-NMR data 2-chloro-3,8-dihydroxy-9H-xanthen-9-one **(15)** measured in Methanol-d_4	81
4.4	The ^{1}H-NMR and ^{13}C-NMR data 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one **(16)** measured in Acetone-d_6	90
4.5	The ^{1}H-NMR and ^{13}C-NMR data 4-chloro-1,6-dihydroxy-9H-xanthen-9-one (**17**) measured in Methanol-d_4	96
4.6	Compressed data analysis of ^{1}H, ^{13}C, FTIR, GC (DI-MS) and yield for compound **21**	111

4.7	Compressed data analysis of ^{1}H, ^{13}C, FTIR, GC (DI-MS) and yield for compound **22**	112
4.8	Percent of inhibition of tyrosinase enzyme by **20** halogenated xanthone derivatives using two substrates (L-tyrosine & L-DOPA)	114
4.9	The antityrosinase activity of active compounds in presence of L-tyrosine substrate (1.0 mM)	119
4.10	The antityrosinase activity of active compounds in presence of L-DOPA substrate (1.0 mM)	120
4.11	K_m and V_{max} values of Potent Compounds **15**, **16** and **17**	125
4.12	IC_{50} value of compounds **15**, **16** and **17** in DPPH assay and FRAP assay	126
4.13	IC_{50} values of HaCaT skin cells treated with compound **16**	129
4.14	Brine Shrimp Lethality Assay (BSLA) test on compound **16**	131
4.15	The morphological characteristics of zebrafish embryos treated with six concentrations of compound **16** at 24, 48 and 72 hpf observed under 5x magnification.	138
4.16	Interactions available on molecular docking analysis of tropolone, kojic acid, compounds **15**, **16, 17**, **12** and **21**, L-tyrosine and L-DOPA with tyrosinase enzyme (PDB ID: 2Y9X)	154

LIST OF FIGURES

Figure		Page
1.1	Xanthone scaffold	1
1.2	General structure of targeted compounds	5
2.1	Structures of naturally derived xanthiones (Nigrolineaxanthone-F), (Brasilixanthone-A) and (Caloxanthone-A)	12
2.2	Natural xanthone derivatives isolated from *Garcinia virgata* stem barks	13
2.3	7-methoxydoxymorellin reported by Reutrakul *et al*., for anticancer activity	14
2.4	1,3-dihydroxyxanthone derivatives tested against Alzheimer's disease	18
2.5	1,2,3-triazole ring of xanthone derivatives that are potent against the A549 lung cancer cells obtained from Wu *et al*., 2019	20
2.6	Di-fluorinated xanthone derivatives and non-fluorinated xanthone, Dodean *et al*., 2008	21
2.7	Di-fluorinated xanthone synthesized for anti-inflammatory breast cancer, Chantarasriwong *et al*., 2019	22
2.8	Halogenated xanthone that shows anticancer properties from Ramakrishnan *et al*., 2023	23
2.9	Synthesized prenylated rings for xanthone scaffold by cyclization technique (Pinto *et al*., 2021)	24
2.10	Reaction diagram of L-tyrosine oxidation forming L-DOPA (Mine *et al*., 2022)	25
2.11	1,3-dihydroxyxanthone derivatives obtained from Zhou *et al*., 2019)	26
2.12	Halogenated xanthone derivatives from (Rosa *et al*., 2021) with antityrosinase activity	27
2.13	Compound **70** with IC_{50} value of 0.448 μM with excellent antioxidant property (Kou *et al*., 2019)	28

2.14	Reported xanthone derivatives on antioxidant activity with IC_{50} values 11.3 μM and 11.1 μM respectively (Cidade *et al*., 2020)	29
4.1	Plausible structures for compounds bearing 4-chlororesorcinol as ring C	70
4.2	^{1}H-NMR spectrum of 2-chloro-3,8-dihydroxy-9H-xanthen-9-one (**15**)	79
4.3	^{13}C-NMR spectrum of 2-chloro-3,8-dihydroxy-9H-xanthen-9-one (**15**)	80
4.4	The HSQC spectrum of compound **15** (500/125 MHz, MeOD)	82
4.5	The HMBC spectrum of compound **15** (500/125 MHz, MeOD)	82
4.6	The FTIR spectrum of compound **15**	83
4.7	DI-MS spectrum of of 2-chloro-3,8-dihydroxy-9H-xanthen-9-one (**15**)	84
4.8	^{1}H-NMR of spectrum of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one **(16)**	85
4.9	^{13}C-NMR spectrum of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one **(16)**	86
4.10	The HSQC spectrum of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one **(16)** (500/125 MHz)	87
4.11	The HMBC spectrum of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one (**16)** (500/125 MHz)	88
4.12	^{1}H-^{1}H homonuclear correlation spectroscopy (COSY) spectrum of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one (**16)** (500/500 MHz)	88
4.13	The FTIR spectrum of compound **16**	91
4.14	DI-MS spectrum of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one (**16**)	92
4.15	^{1}H-NMR spectrum of 2-chloro-3,6-dihydroxy-9H-xanthen-9-one (**17**)	93
4.16	^{13}C-NMR spectrum of 2-chloro-3,6-dihydroxy-9H-xanthen-9-one (**17**)	95

4.17	HMBC spectrum of 2-chloro-3,6-dihydroxy-9H-xanthen-9-one (**17**)	97
4.18	The FTIR spectrum of compound **17**	97
4.19	DI-MS spectrum of 2-chloro-3,6-dihydroxy-9H-xanthen-9-one (**17**)	98
4.20	^{1}H-NMR spectrum of 7-chloro-1-hydroxy-3',3'-dimethylpyrano[3,2-a]xanthen-9-one **(21)**	102
4.21	^{13}C-NMR spectrum of 7-chloro-1-hydroxy-3',3'-dimethylpyrano[3,2-a]xanthen-9-one **(21)**	103
4.22	FTIR spectrum of compound **21**	103
4.23	DI-MS spectroscopy of 7-chloro-1-hydroxy-3',3'-dimethylpyrano[3,2-a]xanthen-9-one **(21)**	104
4.24	^{1}H-NMR spectrum of 8-fluoro-1-hydroxy-3',3'-dimethylpyrano[3,2-a]xanthen-9-one **(22)**	106
4.25	^{13}C-NMR spectrum of 8-fluoro-1-hydroxy-3',3'-dimethylpyrano[3,2-a]xanthen-9-one **(22)**	107
4.26	FTIR spectrum of compound **22**	109
4.27	DI-MS spectroscopy of compound **22**	109
4.28	Lineweaver-Burk Plot of Kinetic Inhibition Analysis of Compound **15**	122
4.29	Lineweaver-Burk Plot of Kinetic Inhibition Analysis of Compound **16**	122
4.30	Lineweaver-Burk Plot of Kinetic Inhibition Analysis of Compound **17**	123
4.31	Plausible radical scavenging mechanism for DPPH with 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one (**16**)	127
4.32	Illustration of Ferric Reducing Antioxidant Power (FRAP) Mechanism with compound **16** as the antioxidant agent	128
4.33	Dose response curve of HaCaT skin cells treated with compound **16**	129
4.34	The survival rate of zebrafish embryo examined after exposure to a series of concentrations of compound **16** from 0 to 120 hpf	132

4.35	The survival rate of zebrafish embryo examined after exposure to a series of concentrations of APAP (positive control) from 0 to 120 hpf	133
4.36	The hatching rate of zebrafish embryo observed after being treated with compound **16** in multiple concentrations from 0 to 120 hpf	134
4.37	The heartbeat rate of zebrafish embryo observed at 96 hpf after being treated with compound **16** in various concentrations	136
4.38	Top score docking poses of compound **15, 16, 17, 12** and **21** (yellow, blue, cyan, brown and orange coloured respectively) on the allosteric site and kojic acid (purple coloured) on the catalytic site of enzyme shown as exemplification for the location of binding sites on the 3D mushroom tyrosinase enzyme structure. Tyrosinase enzyme (PDB: 2Y9X) represented as ribbons and the two spheres represented the copper ions (brown-coloured spheres) located at the catalytic site of the enzyme.	141
4.39	a) Visual representation of docking analysis for compound **15** (yellow coloured) with tyrosinase enzyme on the allosteric site in closed view, two brown spheres indicate the location of the catalytic site in the enzyme b) Color coded interactions of amino acids in the enzyme with compound **15**	142
4.40	a) Visual representation of docking analysis for compound **16** (blue coloured) with tyrosinase enzyme on the allosteric site in closed view, two brown spheres indicate the location of the catalytic site in the enzyme b) Color coded interactions of amino acids in the enzyme with compound **16**	143
4.41	a) Visual representation of docking analysis for compound **17** (cyan coloured) with tyrosinase enzyme on the allosteric site in closed view, two brown spheres indicate the location of the catalytic site in the enzyme b) Color coded interactions of amino acids in the enzyme with compound **17**	144
4.42	Visual representation of docking analysis for compound **12** (brown coloured) with tyrosinase enzyme on the allosteric site in closed view, two brown spheres indicate the location of the catalytic site in the enzyme b) Color coded interactions of amino acids in the enzyme with compound **12**	145

4.43	a) Visual representation of docking analysis for compound **21** (orange coloured) with tyrosinase enzyme on the allosteric site in closed view, two brown spheres indicate the location of the catalytic site in the enzyme b) Color coded interactions of amino acids in the enzyme with compound **21**	146
4.44	a) Visual representation of docking analysis for L-tyrosine substrate with tyrosinase enzyme b) Color coded interactions of amino acids in the enzyme with L-tyrosine substrate	151
4.45	a) Visual representation of docking analysis for L-DOPA substrate with tyrosinase enzyme b) Color coded interactions of amino acids in the enzyme with L-DOPA substrate	152
5.1	The recommended halogenated xanthone derivatives structures	159

LIST OF SCHEMES

Scheme		Page
1.1	General reaction for synthesis of halogenated xanthones **3** and prenylated xanthones **4**	6
2.1	Synthesis of Xanthone via GSS Reaction	15
2.2	Synthesis of Xanthones via Modified Grover, Shah and Shah (GSS) Reaction with Eaton's Reagent	17
2.3	Synthesis of xanthone using phenolic compounds and hydroxybenzoic acid derivatives	18
3.1	General reaction for the synthesis of halogenated xanthone derivatives	35
3.2	General reaction for the synthesis of prenylated halogenated xanthone derivatives from its halogenated xanthone derivatives	53
4.1	Synthesis of xanthone scaffold obtained by study from Varache-Lembege *et al*., 2008	67
4.2	Synthesis of halogenated xanthone derivatives of compound **15** by Eaton's reagent	68
4.3	Proposed general mechanism for synthesis of halogenated xanthone derivatives by using Eaton's reagent in modified GSS method	69
4.4	Synthesis of 2-chloro-3,5,6-trihydroxy-9H-xanthen-9-one (**16**) by Eaton's reagent	85
4.5	Synthesis of 2-chloro-3,6-dihydroxy-9H-xanthen-9-one (**17**) by Eaton's reagent	92
4.6	Synthesis of prenylated halogenated xanthone derivatives from respective halogenated xanthones	99
4.7	Proposed general mechanism of prenylated halogenated xanthone derivatives **21 – 24**	100

LIST OF ABBREVIATIONS

γ	Gamma
α	Alpha
δ	Chemical shift in ppm
UV	Ultraviolet
μM	Micromolar
μg	Microgram
mmol	Milimolar
mL	Mililiter
mg	Miligram
^{o}C	degree celcius
%	percentage
^{13}C	carbon-13
C_3D_6O	deuterated acetone
AChE	Acetylcholinesterase
$CDCl_3$	deuterated chloroform
cm^{-1}	per centimeter
d	doublet
dd	doublet of doublet
DI-MS	Direct Injection-Mass Spectrometry
DMSO	dimethyl sulphoxide
DPPH	2,2-diphenylpicrylhydrazyl (DPPH) assay
FRAP	Ferric ion Reducing Antioxidant Power
FT-IR	Fourier Transform-Infrared spectroscopy
GC-MS	Gas Chromatograph-Mass Spectroscopy
^{1}H	proton

HaCaT	Normal keratinocyte skin cells
hpf	hours post fertilisation
Hz	hertz
IC_{50}	Half-maximal inhibitory concentration
m	multiplet
m/z	Mass-to-charge ratio
CD_3OD	deuterated methanol
t	triplet

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Xanthones are secondary metabolites found in a variety of plant families, fungi, and lichens (Vieira & Kijjoa, 2005). Xanthones, with the IUPAC designation 9H-xanthene-9-one, are usually yellowish-colored heterocyclic compounds having the molecular formula $C_{13}H_8O_2$. The term “xanthone” is a combination of "xanth" is derived from the Greek word which means "yellow" and “one” which refers to the keto group in the structure. Heterocycle moieties play an important role in the design and development of new physiologically and pharmacologically active molecules. As illustrated in **Figure 1.1**, the xanthone scaffold has a dibenzo-γ-pyrone scaffold formed by the coupling of two benzene rings and a pyrone-4-one ring and plays a key role in the development of novel medications with potential biological activities in the pharmaceutical industry.

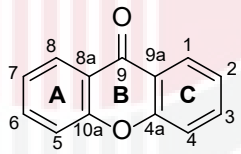


Figure 1.1: Xanthone scaffold

Natural occurring xanthones include various types of substituents at different positions, resulting in a wide range of pharmacological activities. Based on **Figure 1.1**, a carbonyl group and oxygen bridge linking two aromatic rings, giving the appearance of fused ring system. In terms of molecular symmetry, both C-1, C-8 and C-4, C-5 are acidic sites due to the withdrawing effect of the electronegative oxygen atoms (Odrowaz-Sypbiewski *et al*., 2009). In contrast to that, the fused ring structure limits

the flexibility and thereby enhances the stability of the xanthone framework. Carbons 1-4 in the xanthone basic skeleton are allocated to acetate-derived ring **C**, whereas carbons 5-8 are assigned to shikimate-derived ring **A**. For structural elucidation, the remaining carbon atoms are designated as 4a, 5a, 8a, 9 and 9a (Pedro *et al*., 2002).

1.2 Synthetic Approaches to Xanthone

With varied patterns of substitution, isolated xanthones have been successfully extracted from famous natural resources such as *Calophyllum inophyllum* and *Garcinia mangostana*, leading to a wide range of compounds with biological significance. The concentration of xanthones isolated from plants is low in yield, making it challenging to isolate xanthones from their natural resources. As a result of this, xanthone synthesis has been further studied. Synthetic techniques can be used to create new xanthones with varied substituent positions and types on the fundamental building block. Synthetic methods can be divided into two methods, via biosynthesis where enzymatic reaction is used to produce numerous xanthone derivatives and chemical synthesis where catalytic reactions are performed in the laboratory.

1.3 Biological Activities of Xanthone

Xanthone plays an important role in biological active heterocycles which have wide potential in pharmacological field in recent years. Hence, many researchers tend to focus in synthesizing various xanthone derivatives for the development of new lead drug.

Xanthone derivatives are mainly shown in producing as many types of biological activities reported whereby their derivatives can interact with several different

medicinal targets such as antioxidant, anticancer and antibacterial (Li *et al*., 2011). Researchers reported that xanthones which bearing aromatic benzene rings in the structure can produce bioactivities due to the aromatic rings are able imitate the behavior of most inhibitors on the respective bioactivities.

Studies have been conducted showing that the xanthone derivatives possesses biological activities such as antityrosinase (Rosa *et al*., 2021), anticancer (Pedro *et al*., 2002), antioxidant (Wairata *et al*., 2021), antitumor (Luo *et al*., 2013) and anti-melanogenesis activities (Hosseinpoor *et al*., 2021) and more.

Alzheimer's disease is commonly referred to as an illness with multiple factors due to genetic, environmental, and endogenous aspects. Cholinergic neuronal transmission loss and oxidative stress have been recognised as important markers of this Alzheimer's disease. In 2017, it was stated that xanthone derivatives have the potential to be used as dual agents in the treatment of Alzheimer's disease, alongside acetylcholinesterase inhibitors and antioxidants (Cidade *et al*., 2017).

Melanin is the most abundant natural biological polymer pigment in skin and hair (Lam *et al*., 2010). However, excessive formation of melanin can result in dermatological conditions such as freckles, melasma, and lentigo (Kim *et al*., 2008). Although the mechanism of melanogenesis is complex, it has been widely reported that the tyrosinase enzyme may regulate the biosynthetic pathway for the formation of melanin (Gartner *et al*., 2012).

In recent years, Rosa and her colleagues managed to discover that xanthone derivatives are able to inhibit tyrosinase enzyme with a potency in their discovery as a whitening agent (Rosa *et al*., 2021). They also reported that most of their synthesized xanthone derivatives are showing a result as tyrosinase inhibitors with the best results that beats approximately six times lower than the IC_{50} value from the positive control, kojic acid. Previous non-halogenated xanthone derivatives are lacking to be reported in having anti-tyrosinase properties and recently, a study done by Rosa and colleagues in 2021 managed to discover that a halogenated xanthone possesses an antityrosinase activity which can be potential as a skin whitening agent in future. Also, based on our knowledge, prenylated xanthones are yet to be reported to possess an antityrosinase activity too. Therefore, the purpose of this work was to synthesize xanthone scaffolds with halogens, then inducing a cyclic ring into the scaffold, and subsequently with the evaluation of their biological activities, on tyrosinase enzyme in two pathways: monophenolase (L-tyrosine) and diphenolase (L-3,4-dihydroxyphenylalanine; L-DOPA).

Halogenation of compounds are a common approach in drug discovery and a significant number of compounds in clinical development are halogenated (Lu *et al*., 2010; Hernandez *et al*., 2010). Halogen atoms are involved in protein-ligand noncovalent interactions, such as formation of hydrogen bonds and halogen bonds in the enzyme activity (Hernandez *et al*., 2010). Introducing halogen atoms may also contribute to steric effects, through the ability of these bulky atoms to occupy the binding site of molecular targets (Hernandez *et al*., 2010).

Most skin whitening agents in the cosmetic industry nowadays are found to be using toxic ingredients such as hydroquinone and mercury. Although these ingredients can exhibit a high whitening effects, they produce an adverse effect such as inflammation, irritation and blisters towards the users (Owolabi *et al*., 2020). Thus, in our study, we would like to develop a new skin whitening agent with non-toxic properties that are beneficial for the cosmetic industries.

Furthermore, the structure-activity relationships (SARs) on selected xanthones that demonstrates higher activity *in vitro* test will be investigated. Our hypothesis is to create a series of xanthone derivatives by modifying the basic scaffold which are predicted to be a promising lead as whitening agents for pharmaceuticals purposes based on the reported study which shows the activity in helping skin related diseases (Rosa *et al*., 2021). **Figure 1.2** and **Scheme 1.1** illustrates the reaction scheme as well as the general structures of the targeted compounds. Meanwhile, **Table 1.1** lists the structures of all twenty compounds, including ten new compounds.

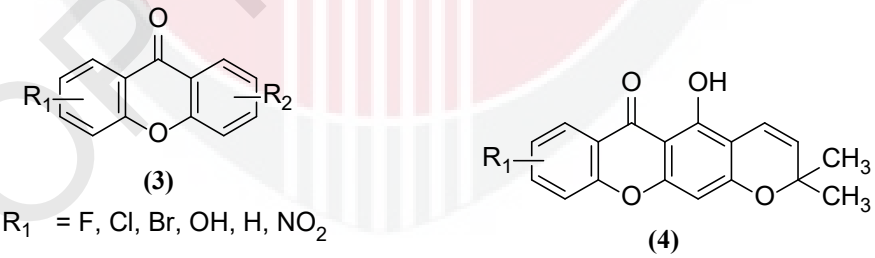
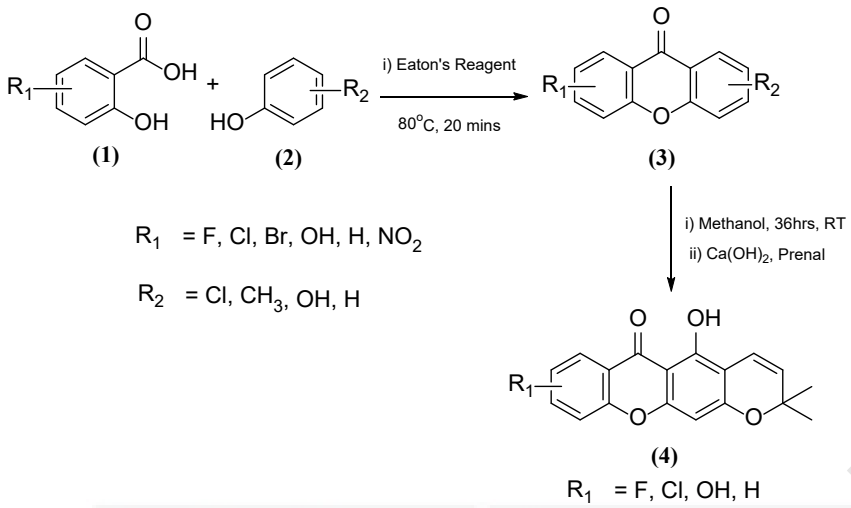


Figure 1.2: General structure of targeted compounds



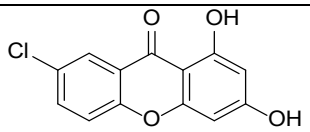
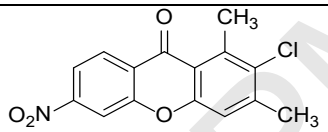
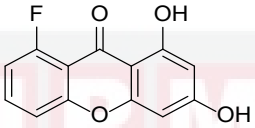
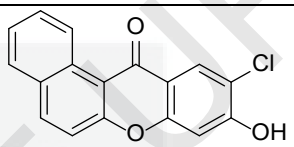
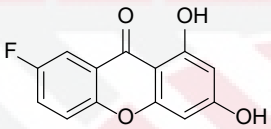
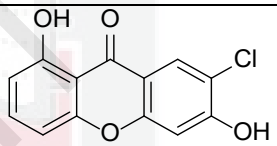
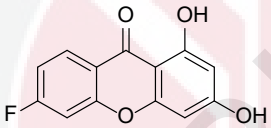
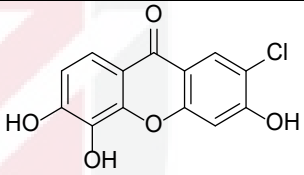
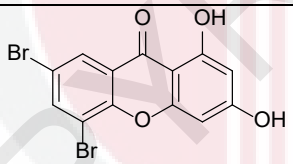
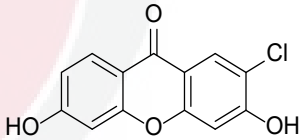
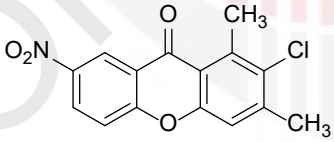
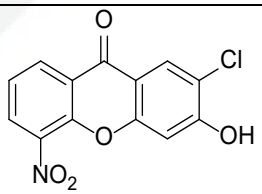
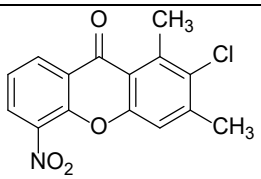
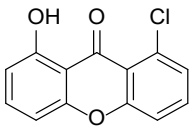
Scheme 1.1: General scheme for synthesis of halogenated xanthones (3) and prenylated xanthones (4)

In this study, we chose halogenated xanthone such as fluorine, chlorine and bromine as different substituents and prenylated xanthone derivatives to discover their potential in inhibiting the tyrosinase enzyme as previous studies also have supported that halogenated xanthone produces this inhibitory effect. For the prenylated derivatives, studies are yet to be reported in this tyrosinase inhibitory, thus, we would like to initiate in discovering their results which can be added into the xanthonoic derivatives of library among the other researchers for future.

Most previous studies reported that compounds that bears an aromatic ring moiety shows a potency in most biological inhibitory properties (Rosa *et al*., 2021; Zhu *et al*., 2013; Haldys *et al*., 2020). Moreover, aromatic rings structurally mimic the tyrosinase substrates which are L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA) that brings an interaction with the hydrophobic tyrosinase enzyme site via van der Waals interactions (Zhu *et al*., 2013). Therefore, xanthone scaffold possesses a great potential

in holding as a tyrosinase inhibitor that are potential for skin whitening agent due to the presence of aromatic ring.

Table 1.1: Structure of synthesized xanthone derivatives

Halogenated xanthones			
5		**13***	
6		**14***	
7		**15***	
8		**16***	
9		**17***	
10*		**18***	
11*		**19**	

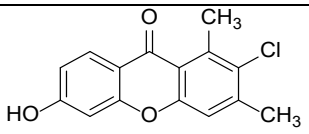
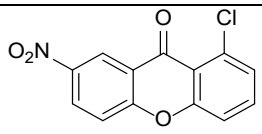
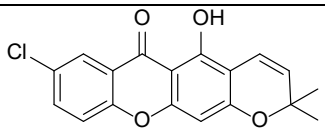
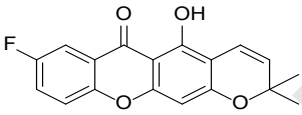
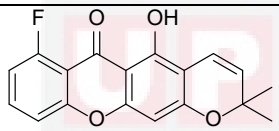
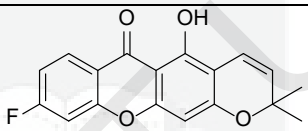
12*		**20***	

Table 1.1: Continued

Prenylated xanthones			
21		**23**	
22		**24**	

*Indicates new compounds

1.4 Problem Statement

Nowadays, in the modern world, a lot of UV radiations are emitted from environments such as natural sunlight and man-made UV rays from the screen of technologies such as smartphones, television and more. The main types of UV rays that are able to affect the skin are UVA rays and UVB rays. People who tend to be more exposed towards the UV rays are at greater risk for skin cancer, melasma, freckles, and age spots which can accelerate skin aging by several years in difference. In addition, continuous exposure of UV rays may speed up the formation of melanin in the skin, DNA damage, gene mutation, impairment of immune system, hyperpigmentation and photoaging (Kim *et al*., 2021; cancer.org, 2023). To achieve a skin whitening effect, tyrosinase inhibitors are introduced in any cosmetic products. Unfortunately, due to the high toxicity of the most known tyrosinase inhibitor such as hydroquinone, kojic acid and

mercury (Burnett *et al*., 2010; Owolabi *et al*., 2020), there is a need in discovering new tyrosinase inhibitors which are safe and non-toxic for the users.

Most isolated xanthones from natural products will have a low yield with a limited type of substituents in the structures, therefore, a synthetic approach in producing xanthones with various positions of substituents leads to new possibilities in discovering new areas of their biological activities with diverse of patterns in substituents.

A previous study done by Rosa and her colleagues in 2021 managed to discover that xanthone derivatives are found to be exhibiting an antityrosinase effect based on the mushroom tyrosinase enzyme study. However, up to these recent years, a small number of studies were reported on halogenated xanthone derivatives as tyrosinase inhibitors. Thus, our study decided in producing varieties of halogenated xanthone derivatives to investigate their tyrosinase inhibitory effect which can also be beneficial among other researchers in tyrosinase inhibitors studies. For prenylated derivatives, our group managed to discover its anticancer bioactivity which were tested on a breast cancer cells and very lacking studies were done for prenylated derivatives on tyrosinase inhibition study. Therefore, our group took this new approach in discovering the tyrosinase inhibitory effects by prenylated derivatives.

Thus, we are aiming in producing a variety of halogenated xanthones and prenylated xanthone derivatives with different designated functional groups on the xanthone scaffold were developed and synthesized to promote their bioactivity in inhibition of tyrosinase enzyme with their antioxidant characteristics. Later, the toxicity of the

potent compounds will be examined, and their SARs will be clarified in order to understand the mechanisms of xanthone interaction with the mushroom tyrosinase enzyme. (Rosa *et al*., 2021; Zhou *et al*., 2019).

1.5 Objectives of Study

- To synthesize and characterize a series of halogenated and prenylated xanthones via spectroscopic methods such as Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FTIR) and Gas Chromatography Mass Spectrometry (GCMS).
- To evaluate the *in vitro* inhibitory activity on mushroom tyrosinase enzyme and antioxidant activity of the synthesized compounds.
- To determine the binding mechanisms of the potent compounds (bioactive) via enzyme kinetic study analysis supported by *in silico* molecular docking analysis.
- To determine the cytotoxicity properties of the potent compounds on normal keratinocyte skin (HaCaT) cell line, brine shrimp assay and zebrafish embryo toxicity test.

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Mohd Razib, S. et al., 2023 (unpublished data)

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