ANTI-PATHOGENIC YEAST *Malassezia globosa* ACTIVITY BY PROPOLIS OF STINGLESS BEE *Geniotrigona thoracica*

Miss Kawisara Konsila

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biotechnology

FACULTY OF SCIENCE

Chulalongkorn University

Academic Year 2022

ฤทธิ์ยับยั้งยีสต์ก่อโรค *Malassezia globosa* โดยพรอพอลิสของชันโรง *Geniotrigona thoracica*

น.ส.กวิสรา ก้อนศิลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	ANTI-PATHOGENIC YEAST *Malassezia globosa* ACTIVITY BY PROPOLIS OF STINGLESS BEE *Geniotrigona thoracica*
By	Miss Kawisara Konsila
Field of Study	Biotechnology
Thesis Advisor	Professor CHANPEN CHANCHAO, Ph.D.
Thesis Co Advisor	Professor PREECHA PHUWAPRAISIRISAN, Ph.D.
	Associate Professor WANCHAI ASSAVALAPSAKUL, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF SCIENCE

(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor CHATCHAWAN CHAISUEKUL, Ph.D.)

..... Thesis Advisor

(Professor CHANPEN CHANCHAO, Ph.D.)

..... Thesis Co-Advisor

(Professor PREECHA PHUWAPRAISIRISAN, Ph.D.)

..... Thesis Co-Advisor

(Associate Professor WANCHAI ASSAVALAPSAKUL, Ph.D.)

..... Examiner

(CHOMPOONIK KANCHANABANCA, Ph.D.)

..... External Examiner

(Assistant Professor Dumrongkiet Arthan, Ph.D.)

กวิสรา ก้อนศิลา : ฤทธิ์ยับยั้งยีสต์ก่อโรค *Malassezia globosa* โดยพรอพอลิสของชันโรง *Geniotrigona thoracica*. (ANTI-PATHOGENIC YEAST *Malassezia globosa* ACTIVITY BY PROPOLIS OF STINGLESS BEE *Geniotrigona thoracica*) อ.ที่ปรึกษาหลัก : ศ. ดร.จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาร่วม : ศ. ดร.ปรีชา ภูวไพรศิริศาล,รศ. ดร.วันชัย อัศวลาภสกุล

Malassezia globosa เป็นยีสต์ที่สามารถก่อโรคผิวหนังเรื้อรังหลายชนิด ทั้งนี้ยาในกลุ่มเอโซลเป็นยารักษาหลัก แต่มีรายงานถึงผลข้างเคียงและการดื้อยาของเชื้อ ดังนั้นจึงควรมีการค้นคว้าและพัฒนายารักษาชนิดอื่นขึ้นมาเพื่อแก้ไขปัญหาดังกล่าว ซึ่งการประยุกต์ใช้ประโยชน์จากพรอพอลิสเป็นอีกหนึ่งทางเลือกที่น่าสนใจ เนื่องจากพรอพอลิสมีฤทธิ์ยับยั้งจุลชีพที่โดดเด่น ในการศึกษานี้ได้ทดสอบฤทธิ์ยับยั้ง *M. globosa* โดยพรอพอลิสของชันโรง *Geniotrigona thoracica* ด้วยการนำพรอพอลิสมาสกัดแยกองค์ประกอบและนำไปทดสอบฤทธิ์ยับยั้งการเติบโตของ *M. globosa* ด้วยวิธี agar well diffusion ร่วมกับ broth microdilution จากนั้นจึงคำนวณค่าความเข้มข้นที่สามารถยับยั้งเชื้อที่ 50% (Inhibitory Concentration, IC_{50}) ด้วยการวิเคราะห์การถดถอยแบบไม่เชิงเส้นตรง ซึ่งพบว่าสารสกัดหยาบด้วย 80% เมทานอลแสดงโซนยับยั้งเชื้อเริ่มต้นที่ 200 มิลลิกรัมต่อมิลลิลิตร (11.83 ± 0.50 มิลลิเมตร) และมีค่า IC_{50} เท่ากับ 2.21 มิลลิกรัมต่อมิลลิลิตร จากนั้นจึงนำสารสกัดหยาบข้างต้นไปสกัดแยกส่วนด้วยตัวทำละลายอินทรีย์ที่มีขั้วต่างกัน พบว่ามีเพียงสารสกัดส่วนเมทานอลเท่านั้นที่มีฤทธิ์ยับยั้งเชื้อ ซึ่งมีโซนยับยั้งเชื้อเริ่มต้นที่ 12.5 มิลลิกรัมต่อมิลลิลิตร (9.00 ± 0.00 มิลลิเมตร) และมีค่า IC_{50} เท่ากับ 1.22 มิลลิกรัมต่อมิลลิลิตร ต่อมาจึงนำไปทำให้บริสุทธิ์ด้วยการใช้คอลัมน์โครมาโทกราฟี พบว่าสารสกัดแฟรกชันที่ 1 มีฤทธิ์สูงที่สุด เกิดโซนยับยั้งเชื้อเริ่มต้นที่ 12.5 มิลลิกรัมต่อมิลลิลิตร (11.83 ± 0.83 มิลลิเมตร) และมีค่า IC_{50} เท่ากับ 185 ไมโครกรัมต่อมิลลิลิตร และเมื่อวิเคราะห์โครงสร้างทางเคมีของสารในโดยใช้นิวเคลียร์แมกเนติกเรโซแนนซ์ พบว่าสารดังกล่าวคือ เมทิลแกลเลท ต่อมาจึงได้ทดสอบฤทธิ์ของสารสังเคราะห์และสารมาตรฐานเมทิลแกลเลท ซึ่งแสดงค่า IC_{50} เท่ากับ 223.7 และ 59.14 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และจากการทดสอบค่าความเข้มข้นที่ต่ำที่สุดในการฆ่าเชื้อซึ่งใช้สารมาตรฐานเมทิลแกลเลทเพราะสารสกัดมีปริมาณจำกัด พบว่ามีค่าเท่ากับ 8 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้ยังได้ทดสอบฤทธิ์ยับยั้งแอกทิวิตีของเอนไซม์ไลเปสของเมทิลแกลเลทโดยใช้สารมาตรฐานร่วมด้วย เนื่องจาก *M. globosa* ไม่สามารถสังเคราะห์กรดไขมันได้เอง การทำงานของเอนไซม์ไลเปสจึงมีสำคัญต่อการอยู่รอดและการก่อโรค ซึ่งพบว่าฤทธิ์ยับยั้งการเติบโตของเชื้อและเอนไซม์ไลเปสนั้นอาจไม่มีความเกี่ยวข้องกัน เพราะที่ความเข้มข้นเดียวกันฤทธิ์ยับยั้งแอกทิวิตีของเอนไซม์ไลเปส (30.25 ± 1.91%) มีค่าต่ำกว่าฤทธิ์ยับยั้งการเติบโตของเชื้อ (57.35 ± 0.59%) รวมทั้งฤทธิ์ยับยั้งแอกทิวิตีของเอนไซม์ไลเปสยังไม่มีแนวโน้มเพิ่มขึ้นเมื่อเพิ่มความเข้มข้นในการทดสอบ แต่อย่างไรก็ตามยังสามารถช่วยลดโอกาสในการก่อโรคที่ความเข้มข้นของสารต่ำได้ การศึกษานี้เป็นการศึกษาแรกที่รายงานถึงฤทธิ์ยับยั้ง *M. globosa* โดยเมทิลแกลเลทซึ่งได้ค้นหาจากผลิตภัณฑ์ธรรมชาติ แสดงให้เห็นว่าพรอพอลิสจาก *G. thoracica* นั้นเป็นอีกแหล่งธรรมชาติที่น่าสนใจในการนำมาค้นหาสารออกฤทธิ์เพื่อพัฒนายาชนิดใหม่สำหรับต้านเชื้อราก่อโรคต่อไป

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6370104123 : MAJOR BIOTECHNOLOGY

KEYWORD: Antimicrobial drug, Bee product, Skin disease

Kawisara Konsila : ANTI-PATHOGENIC YEAST *Malassezia globosa* ACTIVITY BY PROPOLIS OF STINGLESS BEE *Geniotrigona thoracica*. Advisor: Prof. CHANPEN CHANCHAO, Ph.D. Co-advisor: Prof. PREECHA PHUWAPRAISIRISAN, Ph.D.,Assoc. Prof. WANCHAI ASSAVALAPSAKUL, Ph.D.

Malassezia globosa is a lipophilic pathogen which relates to various chronic skin diseases. Azole drugs are main available treatments, but they have been reported with many side effects and microbial drug resistance. Therefore, the alternative medications should be developed. As the antimicrobial activity of propolis is outstanding, this study aims to investigate the potential of propolis from stingless bee, *Geniotrigona thoracica* against the pathogenic yeast. The extraction and characterization of the propolis components were done after the obtained extracts were evaluated for anti-*M. globosa* growth by using agar well diffusion assay and broth microdilution assay. Moreover, Inhibitory Concentration values at 50% (IC_{50}) was estimated by using nonlinear regression analysis. The sample was firstly extracted with 80% methanol (MeOH), then was partitioned with different polarities of organic solvents. The first obtained crude extracts were crude 80% MeOH extract (CME) which exhibited zone of inhibition beginning at 200 mg/ml (11.83 ± 0.50 mm), and its IC_{50} was estimated at 2.21 mg/ml. After CME was partitioned, only crude MeOH partitioned extract (CMPE) showed the activity. The zone of inhibition from CMPE was observed beginning at 12.5 mg/ml (9.00 ± 0.00 mm) and IC_{50} values was estimated 1.22 mg/ml. Hence, CMPE was selected to further purification by column chromatography. The obtained CMPE 1 presented the best activity with zone of inhibition beginning at 12.5 mg/ml (11.83 ± 0.83 mm) and IC_{50} was predicted at 185.0 μg/ml. CMPE 1 was then identified as methyl gallate (MG) by using nuclear magnetic resonance (NMR). Accordingly, our synthetic MG and standard MG were subsequently tested, which the IC_{50} values were 223.7 μg/ml and 59.14 μg/ml, respectively. The Minimal Fungicidal Concentration of MG was 8 mg/ml as standard MG was used as a representative due to the limitation of the extract. Since extracellular lipase is necessary for survival and pathogenicity, the efficiency of MG toward the lipase was additionally investigated. Both colorimetric and agar-based methods were examined. The results suggested that activity of lipase and growth inhibition of MG might be not related, which percentage of lipase inhibition of standard MG (30.25 ± 1.91%) was lower than the growth inhibition (57.35 ± 0.59%) at the same concentration. Either it did not trend to increase in higher concentrations. However, it still supported the reduction of pathogenicity at low concentration. This is the first report that proves the activity against both growth and lipase of *M. globosa* by MG, which is explored from the natural resource. Thus, propolis from *G. thoracica* could be a good candidate source for searching new anti-*Malassezia* or other antifungal agents.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

Words cannot express my deepest appreciation to my advisor, Professor Dr. Chanpen Chanchao, for generous and valuable guidance, assistance, encouragement, inspiration, and believing my abilities throughout this research.

Furthermore, the completion of my dissertation would not have been possible without the support of laboratory facilities, meaningful advice, and technical assistance from my both co-advisors, Professor Dr. Preecha Phuwapraisirisan and Professor Dr. Wanchai Assavalapsakul.

I would like to extend my gratitude to Associate Professor Dr. Chatchawan Chaisuekul, Dr. Chompoonik Kanchanabanca, and Assistant Professor Dr. Dumrongkiet Arthan for serving as my thesis committees, and contributions of expertise and feedbacks.

Additionally, I am also thankful to the 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund) for financial supports. Many thanks should also go to the administrative staffs from the university for corresponding support in document processes.

I'd like to recognize and admire the effort receiving from Phupha farm. Last but not least, I also wish to specially thank the hardworking bees for providing several useful products.

I also had great pleasure of working with all members of Central Molecular Laboratory, Department of Biology, Center of Excellence in Natural Product, Department of Chemistry, Room 2014 Laboratory, Department of Microbiology and my friends in Program of Biotechnology, Faculty of Science, Chulalongkorn University. I must thank for their meaningful helps and kindness friendships.

Finally, I could not have undertaken this journey without my family members who always provide infinite love, supports, motivation, understanding, and profound belief in my potential.

Kawisara Konsila

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS AND DEFINITIONS

ABS	absorbance
CDPE	crude dichloromethane partitioned extract
CHPE	crude hexane partitioned extract
CH_2Cl_2	dichloromethane or methylene chloride
cm	centimeter
CME	crude 80% methanol extract
CMPE	crude methanol partitioned extract
^{13}C NMR	carbon nuclear magnetic resonance
DI	deionized water
Em wavelength	emission wavelength
EtOAc	ethyl acetate
Ex wavelength	excitation wavelength
FLU	fluorescent intensity
g	gram
h	hour
^{1}H NMR	proton nuclear magnetic resonance
Hz	hertz
IC	Inhibitory Concentration
J	coupling constant
KTZ	ketoconazole
l	liter
Log	logarithm based 10
MeOH	methanol
MeOH-*d*4	deuterated methanol
MFC	Minimal Fungicidal Concentration
MG	methyl gallate
MIC	Minimal Inhibitory Concentration
MLNA	modified Leeming-Notman agar

MLNB	modified Leeming-Notman broth
μm	micrometer
μl	microliter
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
N/A	not applicational or not tested
ND	not determined
nm	nanometer
NMR	nuclear magnetic resonance
$O.D_{600}$	optical density at wavelength 600 nm
p-	para
PBS	phosphate buffered saline
PPL	porcine pancreatic lipase
ppm	part per million
psi	pound per square inch
R^2	correlation coefficient
rpm	revolution per minute
SEM	Standard Error of Mean
Syn. MG	synthetic methyl gallate
Std. MG	standard methyl gallate
TLC	thin layer chromatography
TW60	Tween 60
Type I	ultrapure water
UV	ultraviolet
VIC B	Victoria blue
vol	volume
v/v	volume by volume

x	fold
Å	average pore diameter
δ	chemical shift
℃	degree Celsius
/	per
%	percentage
:	ratio
±	plus or minus
≥	greater than or equal to
≤	less than or equal to
>	greater than
<	less than

Positive control	Experiments that are treated with susceptible commercial drugs.
Negative control	Experiments that are treated with the same as all the others, except an inoculum is excluded or replaced with other solutions including 1x PBS and type I water. In this study, it also refers to the experiment, especially agar well diffusion assay which is treated with solvent alone and does not affect to the tested microorganism growth.
Solvent control	Experiments that are treated with the highest final concentration of solvent which is similar to the highest concentration used to dissolve samples.

CHAPTER I
INTRODUCTION

Skin diseases infected by fungi or yeast are one of the vital issues commonly found worldwide, especially in tropical areas where environments support growth of the microorganisms. Skin diseases not only cause physical illness, but also impact to mental health (Hay et al., 2014; Tuckman, 2017). In Thailand, serious concern about the disease situation has also been reported (Chayakulkeeree & Denning, 2017). *Malassezia* yeast is one of pathogenic microorganisms which recently gain more clinical attention. The yeast is the most abundant in human mycobiome, and it can opportunistically cause ranges of chronic skin conditions including seborrheic dermatitis, atopic, dermatitis, folliculitis, psoriasis, and dandruff. The frequent species found in patients are *Malassezia globosa*, *Malassezia restricta*, *Malassezia sympodialis*, and *Malassezia furfur* (Batra et al., 2005; Cabañes, 2014; Lorch et al., 2018; Seifert, 2013). Azole drugs such as ketoconazole, fluconazole, and itraconazole are recognized as a primary effective treatment. Unfortunately, both short- and long-term usage can lead to many side effects including headache, nausea, irritation, hormone-related effects, and liver toxicity. Moreover, azole resistances in *Malassezia* have also been stated (Angileri et al., 2019; Benitez & Carver, 2019; Leong et al., 2021; Rojas et al., 2014) Therefore, the exploration for alternative remedies should be encouraged, particularly natural resources which provide varieties of bioactive compounds.

This study is interested in the application of the bee products, propolis. Propolis is mainly composed of resin from many plant species which are harvested by worker bees or stingless bees. Normally, they use it for constructing or repairing their hives, along with killing pests and pathogens. It is numerous in biological properties such as antimicrobial activity, anti-inflammatory, antioxidant activity, anti-cancer activity, anti-tumor activity, and wound healing (Abdullah et al., 2020; Campos et al., 2015; Desamero et al., 2019; Dutra et al., 2019; Ibrahim et al., 2016; Jongjitaree et al., 2022; Nolkemper et al., 2010; Sforcin, 2016; Umthong et al., 2011; Xool-Tamayo et al., 2020).

There are many factors affecting bioactivities of propolis such as bee species, plant types, environment, and geography. Despite to honeybee, stingless bee has a longer live colony, lower absconding behavior and its management cost is also lower. Nowadays, stingless beekeeping (meliponiculture) is successfully established in Southeast Asia, including Thailand, for both commercial and agricultural aspects. *Geniotigona thoracica* and *Tetragonula pagdeni* are the most famous species in the management (Rattanawannee & Duangphakdee, 2019)

Antimicrobial activity is the most outstanding activity of propolis, so it was utilized as a constituent for supplements, cosmetics, and medicines (Boukraâ et al., 2013). There are multiple reports of antifungal activity of propolis. It effectively inhibits *Candida*, *Trichosporon*, *Geotrichum*, *Saccharomyces*, *Cryptococcus*, *Rhodotorula*, *Microsporum*, and *Trichophyton* (Batac et al., 2020; Gharib & Taha, 2013; Gur et al., 2020; Oliveira et al., 2006; Rattanawannee & Duangphakdee, 2019; Shehu et al., 2016; Veiga et al., 2018). Nonetheless, the study of the activity against *Malassezia* is still limited. There are mostly reports about inhibitory activity of crude extracts to *M. pachydermatis* isolated from dogs, but the mechanism of inhibition is still unknown. According to the limitation, investigation for the effect of propolis to *Malassezia* should be gathered more consideration to the isolates from human, its main active compound, and mechanisms of inhibition.

Since most of *Malassezia* species cannot synthesize fatty acid, they need to rely on the external fatty acid sources. Hence, activity of extracellular lipase is important for their growth and pathogenicity. The yeasts secret lipase to hydrolyze lipid on human skin, then uptake only saturated fatty acids. Accumulation of the remaining unsaturated fatty acid can cause irritation and scalp which lead to various skin disorders (Park et al., 2021; White et al., 2014; Wijaya et al., 2020). Thus, determination of the inhibitory effect to the extracellular lipase is an interesting target mechanism toward this yeast.

Accordingly, this work is inspired to examine the potential of propolis extract from stingless bee, *G. thoracica* against *M. globosa*. The preliminary study exhibited that crude ethanolic extract of propolis from *G. thoracica* inhibited *M. globosa* growth beginning at concentration 120 mg/ml by using agar well diffusion assay. In addition,

it also revealed inhibitory potential of *M. globosa* lipase activity up to 68% at 200 mg/ml. Besides, the usage of crude extract faces limitation of poor solubility at a high concentration, which can lead to the interference and variance of the results. Also, the main active compound from that study is still unknown. Consequently, this research aims to further investigate the bioactive compound of propolis from *G. thoracica* and explore its mechanism of inhibition. This research is extensively focused on more extraction techniques including partition and chromatography. The received extracts from each extraction step are tested for activity of *M. globosa* growth inhibition by using agar well diffusion and broth microdilution assay. They are also determined for activity of *M. globosa* lipase inhibition by both colorimetric method and agar-based method. The most effective part is purified and then analyzed its chemical structure by spectroscopic technique. Hopefully, a new anti-*M. globosa* compound will be obtained and some of its mechanism will be proved. The obtained bioactive compound can be developed to an alternative therapeutic agent, supplement, and additive of cosmetic and personal care products. Furthermore, it can support stingless bee or beekeeping management by adding values to their products, which may lead to protect the important pollinator.

CHAPTER II
LITERATURE REVIEWS

2.1 Biology of stingless bee

2.1.1 Classification

Apart from honeybee (Apini), stingless bee (Meliponini) is one of the largest and the most diverse groups of eusocial insects in the subfamily Apinae. The classification is shown as following:

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Hymenoptera
Family Apidae
Subfamily Apinae
Tribe Meliponini
Genus *Geniotrigona* Moure, 1961
Species *Geniotrigona thoracica* (Smith, 1857)

Approximately 50 genus and more than 400 species of stingless bees have been identified. It is usually found in warm and humid regions of the world including Central and Southern America, Australia, Africa, and Asia. It has been reported that up to 14 genus and 60 species found in Southeast Asia. In Thailand, at least 32 species have been recorded such as *Geniotrigona thoracica* (Smith, 1857), *Heterotrigona itama* (Cockerell, 1918), *Lepidotrigona terminata* (Smith, 1987), *Lepidotrigona ventralis* (Smith, 1857), *Lophotrigona canifrons* (Smith, 1857), *Tetragonilla collina* (Smith, 1857), *Tetragonula melina* (Gribodo, 1893), *Tetragonula fuscobalteata* (Cameron, 1908), *Tetragonula pagdeni*

(Schwarz, 1939), *Tetrigona apicalis* (Smith, 1857), *Tetrigona peninsularis* (Cockerell, 1927), and *Tetrigona melanoleuca* (Cockerell, 1929) (Chuttong et al., 2014; Hrncir et al., 2016; Integrated Taxonomic Information System, 2022; Lumsa-ed et al., 2019; Rasmussen & Cameron, 2010)

2.1.2 Morphology

The basic morphology of stingless bees is similar to other honeybees, except its smaller size, fewer number of wing's veins, and no or reduced sting. The structure of stingless bees is divided into three parts including head, thorax, and abdomen. The antennae, compound eyes, simple eye, and mandibles are important structures in the head part. The thorax part is attached with two pairs of wings and six legs. In workers, their hind legs are modified to corbicular (pollen basket) which is used for collecting pollen and other materials including plants resin. The reduced or non-functional sting structure locates in the abdomen (Figure 2.1) (Jongjitvimol, 2014; Kwapong et al., 2010)

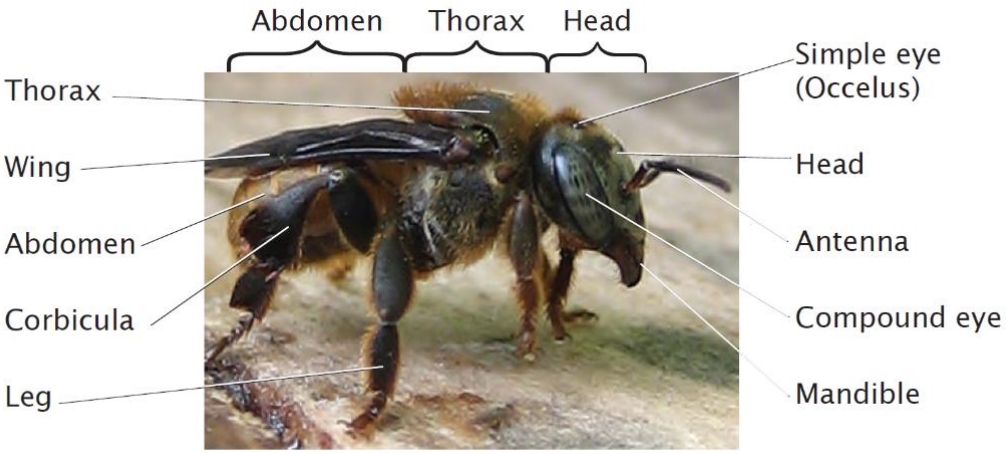


Figure 2.1 Basic morphology of stingless bees (Kwapong et al., 2010).

2.1.3 Nest and colony structures

Stingless bees commonly build their nest in cavities such as stem and branches of living trees, dead logs, and cracked walls. The natural materials are used for nest building such as gums, resins, wax, cerumen (mixture of wax and plant resin), batumen (mixture of resin, mud, and wax) and other chemicals from plant metabolites that are repellent, or antimicrobial. The main structures inside their nest are including [i] hive entrance which particularly comprises with resin. The variety and unique in shape and size are observed in different species, and it can be used to characterize some species. [ii] Brood area which is an important section is enveloped by multiple layers of membranes of cerumen to control an appropriate temperature. [iii] Storage section which is also built by using cerumen is shaped into storage pots for collecting honey, pollen, and bee bread. [iv] Open spaces or left out areas inside the hive are constructed by resins, propolis, and other materials, and [v] nest volume is built by batumen to close the nest apart from the left out or no usage cavity. Like other eusocial honeybees, stingless bees organize a colony with a single queen (large fertile female), several workers (small sterile females), and a few numbers of drones (males). The queen will mate and store sperms from a drone in a different colony in spermatheca. After the eggs are fertilized and laid, they will develop into a queen or worker larvae, whereas the unfertilized eggs will develop into drone larvae. The development of queen or worker larvae depends on the feeding period of royal jelly, which the queen larva has been fed for its entire stage but not the worker larvae. The adult workers will do many activities such as foraging, cleaning, defensing, and larval feeding (Kwapong et al., 2010; Roubik, 2022)

2.1.4 Overview of stingless bee products and managements

The foraging distance for stingless bees is within 2 kilometers from their nests however it is varied in different species. Stingless bees communicate their location through pheromones and sun direction. The workers will gather pollen, nectar, oils, and resins from various plants. Also, they collect water, mud, and

other particles. The products obtained from stingless bees are honey, propolis, wax, and pollen or beebread. Moreover, nucleus colonies may also be stocked or sold to other beekeepers. Examples of commercial stingless bees are *G. thoracica*, *H. itama*, *L. terminata*, and *Tetragonula laeviceps* (Smith, 1857). As stingless bees are less aggressive than honeybees, meliponiculture is easier to manage. In overall, the management cost is lower. Furthermore, stingless bees have low absconding behavior, which they can live in an original colony for many years. From the reasons, meliponiculture trends to successfully establish in many countries including in Southeast Asia. In Thailand, the well-known species, *G. thoracica* and *T. pagdeni* have accomplish raising in artificial hive box for commercial production (Rattanawannee & Duangphakdee, 2019; Rozman et al., 2022).

2.2 Bioactivities of propolis

Propolis is one of bee products originated from plant resin. The workers collect resin from the buds, wounds, fruits, and flowers of many plant species, and then mix them with small amounts of their saliva (enzymes), wax, and pollen. Stingless bees utilize resin in many proposes like honeybees, including building their nest and defensing pathogens, pests, and predators. Not only they use resin to kill enemies, but also they directly apply it on their own bodies to gain the cuticular compounds such as mono-, sesqui-, and triterpenes from the resin. Moreover, resin is also a part for shaping their associating microbial ecosystems. Interestingly, there are studies report that stingless bee propolis is more chemically diverse than honeybee propolis. The differences of physical and chemical properties of propolis depend on many factors such as bee species, harvest seasons, and geographies including plant species and environment. The major groups of chemical constituents found in propolis are flavonoids, phenolic compounds, and terpenes. Some chemicals are only found in propolis which may be caused by the change of chemical structure by the bee enzymes. Hence, the compositions of propolis could be variable in difference species

leading to the broad ranges of its biological properties. Propolis has been used in various aspects including medication since ancient time, because of the efficiency of its antimicrobial, anti-inflammatory, antioxidant, anti-cancer, anti-tumor, and wound healing activities (Athikomkulchai, 2008; Sforcin, 2016; Shanahan & Spivak, 2021).

Accordingly, the bioactivities of the propolis are reviewed and some examples were given as described below.

2.2.1 Antimicrobial activity

Propolis has a strong and wide ranges of antimicrobial activity. It can inhibit both gram-positive and gram-negative bacteria, yeasts, fungi, parasites, and virus as shown in Table 2.1.

Abdullah et al. (2020) showed that ethanolic extract of propolis from the stingless bees *G. thoracica*, *H. itama*, and *Tetrigona binghami* from Brunei exhibited antibacterial activities against two gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) by using disc diffusion assay. The propolis had zone of inhibition depending on the bacterial strain which they approximately ranged from 7-10 mm for *G. thoracica*, 9-13 mm for *H. itama*, and 7-11 mm for *T. binghami*.

Gur et al. (2020) reported that ethanolic extract of Turkish propolis could inhibit 10 bacteria species (*Streptococcus* sp., *P. aeruginosa* DSM 50071, *E. coli* ATCC 25922, *Salmonella typhimurium* NRRL 4463, *S. aureus* ATCC 6538 P, *B. subtilis* ATCC 6033, *Enterobacter aerogenes* CCM 2531, *B. subtilis* subsp. *niger*, *Klebsiella pneumoniae* FMC 5 and *Proteus vulgaris* FMC 11), and two yeast species (*Candida glabrata* ATTC 66032 and *Saccharomyces cerevisiae* UGA 102).

Also, there are many reports showing the activity of propolis against dermatophytes and skin pathogenic yeasts as followed.

Oliveira et al. (2006) proved that alcoholic extract of Brazilian propolis from *Apis mellifera* could inhibit pathogenic yeasts which were isolated from onychomycosis patients including *Candida albicans* (MIC range: 0.0125-0.05 mg/ml), *Candida parapsilosis* (MIC range: 0.0063-0.05 mg/ml), *Candida tropicalis* (MIC range: 0.025-0.05 mg/ml), *Candida lusitanea* (MIC range: 0.0063-0.05 mg/ml), and *Trichosporon* sp. (MIC range: 0.0032-0.0125 mg/ml).

Gharib and Taha (2013) reported that ethanolic extract of Egyptian propolis from *A. mellifera* at concentration 20 mg/ml was efficient to inhibit the pathogenic yeasts. Its best potential was to inhibit *Trichosporon cutaneum* and followed by inhibiting *Cryptococcus neoformans* and *Rhodotorula ruba*, *C. albucans*, and *Geotrichum candidum*, respectively.

Shehu et al. (2016) displayed that aqueous extract of propolis from Malaysian stingless bees, *G. thoracica* had inhibitory activity against *C. albicans* and *Cryptococcus neoformans*. The MICs values obtained from visualization for *C. albicans* and *C. neoformans* were both 1.56 mg/ml, whereas the MICs values obtained from spectrophotometry method were 3.13 mg/ml and 1.56 mg/ml, respectively. They suggested that phenolic and flavonoid compounds such as pinocembrin, morin, rutin, and quercetin were the main compounds of propolis which it might target the pathogens cell wall and cause the cell death.

Veiga et al. (2018) displayed that ethanolic extract of Brazilian green propolis from *A. mellifera* was efficient against *Trichophyton* spp. This propolis was harvested in a native forest with *Baccharis dracunculifolia* and eucalyptus as main plants. It also showed a good result of application in treatment of onychomycosis patients, which completely cured for 56.25% of the patients.

Batac et al. (2020) reported that ethanolic extract of Philippine propolis from stingless bees, *Trigona biroi* exhibited antifungal properties against *Trichophyton rubrum* (MIC at 0.11 g/ml), *Trichophyton mentagrophytes* (MIC at 0.08-0.11 g/ml), and *Microsporum gypseum* (MIC at 0.28 g/ml) which were better than both sunflower and coconut honey.

Moreover, the study of antileishmanial activity of Brazilian propolis from stingless bee *Melipona fasciculata* had been reported. Dutra et al. (2019) revealed that the ethyl acetate fraction of the geopropolis (mixture of resin, soil, slit, and sand particles) was the most active fraction on inhibition of the protozoa, *Leishmania amazonensis* which could cause mucosal and visceral cutaneous infection. They found that gallic and ellagic phenolic acids were the main bioactive compounds which were highly effective on the promastigotes and amastigotes form of *L. amazonensis*. Furthermore, the compounds had a moderate toxicity to mice murine macrophages.

Antiviral activities also have been reported. For example, Nolkemper et al. (2010) showed that the aqueous and ethanolic extract of Czech propolis from *A. mellifera* had high potential for antiviral activity against herpes simplex virus type 2 (HSV-2) at prior to infection. Both aqueous and ethanolic extracts had the inhibitory concentration of 50% (IC_{50}) at 0.0005% and 0.0004% of the extracts, respectively. The main compositions including polyphenols, flavonoids, and phenyl carboxylic acids were identified.

Table 2.1 Summarization of some studies of antimicrobial activities by propolis extracts.

Extract	Pathogen	Inhibitory concentrations[a]	Reference
Ethanolic extract of propolis (*G. thoracica*, *H. Itama* and *T. binghami*)	*B. subtilis and S. aureus*	2 g/l (paper disc)	Abdullah et al. (2020)
Ethanolic extract of Turkish propolis	*Streptococcus sp.*	- [b]	Gur et al. (2020)
	P. aeruginosa		
	E. coli		
	S. typhimurium		
	S. aureus		
	B. subtilis ATCC 6033		
	B. subtilis subsp.		

Ethanolic extract of Turkish propolis (Cont.)	*niger*	- [b]	Gur et al. (2020)
	E. aerogenes		
	K. pneumoniae		
	P. vulgaris		
	C. glabrata		
	S. cerevisiae		
Alcoholic extract of Brazilian propolis (*Apis mellifera*)	*C. albicans*	MIC: 0.0125-0.05 mg/ml	Oliveira et al. (2006)
	C. parapsilosis	MIC: 0.0063-0.05 mg/ml	
	C. lusitanea		
	C. tropicalis	MIC: 0.025-0.05 mg/ml	
	Trichosporon sp.	MIC: 0.0032-0.0125 mg/ml	
Ethanolic extract of Egyptian propolis (*A. mellifera*)	*T. cutaneum*	20 mg/ml	Gharib and Taha (2013)
	C. neoformans		
	R. ruba		
	C. albucans		
	G. candidum		
Aqueous extract of propolis from Malaysian (*G. thoracica*)	*C. albicans*	MIC: 3.13 mg/ml	Shehu et al. (2016)
	C. neoformans	MIC: 1.56 mg/ml	
Ethanolic extract of Brazilian green propolis	*Trichophyton* spp.	- [b]	Veiga et al. (2018)
Ethanolic extract of Philippine propolis (*Trigona biroi*)	*T. rubrum*	MIC: 0.11 g/ml	Batac et al. (2020)
	T. mentagrophytes	MIC: 0.08-0.11 g/ml	
	M. gypseum	MIC: 0.28 g/ml	
Ethyl acetate fraction of Brazilian propolis extract (*M. fasciculata*)	*L. amazonensis*	- [b]	Dutra et al. (2019)
Aqueous extract of Czech propolis (*A. mellifera*)	Herpes simplex virus type 2 (HSV-2)	IC_{50}: 0.0005%	Nolkemper et al. (2010)
Ethanolic extract of Czech propolis (*A. mellifera*)		IC_{50}: 0.0004%,	

[a] Inhibitory concentrations refer to MICs, ICs and concentration exhibiting zone of inhibition.

[b] The symbol "-" indicates the values were not reported.

2.2.2 Anti-inflammatory activity

Inflammation can be activated by many factors like pathogens, damaged cells, irritants, and free radicals. Thus, the anti-inflammatory activity of propolis has also been examined via many key inflammatory mediators and enzyme assay.

Xool-Tamayo et al. (2020) investigated both *in vitro* and *in vivo* anti-inflammatory activity of ethanolic extract of Mayan propolis from Yucamiel propolis paste. They presented that the extract reduced of pro-inflammatory cytokines expression including IL-1β, IL-6, and TNF-α at 145 pg/ml, 350 pg/ml, and 210 pg/ml, respectively. It also increased the anti-inflammatory cytokines including IL-10 and IL-4 at 833 pg/ml and 446 pg/ml, respectively. The higher variability of macrophages was observed at 106% in concentration 50 μg/ml. Moreover, the percentage of swelling inhibition on paw and ear of edema mice was evaluated. They found that propolis presented the inhibition of edemas at short times when using 50 mg/kg dose. In 1-3 h range, the propolis extract exhibited the maximum percentage of inhibition on the paw and ear by 9% and 22% , respectively. The major compound groups of the propolis were phenolic compounds, alcohol, and terpenes.

Campos et al. (2015) determined the activity through the indirect way or hyaluronidase enzyme which degradation of hyaluronic acid by the enzyme could lead to bone loss, inflammation, and pain. They displayed that ethanolic extract of Brazilian propolis from stingless bee, *Tetragonisca fiebrigi* could inhibit the hyaluronidase enzyme in dose-dependent manner which had the percentage of the enzyme inhibition up to 43% at 75 mg/ml. Additionally, the main components found in the propolis were pinocembrin, pinobanksin-3-O-acetate, and pinobanksin-3-O-propionate.

2.2.3 Antioxidant activity

The antioxidant activity can prevent or treat diseases which associate with oxidative stress. The common components in propolis, flavonoids and phenolic compounds, have been reported as the antioxidants that could inhibit lipid oxidation through radical scavenging. For example, the antioxidant activity of methanolic extract of Malaysian propolis from stingless bee species, *Heterotrigona itama* and *Geniotrigona thoracica* were examined. They found that the extract of *H. itama* (IC_{50} at 15.0 ± 0.21 μg/ml) had a higher antioxidant activity than *G. thoracica* (IC_{50} at 270.0 ± 0.19 μg/ml). The extract of *H. itama* also showed more nitric oxide scavenging activity. From phytochemical screening, both extracts presented terpenoids, flavonoids, phenols, and essential oils but steroids, saponin, and coumarins were only found in the extract of *H. itama* (Ibrahim et al., 2016).

2.2.4 Anticancer activity

Cancer is found as a major cause of death worldwide. Since the resistance from the common treatment, chemotherapy has been reported to have side effects. Thus, new anticancer drug development is needed.

Umthong et al. (2011) utilized Thai propolis from the stingless bee, *Tretagonula laeviceps* as a searching resource. They reported that hexane extract of the propolis had an antiproliferative activity against the five tested cancer cell lines including colon (SW620), breast (BT474), hepatic (Hep-G2), lung (Chago), and stomach (Kato-III) tissue cancers in contrast to the normal cell lines (CH-liver cells and HS27 fibroblast cells). Furthermore, more enrichment and purification of propolis extract through column chromatography and size exclusion chromatography could increase the activity. The obtained fraction number 3 of the extract eluted with 30% (v/v) CH_2Cl_2 in hexane showed the highest antiproliferative activity on cancer with IC_{50} value ranging from 4.09-14.7 μg/ml.

Desamero et al. (2019) found that ethanolic extract of Philippine propolis from stingless bee, *Tetragonula biroi* presented tumor suppression in *in vivo* models of differentiated-type gastric cancer model, A4gnt KO mice through regression of macroscopic and histological lesions. There are more than 500 phytochemical constituents which were found in the propolis such as carbohydrates, steroids, alkaloids, anthraquinones, phenols, and terpenoids. Accordingly, 15 compounds (guaiol, tibolone, andrographolide, gallic acid, β-eudesmol, danthron, ginkgolide-B, cinnamic acid, colchicine, protocatechuic acid, ginkgolic acid, rhodoxanthin, pterostilbene, rosmanol, and butylated hydroxytoluene) were suggested as candidates for anti-cancer activity.

2.2.5 Wound healing property

Propolis has been proved for wound healing in *in vitro*, various *in vivo* models, and in clinical trials. For example, Jongjitaree et al. (2022) demonstrated wound healing property of three fractions obtained from ethanolic extract of Thai propolis from *Tetragonula fuscobalteata* [*n*-hexane extract of propolis (HEP), ethyl acetate extract of propolis (EEP), and aqueous extract of propolis (AEP) fractions] through human gingival fibroblast (HGF) proliferation, migration, and *in vitro* wound healing. The minimum concentrations of 3 propolis extracts which enhanced cell proliferation were 15.62, 40, and 400 μg/ml for HEP, EEP, and AEP, respectively. According to the concentration, only two extracts, HEP and EEP, significantly increased cell migration. However, the results from scratch assay showed that all extracts had the efficiency to induce HGF wound healing activity by increasing the percentage of wound closure. Also, the chemical analysis was observed for polyphenols, flavonoids, and triterpenoids in the propolis samples. Moreover, the antimicrobial activity of propolis is the important factors that help reducing the biofilm formation leading to promote further healing processes (Oryan et al., 2018).

2.3 Impacts of skin diseases

Skin diseases can commonly occur to people in all age groups and gender around the world. According to the Global Burden of Disease report in 2010-2013, they were ranked 4th in leading cause of nonfatal disease burden worldwide. Fungal skin diseases were ranked 4th in range of the top 10 most prevalent diseases globally in 2010 which evaluated from 15 categories of skin disease of 187 countries from 1990 to 2010. Moreover, other skin and subcutaneous diseases and acne vulgaris were reported in the range (Hay et al., 2014; Seth et al., 2017). Also, the serious fungal infections in Thailand have been reported. The study from Chayakulkeeree and Denning (2017) presented that about 1.2 million of the population were affected by serious fungal infections, which the estimations of the burden based on size of the non-risk and risk population (such as HIV infection, organ transplantation, chronic pulmonary diseases, and intensive care unit admission) and available epidemiological databases. Also, there are many reports suggesting that the diseases have psychological impact, especially psoriasis, eczema, and skin cancer. The visible symptoms such as psoriasis can lead to depression, anxiety, and suicidality (Tuckman, 2017).

2.4 Biology of *Malassezia* genus

Malassezia species belong to phylum Basidiomycota, subphylum Ustilagomycotina, class Malasseziomycetes, order Malasseziales, family Malasseziaceae, and genus *Malassezia*. According to the report from Lorch et al. (2018), there are 18 described species including *M. arunalokei, M. brasiliensis, M. caprae, M. cuniculi, M. dermatis, M. equina, M. furfur, M. globosa, M. japonica, M. nana, M. obtuse, M. pachydermatis, M. psittaci, M. restricta, M. slooffiae, M. sympodialis, M. vespertilionis*, and *M. yamatoensis*. *Malassezia* species can colonize on both human and animal skin. These yeasts are the most abundant in human mycobiome. They are a part of human normal flora which in circumstance can cause skin disease depending on ages, genders, personal health conditions, and environment. The commensal yeasts associate with many skin disorders such as seborrheic dermatitis, atopic dermatitis, folliculitis, pityriasis versicolor, and psoriasis, whereas their pathogenicity is still unclear.

M. globosa, *M. restricta*, *M. sympodialis*, and *M. furfur* are the most frequent species found in infected human skin, but only *M. globosa* and *M. restricta* are also found in healthy human skin since the beginning of birth. Recently, some species including *M. furfur*, *M. sympodialis*, and *M. pachydermatis* are reported in fungemia or bloodstream infections (Boekhout et al., 2010; Cabañes, 2014; Findley et al., 2013; Rhimi et al., 2020).

Almost of the yeasts are lipid-dependent species, except for *M. pachydermatis*. Since they need to utilize fatty acids from other sources, they are particularly found on the sebum rich of body areas such as trunk, face, and head region. They cannot produce their own fatty acid due to the lack of genes encoding fatty acid synthase. Thus, the secretion of hydrolase enzymes including lipases and phospholipases C are important for survival and pathogenesis roles. The yeasts secret lipases to hydrolyze lipid on human skin resulting in achieving both saturated and unsaturated fatty acids. Only saturated fatty acids are consumed for the yeast growth and survival including cell wall formation. The left out unsaturated fatty acids can trigger inflammation and dandruff formation. For example, [i] oleic acid can irritate and has effects on keratinocytes, and [ii] arachidonic acid can produce proinflammatory eicosanoids which cause inflammation and skin barrier disruption (Figure 2.2). The common species frequent caused dandruff are *M. furfur*, *M. globosa*, and *M. restricta*. Moreover, *M. globosa* also has the highest lipase activity (Park et al., 2021; Sparber & LeibundGut-Landmann, 2017; White et al., 2014; Wijaya et al., 2020). The lipases activity may be considered as one of the main targets to inhibit the yeasts.

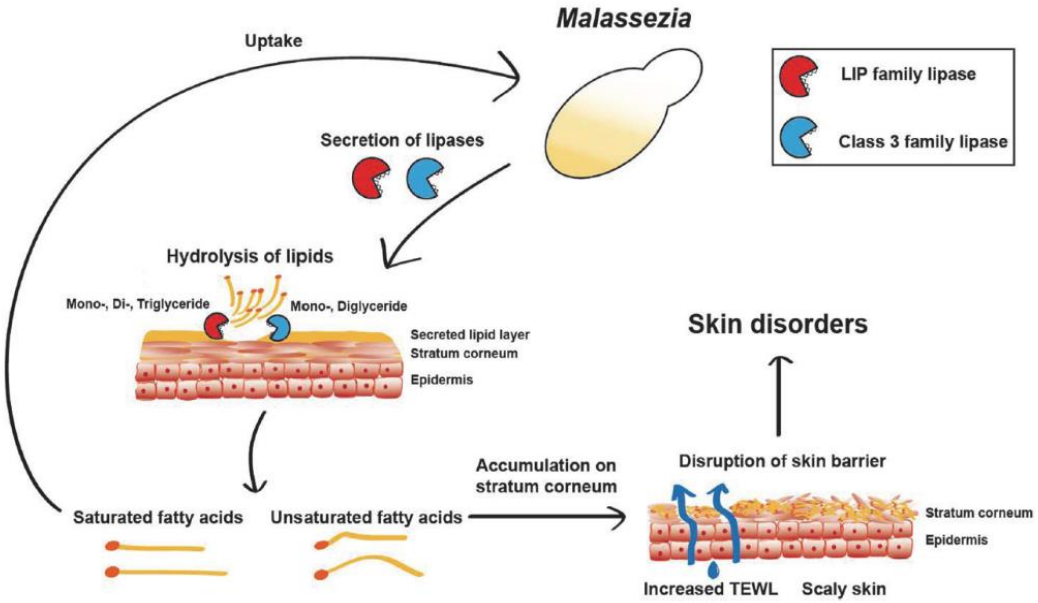


Figure 2.2 The roles of extracellular lipase of *Malassezia* toward its growth and pathogenicity (Park et al., 2021).

2.5 Azole drugs

The common antifungal agents for mycoses treatments are polyene, azoles, pyrimidine analog, echinocandins, and allylamine. The azole drugs have been reported as a potent treatment of *Malassezia* infections. They are divided into two groups which are [i] triazoles including fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole, and [ii] imidazoles including ketoconazole. There are reports suggested that ketoconazole, albaconazole, voriconazole, and itraconazole present the most effective activity (Ashbee, 2007; Ashley & Perfect, 2022, September 14; Pumirat et al., 2013; Rojas et al., 2014). Azoles can disrupt fungal membrane and fungal cell growth through inhibition of C-14 α-demethylase and blocking the demethylation of lanosterol to ergosterol (Makvandi et al., 2021). However, azoles can cause abdominal pain, nausea, vomiting diarrhea, headache, and irritating. The long-term usage can lead to hepatotoxicity and many hormone-relating effects such as gynecomastia, alopecia, hypokalemia, hyponatremia, and adrenal insufficiency. Also, neuropathies, pancreatitis, phototoxic reactions, and skin cancer have been reported in some azoles (Benitez &

Carver, 2019; Lee & Goldman, 1997). Nonetheless, the multi-azole resistance in *Malassezia* yeast has occurred in *M. dermatitis*, especially itraconazole and ketoconazole (Angileri et al., 2019).

2.6 Methods for investigating susceptibility of microorganisms

There are many methods for *in vitro* investigation of antimicrobial activity which present different strengths and weaknesses. Hence, two kinds of methods are used in this study to achieve the reliable results, including agar diffusion and broth dilution described as following.

2.6.1 Agar well diffusion assay

The diffusion assays are qualitative method which is performed on agar plate. The assays are widely used because they provide simplicity, flexibility, easy interpretation, and low cost with no need of any special equipment. They are divided to many types such as agar disk-diffusion, antimicrobial gradient (Etest), agar well diffusion, and cross streak method. The agar well diffusion is performed in this study. Briefly, [i] the culture suspensions are inoculated and spread entirely on agar medium, [ii] then, the holes are punched with sterile cork borer or tips, [ii] and the test samples are added into each hole. After incubation, diffusion of the test samples will occur. The test samples which show antimicrobial activity will inhibit growth of the microbe and the zone with no colony growth (zone of inhibition) will be observed and measured. Nonetheless, these methods have some disadvantages including lacking automation of the test and the results may be not accurate in some condition due to the slow growing bacteria (Balouiri et al., 2016; Reller et al., 2009).

2.6.2 Broth microdilution

Broth microdilution methods are commonly used because of its rapid, reproducibility, convenience, automate, and high throughput detections. Furthermore, it requires only few amounts of test sample. It is a quantitative

method which can determine inhibition values including Minimal Inhibitory Concentration (MIC), and Minimal Bactericidal or Fungicidal Concentration (MBC or MFC) which is the lowest concentration that kills 98-99.9% of the tested microorganism. Briefly, this method is performed in 96-well plate at suitable volume (approximate 0.1-0.2 ml). A test sample in various conditions is diluted with culture media by fold dilution method and then they are added into each well. After that, each well of the plate is filled with appropriate microbial suspensions, followed by incubation step. Absorbance or optical density is measured to further interpret the results. Moreover, colorimetric methods based on dye reagents as indicator of growth detection have been developed, such as resazurin or Alamar blue dye. Resazurin dye is an oxidation-reduction indicator. It is normally a blue dye which has non-fluorescent and non-toxic properties. It can detect the viable cells by the activity of oxidoreductases of the cell which reduce the blue dye to resorufin or pink and fluorescent dye. The results from resazurin assay can be visually observed or further calculated from relative fluorescent intensity measured by spectrophotometry. Thus, the standardized or appropriate conditions for tested microorganism should be considered in broth microdilution assay (Balouiri et al., 2016; Sarker et al., 2007).

2.6.3 Inhibitory Concentration (IC)

Apart from MICs, ICs values can be estimated inhibitory concentration at the target response percentage when the response depends on dose. IC_{50} is normally reported when a concentration of the response reaches 50%. The models of dose response used to estimate the ICs values can be both linear and nonlinear models. Since the antimicrobial activity values have random variation, the selected model should match their nature of the response which can illustrate their dose-response pattern. Thus, nonlinear regression analysis has been used to estimate ICs values of antimicrobial agents, including four-parameter logistic model with variable slope, rather than linear regression

analysis (King & Krogstad, 1983; Lyles et al., 2008). A sigmoidal curve (Figure 2.3) with variable slope (hill slope) and constant difference (Top - Bottom = 100) of 100 between the top (percentage of response at high concentrations) and the bottom (percentage of response when inhibitor is absent) is fitted to the formular as following (GraphPad Software, 2022; Rautenbach et al., 2006):

$$Y = \text{Bottom} + \left[\frac{\text{Top}-\text{Bottom}}{1 + 10^{[(\text{LogIC50}-x)*\text{HillSlope}]}}\right]$$

There are many software packages for the analysis including Graphpad Prism.

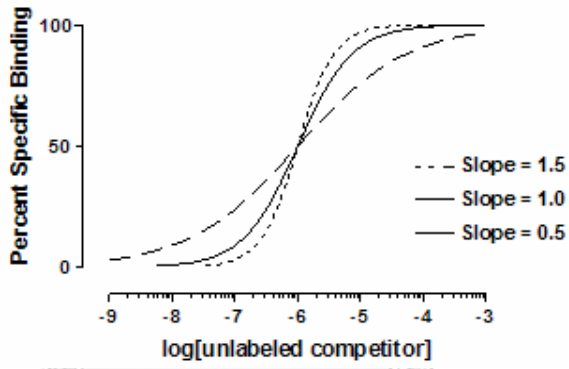


Figure 2.3 Example of sigmoidal dose-response with variable slope curve (GraphPad Software, 2022).

2.7 Methods for investigating activity of lipase

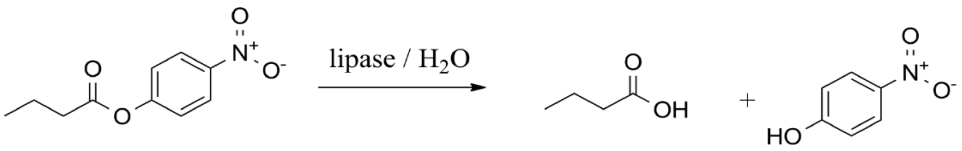
The activity of lipase can be examined directly from microorganisms and their isolated enzyme. There are many methods for detecting lipophilic activity of microorganism and measuring the activity of lipase in both forms of crude and purified enzymes.

The detection for lipophilic activity is normally based on solid media which can be categorized to at least two methods including methods based on the changes in appearance of lipase substrates, and indicator dyes. For lipase substrates such as Tweens, tributyrin or triolein can be used for the screening by in cooperating with

diffusion assay. After incubation, lipase activity is observed by clear zones, turbid zones, or opaque halos zones. Moreover, the addition of indicator dyes can enhance more visualization of lipolytic activity. The dyes such as Victoria blue B, Spirit blue, Nile blue sulfate, and night blue are supplemented with substrates for the detection. For example, the presence of Victoria blue B can present the darker transparent zone against an opaque background, while, in some case studies, hazy zone can be observed (Samad et al., 1989; Thomson et al., 1999).

To access the activity of crude or purified lipases, several methods such as titrimetric methods, colorimetric methods, fluorometric methods, turbidimetric methods, chromatographic methods, and immunoassay can be used. In this study, colorimetric methods are selected and performed because of the speed, sensitivity, inexpensive cost, and convenience of assays. The synthetic substrates, particularly *p*-nitrophenyl derivatives of fatty acids, are frequently used which lipases can hydrolyze the substrate and then releases *p*-nitrophenol. The product, *p*-nitrophenol (anionic form, *p*-nitrophenolate), can be noticed as yellowish coloration and can be measured spectrophotometrically in the range of 405-410 nm in an alkaline buffer (Figure 2.4) (Pohanka, 2019; Stoytcheva et al., 2012; Thomson et al., 1999).

Enzymatic hydrolysis:



The form of *p*-nitrophenol in alkaline condition:

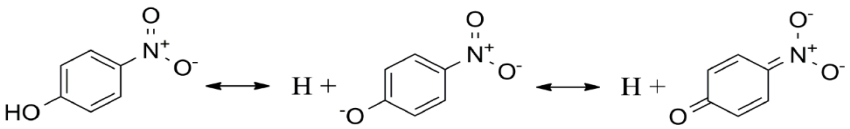


Figure 2.4 The hydrolysis steps of *p*-nitrophenyl substrate by lipase and the formation of its product, *p*-nitrophenol in alkaline condition.

2.8 Chromatography techniques

Chromatography is a qualitative and quantitative analysis. This technique is widely applied in multiple fields such as pharmaceutical science, environmental science, police work, forensic science, archeology, biomedical research, and food science. It is an important technique using for separation, identification, and purification of the components in mixtures. The principle of this technique is based on [i] stationary phase which is a solid phase or liquid phase coated on solid surface, [ii] mobile phase which is liquid phase or gaseous components, and [iii] separated molecules. After the mixtures are applied onto stationary phase, the molecules components will be separated from each other by the interaction with mobile phase. There are many factors affecting the separation process such as molecular characteristics, polarity, molecular weights of molecules and types of mobile phase, and stationary phase. Chromatography is divided into many types including column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography (Coskun, 2016; Kuiper et al., 2019).

2.9 Spectroscopy techniques

Spectroscopy refers to the observation of interaction between matter or particles and electromagnetic radiation as a function of wavelength. It is measured by radiant energy absorbed or emitted from the test samples. There are many types of spectroscopies such as optical spectroscopy [ultraviolet (UV), visible, and infrared (IR)], Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectroscopy, Raman spectroscopy, and fluorescence spectroscopy.

NMR spectroscopy is an absorption spectroscopy which can determine the structure of compounds by analysis of magnetic properties from the certain atomic nuclei to distinguish the difference of local electron environment of hydrogen, carbon, or others in the compounds. There are multiple types of nuclei for NMR detection including ^{1}H (proton), ^{13}C (carbon 13), ^{15}N (nitrogen 15), ^{19}F (fluorine 19) which ^{1}H and ^{13}C are commonly used. Since nuclei have spins and electrical charges, the absorption and

transferring of radio frequency energy can be occurred when applying external magnetic field. The nuclei can have resonance by transferring nuclear energy from ground state to higher state levels followed by turning back to the ground state. Later, the energy is emitted, and it can be measured as a frequency signal by detector. Chemical shift (δ, units of parts-per-million or ppm) is the frequency shift of the nuclei that is shielded by neighboring molecules which are measured by the different frequency of a sample signal relative to a signal of standard compound (BYJU'S, n.d.; LibreTexts libraries, 2022; Weebly, 2009).

CHAPTER III
MATERIALS AND METHODS

3.1 Sample collection

Raw propolis of stingless bee, *Geniotrigona thoracica*, was collected from a local stingless bee farm, Phupha Farm, at Amphawa district, Samut Songkhram province, Thailand in January 2019. Character and weight were recorded. It was stored at -20 ℃ with light protection by covering with aluminum foil until used. In addition, plants in surrounding area were recorded.

3.2 Crude extraction and partition

This extraction method was modified from Yang et al. (2011) and Boonsai et al. (2014). The raw propolis (145-150 g) was cut into small pieces, approximately 2 cm x 2 cm. Then, it was extracted with 80% (v/v) methanol (MeOH) at ratio 1:6 (g/ml) and was incubated at 25 ℃ for 20 h in the dark and shaking condition at 100 rpm (SI-23MC, Bioer Technology, China). After that, the soluble part was separated and centrifuged at 7,000 rpm, 4 ℃ for 15 min (Sorvall RC 6 Plus Centrifuge, Thermo Scientific, USA), and the supernatant was collected. The remaining solid was re-extracted twice with the same process described. All supernatants were combined and evaporated at 40-45 ℃ under reduced pressure (rotary evaporator, Heidolph, Germany). Finally, crude 80% MeOH extract of *G. thoracica* propolis (CME) was obtained and stored in the dark at -20 ℃ for further usage.

The CME was partitioned by three organic solvents, namely hexane, dichloromethane (CH_2Cl_2), and MeOH (low to high polarity, respectively). Firstly, the CME was dissolved in MeOH, and then hexane was added with the same volume of the MeOH. The mixture was mixed and was poured into a separating funnel. The upper phase or hexane part was collected after the two phases were separated. The lower phase or MeOH part was re-partitioned again with hexane at least 2 times as previously described. All received hexane parts were pooled and evaporated at 40-45 ℃ under reduced pressure to obtain crude hexane partitioned extract (CHPE). The remaining

MeOH part was further partitioned with equal volume of CH_2Cl_2 with the same manner. According to the step, CH_2Cl_2 part was obtained from the lower phase while MeOH part was obtained from the upper phase. After each part was separately pooled and evaporated, crude CH_2Cl_2 partitioned extract (CDPE) and crude MeOH partitioned extract (CMPE) were received. Character and weight of all partitioned crudes were recorded. They were stored in the dark at -20 ℃ for further usage.

The CME, CHPE, CDPE, and CMPE were tested for anti-*Malassezia globosa* activity as assays described in sections 3.5, 3.9 and 3.11.

3.3 Tested microorganism and culture conditions

The selected microorganism in this study was a skin pathogenic yeast *Malassezia globosa*, Guého & Guillot (1996) purchased from American Type Culture Collection (MYA-4889, ATCC), USA. All experiments with the yeast were performed under biosafety level 2 guidelines. Culture condition in this study was performed by following ATCC recommendation. The yeast was grown in modified Leeming-Notman broth (MLNB) or modified Leeming-Notman agar (MLNA) Culture stock was stored in MLNB mixed with 18% glycerol at -80 ℃.

The *M. globosa* stock was streaked on MLNA and was incubated at 30 ± 2 ℃ for 7-10 days (Incucell incubator, MMM Medcenter, Germany). After that, a single colony (1-2 mm) was picked. It was then transferred to MLNB (20 ml) and was also taken to observe microscopic feature under light microscope. The culture was incubated at 30 ℃, 200 rpm (New Brunswick Innova 44 incubator Shaker, Eppendorf, Germany) for 7 days before used. The working culture was used within three subcultures or five passages from ATCC reference culture, which was recommended by ATCC (Figure 3.1).

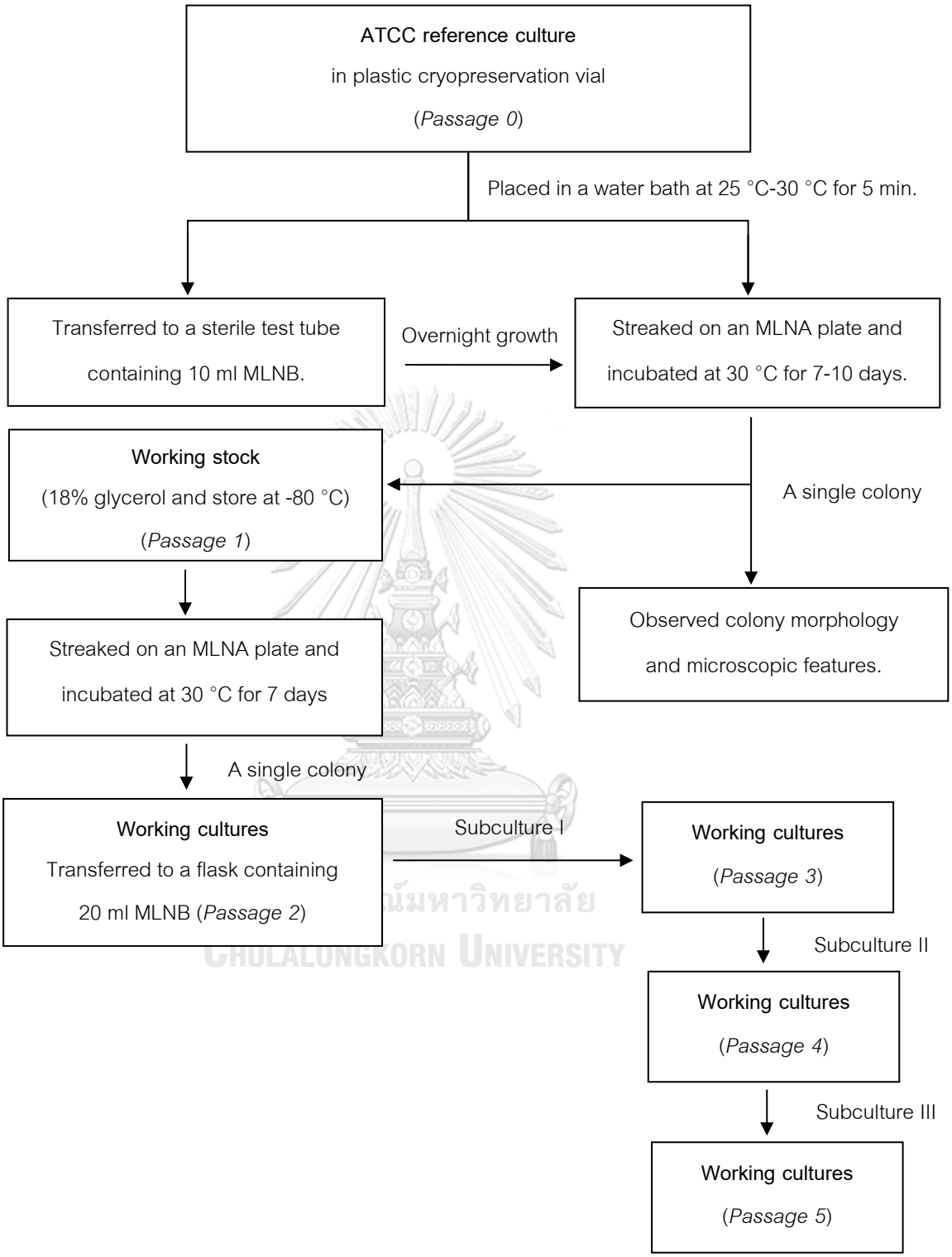


Figure 3.1 Culture maintaining system in this study (demonstrated as one replicate of each working stock and working culture).

3.4 Preparation of yeast cell suspension

After reaching 7 days of incubation, the yeast cells were harvested by transferring 1 ml of working culture into a microcentrifuge tube and centrifugation at 12,000 rpm, room temperature for 5 min. The yeast cells were collected and washed twice by resuspending with sterile phosphate buffered saline (1x PBS, pH 7.2-7.4). The optical density at 600 nm ($O.D._{600}$) was measured and adjusted to be approximately 0.1, 0.5, and 1 using spectrophotometer (Thermo Scientific, USA) as PBS alone was considered as blank. Yeast cell suspensions from each O.D were strained at ratio 1:1 with 0.01% (w/v) methylene blue solution [methylene blue in 2% (w/v) sodium citrate solution] and were counted using hemocytometer (BOECO, Germany) under light microscope (Figure 3.2). A graph representing the relationship between $O.D._{600}$ (X-axis) and cell number (Y-axis) was conducted using Microsoft excel 365. The obtained formular was used to calibrate the yeast density (cell/ml) for further experiments.

After staining with methylene blue, the dead yeasts were stained with blue color. Therefore, only the alive yeast cells without the staining were counted. Yeast cell density (cells/ml) was calculated as the following formula:

$$\text{Yeast cell density (cells/ml)} = \frac{(A/B)}{C} \times D$$

where: A is identified as total number of alive yeast cells.

B is identified as number of hemocytometer squares.

C is identified as volume of a hemocytometer square.

D is identified as dilution factor (optional).

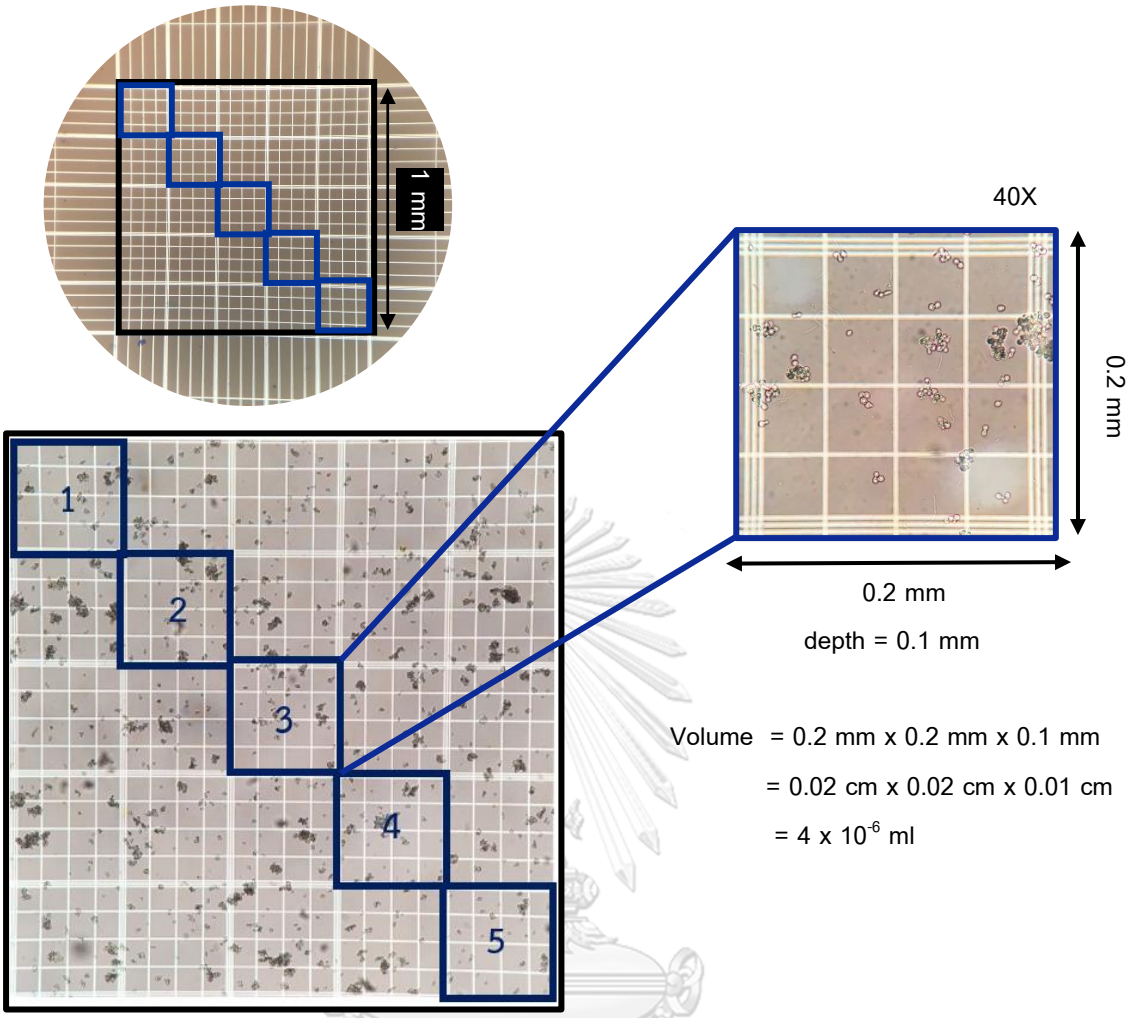


Figure 3.2 A representative of yeast cell counting pattern using hemocytometer.

3.5 Primary screening of antimicrobial activity against *M. globosa*

The obtained propolis extracts were primarily tested for inhibitory activity of *M. globosa* growth by agar well diffusion assay modified from Hendi et al. (2011). Yeast cell suspension at 2 $O.D._{600}$ (approximate density at 4×10^6 cells/ml) was prepared. Then, 100 μl of the suspension (to final density 4×10^5 cells/ml) was dropped and swabbed by sterile cotton swab on a culture plate containing 20 ml of MLNA. After the surface of inoculated plate was dried, 4-5 wells were made by using sterile cork borer (diameter 8 mm).

The tested extracts were prepared at various concentrations by two or four-fold dilution method using dimethyl sulfoxide (DMSO) as a solvent. Then, 100 μl of the

prepared extracts were added in to each well. A commercial drug, Ketoconazole (KTZ) at 2 or 4 μg/ml was used as a positive control. Besides, DMSO alone was used as negative control. Tested plates were incubated at 30 ± 2 ℃ for 7-14 days. The area around each well which shown no growth was considered as a zone of inhibition. The diameter of zone of inhibition was measured in mm. The results were reported as mean ± Standard Error of Mean (mean ± SEM) and percentage of growth inhibition compared to KTZ was calculated (Khalid et al., 2017) as the following formula:

$$\text{Percentage of growth inhibition (\%)} = \left(\frac{A}{B}\right) \times 100$$

where: A is defined as zone of inhibition of tested sample (mm).

B is defined as zone of inhibition of standard drug, KTZ (mm).

3.6 Isolation and purification of bioactive compound

The crude partitioned extract, that displayed the best antimicrobial activity after screening in section 3.5, was selected to enrich and purify by chromatography techniques.

3.6.1 Silica gel 60 Å column chromatography

The selected active crude partitioned extract was firstly optimized for suitable solvent systems to elute a column by using thin layer chromatography or TLC method (section 3.6.2).

The column chromatography method was modified from Khongkarat et al. (2020). A glass column at volume 500 ml (NK Laboratory, Thailand) was packed with 150 g of silica gel 60 Å (particle size 0.063–0.200 mm for column chromatography) (Merck, Germany) by using CH_2Cl_2 as a solvent. The selected crude partitioned extract (4.64 g) was dissolved in 1:1 (v/v) MeOH : CH_2Cl_2 until it was not sticky. Then, it was mixed with silica gel 60 Å and was evaporated until dry. The prepared extract was placed over the packed silica gel 60 Å column, followed by silica gel 60 Å and cotton wools. The packed column was eluted by

[i] 2 l of 1:19 (v/v) MeOH : CH_2Cl_2, [ii] 1 l of 1:4 (v/v) MeOH : CH_2Cl_2, [iii] 2 l of 1:1 (v/v) MeOH : CH_2Cl_2 and [iv] 2 l of MeOH, respectively. The eluted fractions from the solvents [i] and [ii] were collected 10 ml/fraction each and eluted fractions from the solvent [iii] and [iv] were collected 200 ml/fraction each. All obtained fractions were evaporated at 40-45 ℃ under reduced pressure. Then, each fraction was checked for chemical profile by TLC (section 3.6.2). The fractions which shown the same chemical profile were combined and stored in the dark at -20 ℃ for further usage.

The received fractions were tested for anti-*M. globosa* activity as assays described in sections 3.5, 3.9, and 3.11.

3.6.2 Thin layer chromatography (TLC)

TLC Silica gel 60 F_{254} plates (Merck, Germany) size 2 cm x 5 cm were prepared. The tested extracts were dissolved with appropriate solvent such as MeOH, 1:1 (v/v) MeOH : CH_2Cl_2, and ethyl acetate (EtOAc). Then, they were spotted by a capillary tube on a TLC plate at the starting position 0.5 cm above the bottom edge while a solvent front line was set at 0.5 cm lower from the upper edge. After the sample spots were air dried, the TLC plate was transferred into a glass chamber containing a suitable solvent such as [i] MeOH, [ii] CH_2Cl_2, [iii] EtOAc, [iv] MeOH : CH_2Cl_2 at 1:19, 1:9, 1:4, 1:1 (v/v), and [v] MeOH : EtOAc at 1:19, 1:9, 1:4, 1:1 (v/v). The chamber lid was closed until the solvent reached solvent front line. Later, the TLC plate was removed and air dried. Results of chemical profiles were conducted by visualization the TLC plate [i] using ultraviolet light (UV) at wavelength 254 nm and/or [ii] using anisaldehyde dripping followed by heating on a hot plate.

3.7 Chemical structure analysis

The most active enrich or pure fraction was sent to analysis service at Department of Chemistry, Faculty of Science, Chulalongkorn University for chemical structure analysis by Nuclear Magnetic Resonance (NMR). Briefly, the sample (approximately 5-20 mg)

was completely dissolved in MeOH-*d*4 (Eurisotop, Cambridge Isotope Laboratories, Inc., USA) and was transferred to an NMR tube. Then, the sample was operated for ^{1}H at 500 MHz and ^{13}C at 125 MHz by using Jeol JNM-ECZ500R NMR spectrometer. The NMR spectra, chemical shifts in δ (ppm), and *J* coupling values (Hz) were reported and compared to reference reports.

3.8 Chemical synthesis

Synthesis method of methyl gallate (MG) modified from Maximo da Silva et al. (2015) was conducted. The gallic acid (GA, MW 170.12) provided from Department of Chemistry, Faculty of Science, Chulalongkorn University at 1.015 g was completely dissolved in MeOH anhydrous (10 ml). Later, concentrated sulfuric acid (1 ml) and molecular sieves, 4 Å 1.6-2.6 mm (Sigma-Aldrich, USA) were added. The mixture was heated at reflux condition for 5-10 h. TLC analysis was done (section 3.6.2) along and at the end of reaction by using 1:9 (v/v) MeOH : CH_2Cl_2 as a solvent. The selected active fraction, and GA were used for comparing TLC pattern. The obtained product was cooled down and evaporated. Then, EtOAc or MeOH was added, and the product solution was filtered through filter paper. After that, water was added to remove acidity in the solution. The product solution was then mixed with sodium sulfate anhydrous (Supelco, Merck, Germany) to separate the water. The lower part of the mixture containing water was removed, and the remaining was evaporated at 40-45 ℃ under reduced pressure. The obtained compound was further purified by Silica gel 60 Å column chromatography as previously described in section 3.6.1, except for the mobile phases were changed to 2:23 (v/v) MeOH : CH_2Cl_2. Also, TLC analysis was performed along as mentioned above.

The synthetic MG was tested for antimicrobial activity against *M. globosa* as assays described in sections 3.5 and 3.9.

3.9 Broth microbroth dilution assay

The extracts which shown the best antimicrobial activity from primary screening were selected and further examined for Minimal Inhibitory Concentrations (MIC) and Minimal Fungicidal Concentrations (MFC) by method modified from Far et al. (2018) and Gucwa et al. (2018). MIC values were evaluated by using broth microdilution method in 96-well plate at final volume 200 μl. Tested extract stock was prepared at concentration 10x of the primary screening by dissolving in DMSO. Then, they were diluted in microcentrifuge tube to various concentrations (2x of final concentrations) by two or four-fold dilution method using 2x MLNB or 5% (v/v) Tween 60 in 2x MLNB as a diluent. The prepared test extract at any concentration (100 μl) was added into a well of 96-well plate. Later, 100 μl of yeast cell suspensions at 0.2 $O.D._{600}$ (approximately density at 5 x 10^5 cells/ml) were added in each well (to final density at 1-2 x 10^5 cells/ml) and were mixed gently with the extracts. Besides, sterile culture medium (2x MLNB) supplemented with extract without inoculation (replaced by 1x PBS) was considered as control or background for each concentration. Sterile 2x MLNB mixed with appropriate solvent with and without inoculation were used as solvent control and background of the control, respectively. Positive control or KTZ at concentrations 0.0025-0.8 mg/ml (in the same appropriate solvent as tested extracts) were performed along. The 96-well plates were incubated at 30 ℃, 80 rpm (LSI-3016R, LabTech, Thailand). After 12 h of incubation, 0.02% (v/w) resazurin at 30 μl was added to each well and were gently mixed. Then, the plates were incubated again at the same condition for further 36 h (total 48 h). Finally, the plates were carried out and measured fluorescent intensity for each well by using microplate reader (EnSight Multimode Plate Reader, PerkinElmer, USA) which was operated at Ex wavelength of 540 nm and Em wavelength of 590 nm. The percentage of growth inhibition was calculated as a formula below:

$$\text{Percentage of growth inhibition (\%)} = \left(\frac{[\text{FLU(A}-\text{a)}]-[\text{FLU (B}-\text{b)}]}{\text{FLU(A}-\text{a)}} \right) \times 100$$

where: A is defined as fluorescent intensity of solvent control.

B is defined as fluorescent intensity of tested treatment.

a is defined as fluorescent intensity of negative control of solvent control.

b is defined as fluorescent intensity of negative control of tested treatment.

The results of MIC50 and MIC90 were defined by the lowest concentration of tested extracts which had percentage of growth inhibition reached 50% and 90% in the experiment.

The aliquots (20 μl) from each treatment well which displayed percentage of inhibition at least 80% were transferred and dropped on MLNA plate. The culture plates were incubated at 30 ± 2 ℃ for 5-10 days. The lowest concentration which appeared no growth of colony was determined as MFC.

3.10 Estimation of Inhibition Concentration (IC)

Inhibition concentration (IC) at 50% and/or 90% (IC_{50}, IC_{90}) were calculated by using a non-linear regression analysis. A graph between percentage of inhibition (Y-axis) and log concentrations (X-axis) of the tested extracts was plotted, and ICs values and correlation coefficient (R^2) were analyzed by using GraphPad Prism 8.

3.11 Inhibition of *M. globosa* lipase activity

3.11.1 Assay of lipase activity by spectrophotometric method

A quantitative assay for inhibition of lipase activity was conducted by using the method modified from Sivasankar et al. (2017) and Honnavar et al. (2018). The reaction was composed of 1:9 (v/v) of crude lipase : reaction mixture. Crude lipase from *M. globosa* was prepared from the MLNB culture (5 ml) which was inoculated with yeast cell suspensions at 2 $O.D._{600}$ (approximately 4 x 10^6 cells/ml) and was cultured at 30 ℃, 200 rpm for 7 days. The inoculated culture was centrifuged at 4 ℃, 6,000 rpm for 10 min (centrifuge 5804R, Eppendorf, Germany).

Then, the supernatant was recovered and filtered through 0.22 μm polyvinylidene fluoride (PVDF) syringe membrane filter. From previous steps, cell free culture supernatant (CFCS) or crude lipase was obtained, and it was kept on ice until used. The reaction mixture was prepared comprising of [i] 1x vol. of substrate solution containing 0.3% (w/v) *p*-nitrophenyl palmitate (*p*NPP) in propanol and [ii] 9x vol. of reaction buffer [0.2% (w/v) sodium desoxycholate and 0.1% (w/v) gummi arabicum in 50 mM Na_2PO_4 buffer (pH 8.0)]. Tested extract stocks were prepared in DMSO and were then diluted at various concentrations (2x of the final concentration) by using the reaction buffer or reaction buffer supplemented with an appropriate ratio of Tween 60 as a diluent.

The assay was performed in 1.5 ml microcentrifuge tubes in total volume of 100 μl. Firstly, 81 μl of the reaction buffers supplemented with the tested extracts were prewarmed at 30 ℃, 80 rpm for 30 min and they were then mixed with 10 μl of the crude lipase. The mixtures were incubated at 30 ℃, 80 rpm for another 30 min. After that, the substrate solution at 9 μl was added and incubated at 30 ℃, 80 rpm for 2 h. Later, 200 μl (2x vol.) of 1 M Tris-HCl (pH 8.0) was added for stabilizing the pH-dependent *p*NP (*p*-Nitrophenol). The mixtures were centrifuged at 4 ℃, 12,000 rpm for 5 min. The supernatants at 200 μl were collected and transferred to 96 well plate. The plate was taken to measure absorbance at 410 nm by using microplate reader. Besides, the reaction containing reaction mixture supplemented with each extract concentration without crude lipase (replaced by sterile Type I water) was considered as a negative control or background. The reaction containing reaction mixture mixed with an appropriate solvent with and without crude lipase were used as solvent control and background of the control, respectively. Also, the reaction with porcine pancreatic lipase (final concentration 0.02 mg/ml) was performed along to indicate an availability of the assay. Percentage of lipase inhibition was calculated by the following formular:

$$\text{Percentage of lipase inhibition (\%)} = \left(\frac{[\text{ABS}(\text{A}-\text{a})]-[\text{ABS}\ (\text{B}-\text{b})]}{\text{ABS}(\text{A}-\text{a})}\right) \times 100$$

where: A is defined as absorbance of solvent control.

B is defined as absorbance of tested treatment.

a is defined as absorbance of negative control of solvent control.

b is defined as absorbance of negative control of tested treatment.

3.11.2 Detection of extracellular lipase by plate assay method

A qualitative assay for lipase detection was performed to ensure lipophilic activity of the yeast by using modified methods from Samad et al. (1989) and Cania et al. (2020). Agar medium plates supplemented with indicator dye (TW60-Vic B agar) were prepared composing of 2.5% agar, 2% Tween 60 (lipase substrate), and 0.01% Vitoria Blue B (indicator). Additionally, agar medium without indicator dye (TW60 agar) were prepared by using Ramnath et al. (2017) method. Later, they were punched a well by using sterile cork borer with diameter 4 mm. Yeast cell suspension at 2 $O.D._{600}$ (approximate density at 4 x 10^6 cells/ml) was reduced volume from 1 ml to 100 μl to obtain suspension at density at 10^7 cells/ml. Then, 50 μl of the suspension (approximate final density at 10^6 cells/ml) was transferred to each well of the agar mediums. The agar medium plates were incubated at 30 ℃, 1-2 days for TW60-Vic B agar and 10-15 days for TW60 agar. The result of hazy or darker clearance zone was visualized in TW60-Vic B agar, while zone of calcium precipitation was observed in TW60 agar.

In addition, the most active fraction after enrichment and purification was also tested in this assay to confirm the results of section 3.11.1. The experiment was performed in 1.5 ml microcentrifuge tube at total volume 1 ml. Briefly, the tested fraction stock was prepared in DMSO (at 100x of final concentration) and was diluted in 2x MLNB to 2x of final concentration. Yeast cell suspension at density at 10^7 cells/ml was prepared as mentioned above. Then, 1:1 (v/v) of 2x MLNB supplemented with tested fraction : yeast cell

suspension was mixed and incubated at 30 ℃, 200 rpm for 24 h and 48 h. After reached each time point, the samples were washed twice with PBS by centrifugation at 12,000 rpm, room temperature for 5 min. The suspensions (100 μl of the sample in total 1 ml 1x PBS) were measured $O.D_{600}$. Then, they were adjusted density and added in each well of TW60-Vic B agar at final density of approximate 10^6 cells/ml as previously described. The agar medium plates were incubated at 30 ℃, for 1-2 days. The results of hazy or darker clearance zone were visualized and scored.

3.12 Data analysis

Each experiment was done in triplicates and was separately repeated at least two times. The results were reported as mean ± Standard Error of Mean (mean ± SEM) determined by using Microsoft excel 365. Regression analysis was analyzed by using Microsoft excel 365 or GraphPad Prism 8. Statistical significance of the data was calculated by one-way ANOVA using IBM SPSS version 28, in which p value ≤ 0.05 was accepted as a significant difference. Also, evaluations of normality test and homogeneity of variance test were considered.

CHAPTER IV
RESULTS

4.1 Propolis sample

An area around the stingless bee farm was surrounded by various fruit trees, such as *Cocos nucifera* L. (coconut), *Citrus maxima* (Burm.) Merrill (pomelo), *Mangifera indica* (mango), *Itchi chinensis* Sonn. (lychee), *Musa acuminata* (banana), *Artocarpus heterophyllus* Lam. (jack fruit), and *Tamarindus indica* L. (tamarind) (Figure 4.1 A-B). The raw propolis from stingless bee, *G. thoracica* was collected from the hive entrance, edges, open space, and inside their hives (cerumen and pots) as example shown in Figure 4.1 D-F, respectively. The obtained propolis appeared as a black resin (Figure 4.1 C).

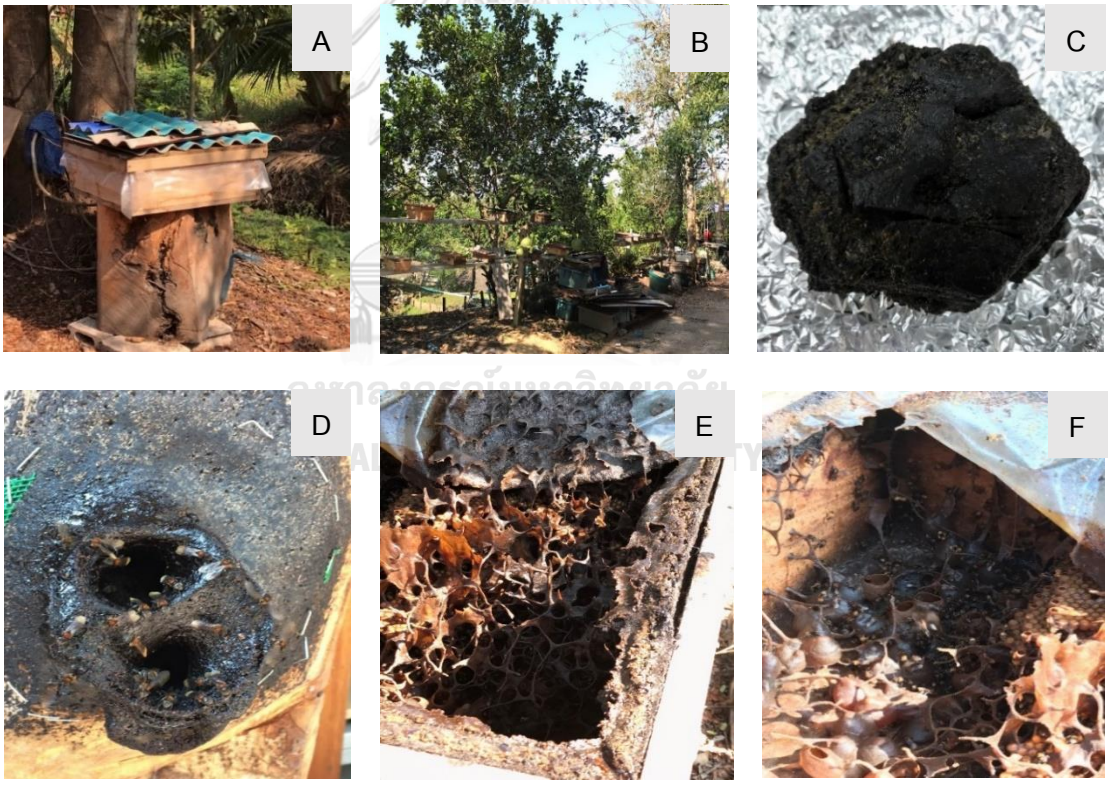


Figure 4.1 Propolis collecting site. It shows (A) a man-made wooden hive using for meliponiculture, (B) overview of the area around stingless bee farm, Phupha farm, (C) raw propolis sample of *G. thoracica* and (D) the position of propolis at the entrance, (E) edges or open space, and (F) cerumen of honey and pollen pots within the hives.

4.2 Propolis crude extract

The dark brown and sticky resin, CME, was obtained after extraction with 80% MeOH. It was recovered at 22.41 g, 15.46% of the raw propolis. According to the partitioning of CME step, CHPE, CDPE, and CMPE were obtained with 2.54 g (1.75%), 7.89 g (5.44%), and 2.56 g (1.77%), respectively. All portioned extracts appeared as sticky resin with different in colors as described in Table 4.1. Then, all crude extracts were primarily tested for antimicrobial activity against *M. globosa*.

Table 4.1 Appearance, weight (g), and yield (%) per an extraction batch of obtained crude propolis extracts.

Extracts	Type of solvent	Appearance	Weight (g)	Yield[a] (%)
CME	80% MeOH	Dark brown, sticky resin	22.41	15.46
CHPE	hexane	Yellow, sticky resin	2.54	1.75
CDPE	CH_2Cl_2	Red brown, sticky resin	7.89	5.44
CMPE	MeOH	Dark brown, sticky resin	2.56	1.77

[a] The values were calculated comparing to 145 g of the raw propolis.

4.3 Characteristic features and density of the yeast inoculum

Before performing anti-*M. globosa* experiments, the yeast was taken to observe macro- and micro-characteristic features after reached the incubation period. At day 7, a single colony of the yeast was observed with 1-2 mm in diameter. It appeared as a raised shiny and creamy colony, with a slightly lobated margin. After reached day 20 or longer, the colony spread out (3-5 mm in diameter), it was flatter and more wrinkled surface to cerebriform, with a raised point at the center (Figure 4.2 A-B). A microscopic feature of the yeast cell was shown as a spherical or round shape with a narrow base of their budding site (Figure 4.2 C-D).

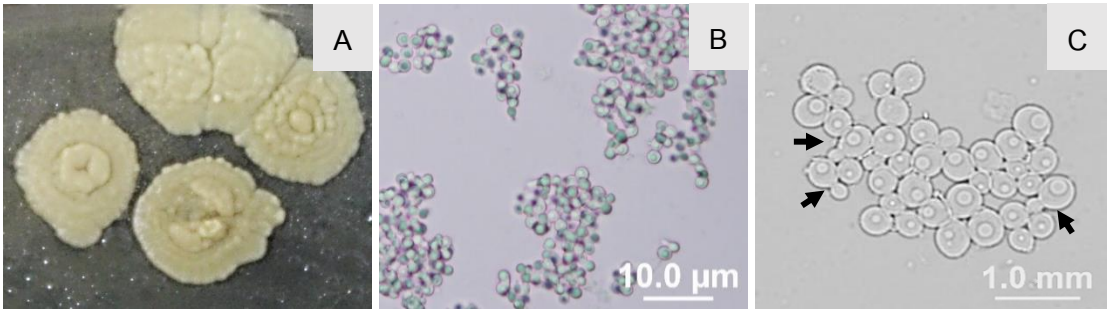


Figure 4.2 Morphology of *M. globosa* (A) colonies appearance after incubation for 20 days and microscopic feature of *M. globosa* culture in broth (MLNB) for 7 days at (B) 40x and (C) 100x magnification. The black arrow indicated budding sites with a narrow base.

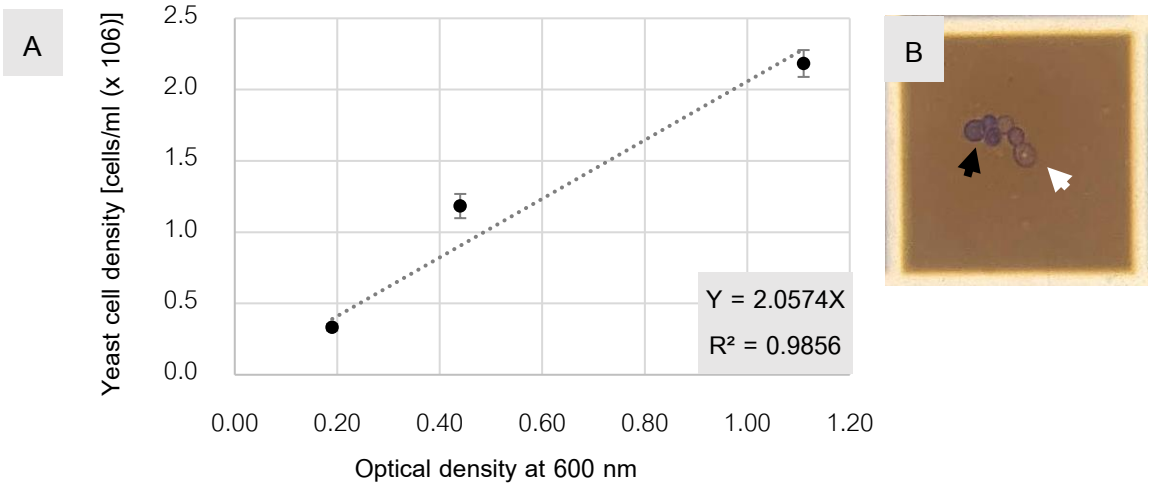


Figure 4.3 Density of yeast cell suspension. In (A), yeast cell density [cells/ml (x 10^6)] at different $O.D._{600}$ was estimated from the graph and, in (B), the difference between dead (a black arrow) and alive yeast cell (a white arrow) by using methylene blue staining method was shown. Error bars represent SEM.

In addition, a graph between relationship of $O.D._{600}$ (X-axis) and cell number (cells/ml) (Y-axis) was generated (Figure 4.3). Also, linear regression and correlation coefficient (R^2) were analyzed. The graph had R^2 at 0.9856, and its formular was Y = 2.0574X. The formular was then used for estimation and preparation of yeast cell suspension before conducting experiments.

4.4 Primary screening of antimicrobial activity of crude extracts

The CME, CHPE, CDPE, and CMPE were screened antimicrobial activity by agar well diffusion. The diameters of zone of inhibition (mm) and percentage of growth inhibition (%) compared to KTZ were reported (Table 4.2).

Zone of inhibition from CME was observed at 11.83 ± 0.50 mm (56.00 ± 3.69 %) and 12.00 ± 0.67 mm (56.61 ± 1.76 %) from 200 and 400 mg/ml, respectively. Meanwhile, it was not observed at concentrations 50 and 100 mg/ml. After the antimicrobial activity against *M. globosa* of CME was confirmed, the CME was then passed through another crude extraction step, partition. Accordingly, CHPE, CDPE, and CMPE were obtained and screened for antimicrobial activity to select the best part of the partitioned crude. At the same concentrations 50, 100, and 200 mg/ml, only CMPE exhibited zone of inhibition at 10.33 ± 0.47 mm (46.88 ± 2.39 %), 11.14 ± 1.26 mm (50.43 ± 3.68 %), and 13.58 ± 1.37 mm (61.04 ± 2.67 %), respectively. Moreover, it also presented zone of inhibition at 12.5 and 25 mg/ml at 9.00 ± 0.00 mm (40.70 ± 0.98%) and 10.00 ± 0.82 mm (45.69 ± 0.47%), respectively (Table 4.2, Figure 4.4, and Appendix D; Table D3). Therefore, the CMPE was chosen for further purification.

Table 4.2 Diameter of zone of inhibition and percentage of growth inhibition of CME, CMPE, CHPE, and CDPE obtained from agar well diffusion assay.

Extracts	Concentrations (mg/ml)	Diameter of zone of inhibition (mm)	Percentage of growth inhibition[a] (%)
CME[b]	50	0.00 ± 0.00	0.00 ± 0.00
	100	0.00 ± 0.00	0.00 ± 0.00
	200	11.83 ± 0.50	56.00 ± 3.69
	400	12.00 ± 0.67	56.61 ± 1.76
	4 μg/ml KTZ	21.17 ± 0.50	
CMPE[c]	50	10.33 ± 0.47	46.88 ± 2.39
	100	11.14 ± 1.26	50.43 ± 3.68
	200	13.58 ± 1.37	61.04 ± 2.67
	4 μg/ml KTZ	22.11 ± 1.26	
CHPE[b]	50	0.00 ± 0.00	0.00 ± 0.00
	100	0.00 ± 0.00	0.00 ± 0.00
	200	0.00 ± 0.00	0.00 ± 0.00
	4 μg/ml KTZ	23.00 ± 1.00	
CDPE[b]	50	0.00 ± 0.00	0.00 ± 0.00
	100	0.00 ± 0.00	0.00 ± 0.00
	200	0.00 ± 0.00	0.00 ± 0.00
	4 μg/ml KTZ	23.17 ± 0.83	

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E1-2, and 4-5).

[b] The result was obtained from 2 separately repeats with 3 replicates each.

[c] The result was obtained from 3 separately repeats with ≥3 replicates each.

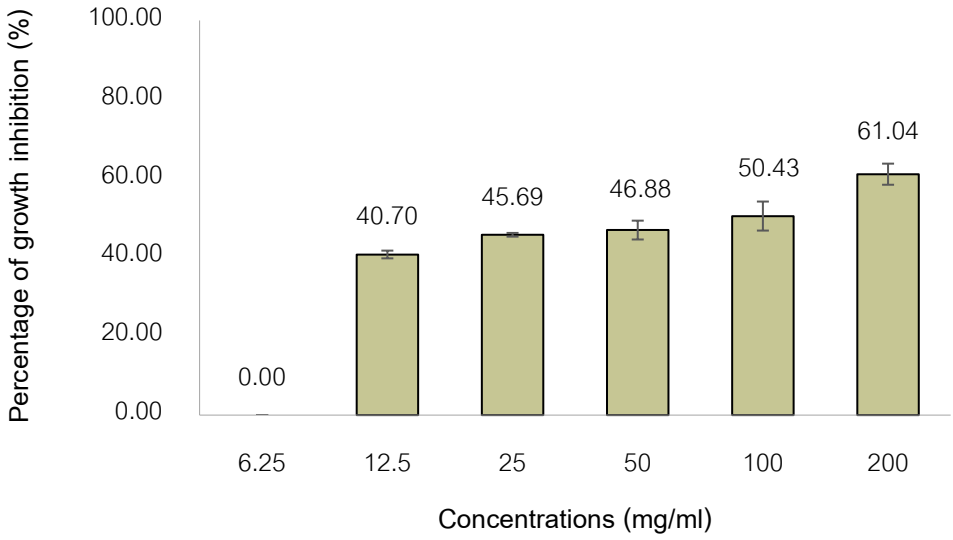


Figure 4.4 Percentage of growth inhibition of CMPE at concentration 6.25-200 mg/ml using agar well diffusion assay. The values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E2-3). Error bars represent SEM.

4.5 Purification and isolation of bioactive compound from CMPE

Since the CMPE presented the best antimicrobial activity from the primary screening, it was further purified to isolate its bioactive compound by using chromatography techniques. After that, the obtained extracted fractions were examined for antimicrobial activity.

4.5.1 CMPE fractions from silica gel 60 Å column chromatography

After the CMPE was purified by silica gel 60 Å column chromatography, three extracted fractions, CMPE 1, 2, 3 were recovered at 0.24 g (0.17%), 0.68 g (0.47%), and 1.98 g (1.37%), respectively. Each fraction had different appearances, in which CMPE 1 appeared as crystal, whereas CMPE 2 was oily liquid, and CMPE 3 was sticky resin as described in Table 4.3.

Table 4.3 Appearance, weight (g), and yield (%) per an extraction batch of obtained extract fractions from CMPE (CMPE 1-3).

Extracts	Type of solvents	Appearance	Weight (g)	Yield[a] (%)
CMPE 1	1:19 (v/v) MeOH : CH_2Cl_2	Yellow-brown, sticky liquid with white solid	0.24	0.17
CMPE 2	1:4 (v/v) MeOH : CH_2Cl_2	Red-brown, oily liquid	0.68	0.47
CMPE 3	1:1 (v/v) MeOH : CH_2Cl_2 and MeOH	Dark-brown, sticky resin with slightly white and oily liquid	1.98	1.37

[a] The values were calculated by comparing to 145 g of the raw propolis.

4.5.2 Thin layer chromatography of fractions CMPE 1-3

TLC analysis was conducted along the purification steps. The TLC patterns of fractions CMPE 1-3 are shown in Figure 4.5. Solvent system at 1:19 (v/v) MeOH : CH_2Cl_2 was used to analyze and detect CMPE 1 while, 1:4 (v/v) MeOH : CH_2Cl_2 was used for CMPE 2-3. Thus, TLC pattern of CMPE 2 and 3 were long smear bands. It indicated that they contained more than one chemical compositions. Hence, CMPE 1 exhibited one intense spot, indicating that it could contain a single chemical composition or more than one with similar in polarity and molecular weight.

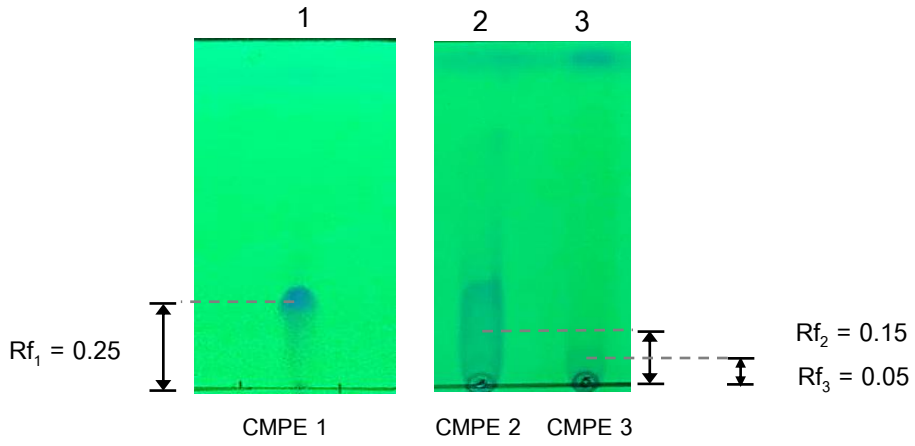


Figure 4.5 A TLC pattern of CMPE fractions. Lanes 1-3 were CMPE 1, 2, and 3 under UV 254 nm visualization, respectively. The mobile system for CMPE 1 was 1:19 MeOH : CH_2Cl_2 while the mobile system for CMPE 2 and 3 were 1:4 MeOH : CH_2Cl_2.

4.5.3 Examination of antimicrobial activity of fraction CMPE 1-3

Antimicrobial activity of CMPE 1-3 at concentration 3.13, 12.5, and 50 mg/ml were screened, and its results were reported as shown in Table 4.4. Both CMPE 1 and 3 exhibit zone of inhibition beginning at 12.5 and 50 mg/ml in which the zones from CMPE 3 were smaller and less stable. In contrast, CMPE 2 did not present antimicrobial activity at the mentioned concentrations. The zone of inhibition at 12.5 and 50 mg/ml from the CMPE 1 were observed at 11.83 ± 0.83 mm (46.36 ± 3.54%) and 21.67 ± 1.00 (84.97 ± 4.46%), while CMPE 3 were observed at 9.50 ± 0.50 mm (40.57 ± 5.26%) and 10.50 ± 0.50 mm (45.85 ± 4.53%), respectively.

Additionally, CMPE 1 also presented percentage of growth inhibition greater than the extracts from previous extraction steps, CME and CMPE, when comparing at the same concentration 50 mg/ml. Besides, the percentage of inhibition of CMPE 3 was slightly lower but was not significantly different from CMEP (Figure 4.6) ($p > 0.05$). Thus, more purification steps could isolate and improve the activity of extract parts.

Table 4.4 Diameter of zone of inhibition and percentage of growth inhibition from fractions CMPE 1-3 at concentrations 3.13-50 mg/ml obtained from agar well diffusion assay.

Extracts	Concentrations (mg/ml)	Diameter of zone of inhibition (mm)	Percentage of growth inhibition[a] (%)
CMPE 1[b]	3.13	0.00 ± 0.00	0.00 ± 0.00
	12.5	11.83 ± 0.83	46.36 ± 3.54
	50	21.67 ± 1.00	84.97 ± 4.46
	2 μg/ml KTZ	25.50 ± 0.17	
CMPE 2[b]	3.13	0.00 ± 0.00	0.00 ± 0.00
	12.5	0.00 ± 0.00	0.00 ± 0.00
	50	0.00 ± 0.00	0.00 ± 0.00
	2 μg/ml KTZ	24.83 ± 0.50	
CMPE 3[c]	3.13	0.00 ± 0.00	0.00 ± 0.00
	12.5	9.50 ± 0.50	40.57 ± 5.26
	50	10.50 ± 0.50	45.85 ± 4.53
	2 μg/ml KTZ	25.39 ± 0.28	

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E6-8).

[b] The result was obtained from 2 separately repeats with 3 replicates each.

[c] The result was obtained from 3 separately repeats with ≥2 replicates each.

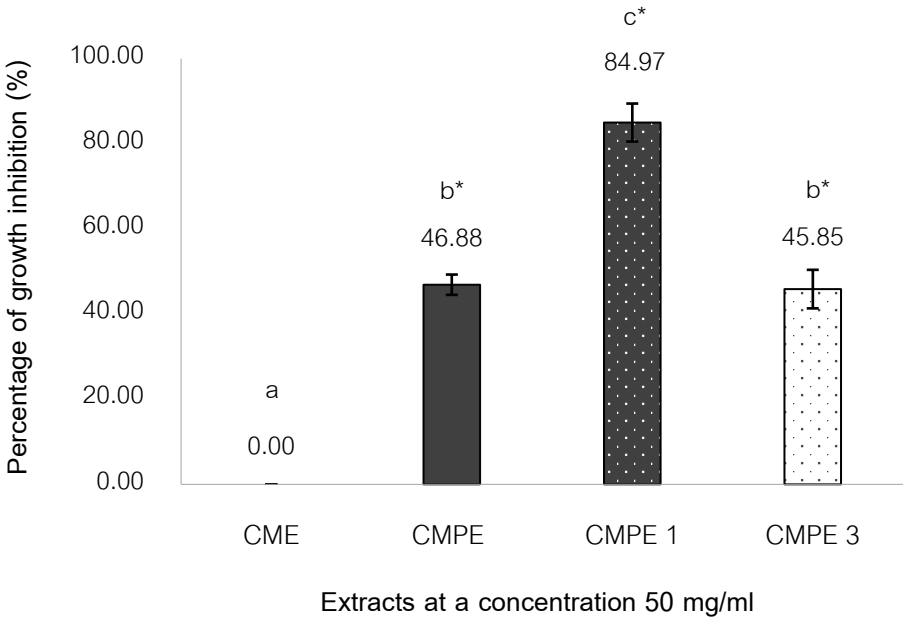


Figure 4.6 Percentage of growth inhibition of CME, CMPE, CMPE 1, and CMPE 3 at concentration 50 mg/ml using agar well diffusion assay. Error bars represent SEM. Symbol " * " and letters indicate that data is significantly different from control (DMSO alone) and each type of extract, respectively ($p \leq 0.05$; One-way ANOVA, Games-Howell).

4.6 Chemical structure analysis of fraction 1 from CMPE by NMR

Since CMPE 1 revealed the most potent antimicrobial activity and displayed a single spot on TLC after purification, the chemical structure was further analyzed by NMR technique. The data was reported as following: ^{1}H NMR (500 MHz, MeOH-d_4) δ 7.03 (s, 2H), 3.81 (s, 3H); ^{13}C NMR (125 MHz, MeOH-d_4) δ 169.03, 146.51, 139.77, 121.44, 110.02, 52.26 and the obtained ^{1}H and ^{13}C NMR spectra are shown in Figure 4.7 A and B, respectively. According to the information, the bioactive compound or CMPE 1 was identified as methyl gallate (Methyl 3,4,5-trihydroxybenzoate, $C_8H_8O_5$) as in Figure 4.8.

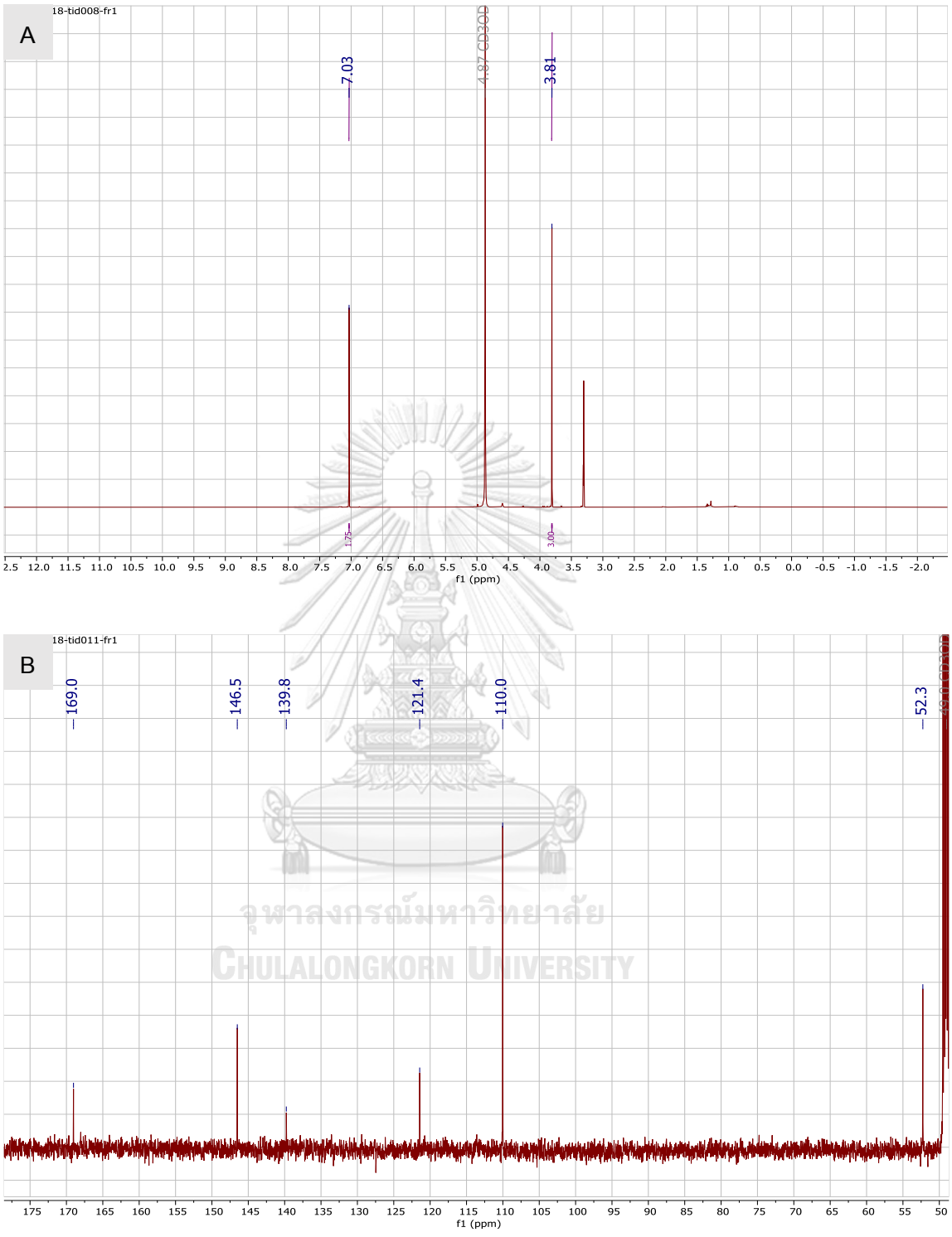


Figure 4.7 NMR spectra of methyl gallate from (A) ^{1}H and (B) ^{13}C NMR.

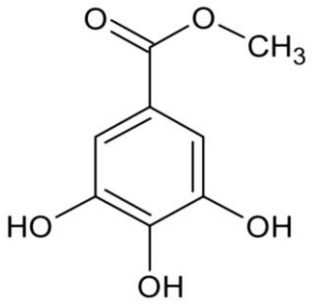


Figure 4.8 Chemical structure of methyl gallate (Bebout & Pagola, 2009).

4.7 Chemical synthesis of methyl gallate (MG)

4.7.1 Purification of synthetic MG by chromatography techniques

Since MG was identified as the active compound, our in-house synthesis of MG was conducted. After synthesis and purification steps, pale yellow into white fine solid was obtained at 0.09 g (8.87%) from 1.015 g GA. TLC pattern of synthetic MG compared to extracted MG (CMPE 1) is presented in Figure 4.9 B.

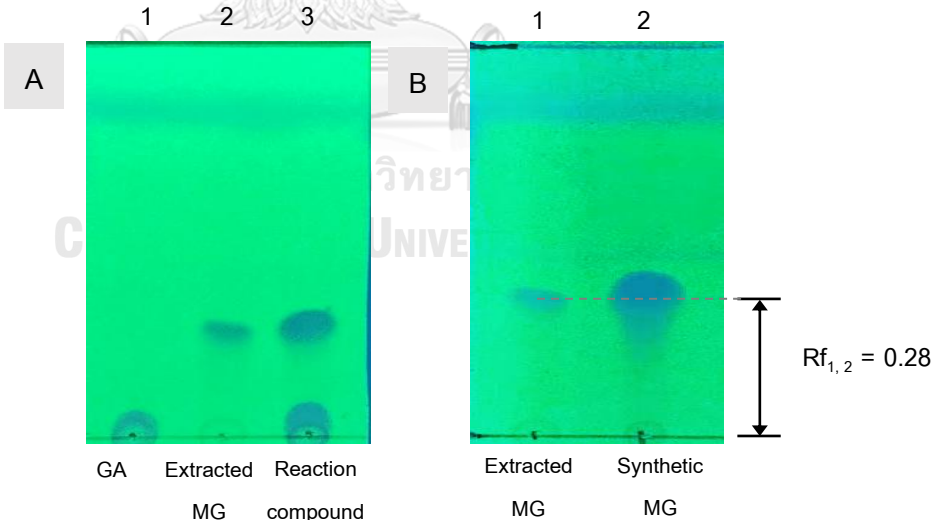


Figure 4.9 A representative of TLC pattern from chemical synthesis of methyl gallate. In (A), lanes 1-3 were gallic acid or GA, extracted MG, and compound from synthesis reaction before purification step. In (B), lanes 1-2 were the extracted MG and synthetic MG after purification to under UV 254 nm visualization. Both mobile systems were 2:23 MeOH : CH_2Cl_2.

4.7.2 Antimicrobial activity of synthetic and standard MG

Synthetic MG was further tested the antimicrobial activity compared to the extracted and standard MG (purity > 98.0%, TCI, Tokyo). The synthetic MG presented zone of inhibition beginning from 12.5 and 50 mg/ml at 14.00 ± 1.63 mm (52.33 ± 3.75%) and 21.33 ± 1.25 mm (79.97 ± 1.78%), respectively (Table 4.5) which were resemble manner and not significantly different from extracted MG or CMPE 1 ($p > 0.05$). Interestingly, only standard MG displayed the zone of inhibition 11.00 ± 0.82 mm (39.73 ± 2.40%) at 3.13 mg/ml. The standard MG also presented significantly higher antimicrobial activity ($p \leq 0.05$) against *M. globosa* at concentration 3.13-50 mg/ml (Figure 4.10), which might be caused by an effect from the higher purity of the compounds.

Table 4.5 Diameter of zone of inhibition and percentage of growth inhibition from synthetic MG and standard MG at concentrations 3.13-50 mg/ml obtained from agar well diffusion assay.

Extracts	Concentrations (mg/ml)	Diameter of zone of inhibition (mm)	Percentage of growth inhibition[a] (%)
Synthetic MG[b]	3.13	0.00 ± 0.00	0.00 ± 0.00
	12.5	14.00 ± 1.63	52.33 ± 3.75
	50	21.33 ± 1.25	79.97 ± 1.78
	2 μg/ml KTZ	26.67 ± 1.25	
Standard MG[b]	3.125	11.00 ± 0.82	39.73 ± 2.40
	12.5	16.67 ± 1.25	60.19 ± 3.58
	50	27.67 ± 1.25	99.96 ± 2.97
	2 μg/ml KTZ	27.67 ± 0.47	

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E9-10).

[b] The result was obtained from 3 replicates.

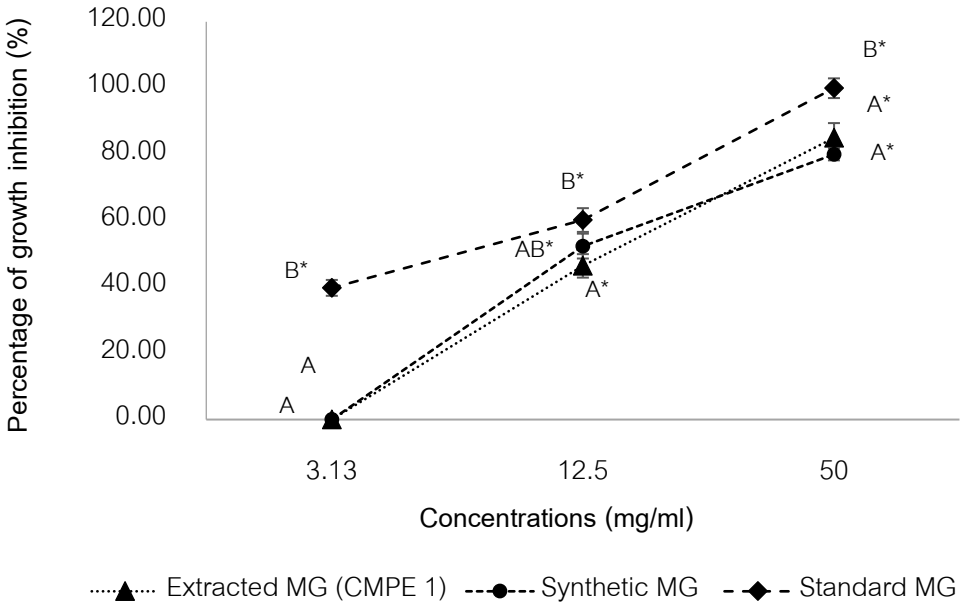


Figure 4.10 Percentage of growth inhibition of extracted MG (CMPE 1), synthetic MG, and standard MG at concentrations 3.13-50 mg/ml using agar well diffusion assay. Error bars represent SEM. Symbol " * " and capital (uppercase) letters indicate that data is significantly different from control within each extract group (DMSO alone), and each concentration between the different group of extracts, respectively ($p \leq 0.05$; One-way ANOVA, Dunnett's T3).

Additionally, the appearances of zone of inhibition from an agar well diffusion assay treated with each sample are illustrated as shown in Figure 4.11-4.12.

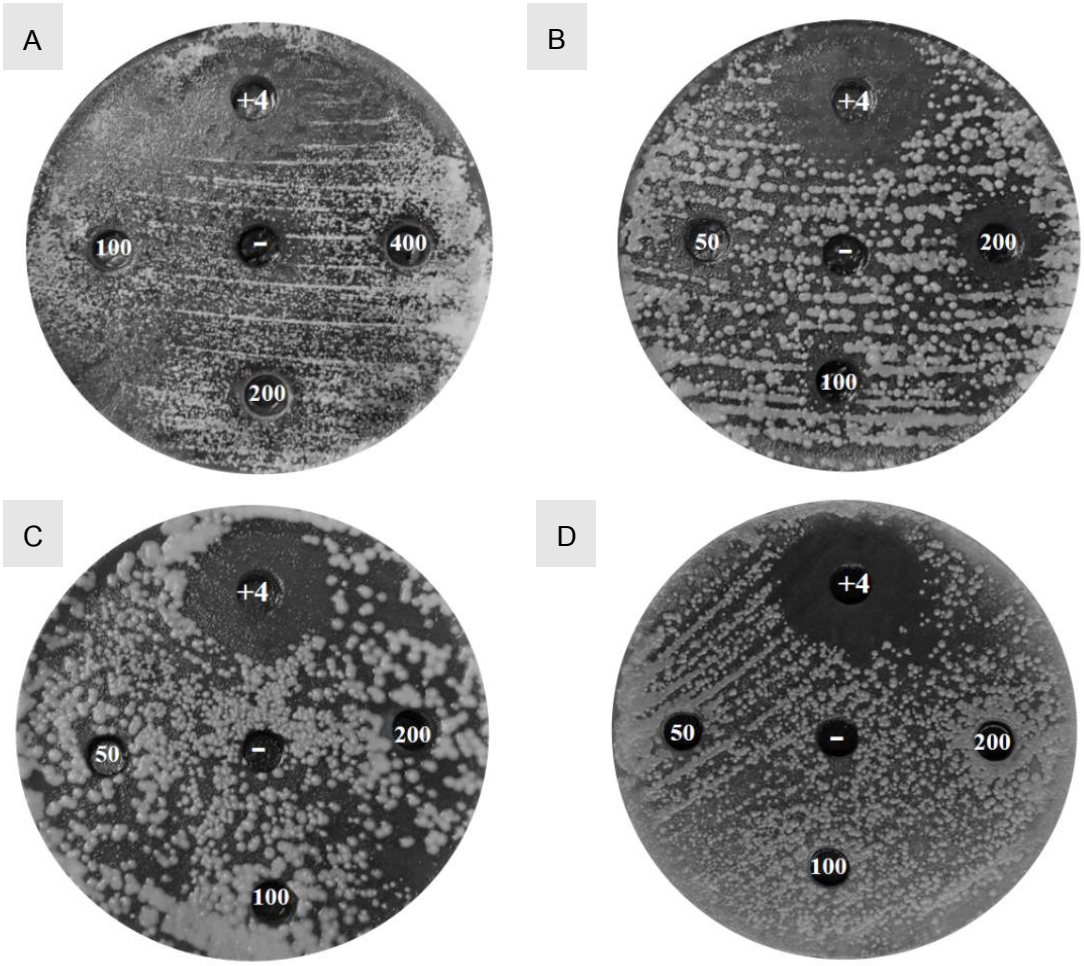


Figure 4.11 A representative of results from an agar well diffusion assay of (A) CME, (B) CMPE, (C) CDPE, and (D) CHPE. The symbols "+4" and "–" represent positive control (4 μg/ml KTZ) and negative control (DMSO), respectively. The numbers represent extract concentrations (mg/ml). Results of tested plates were observed and collected in 7-14 days depending on growth pattern in which could affect by the stability of extracts. Hence, size and appearance of the colonies may be different.

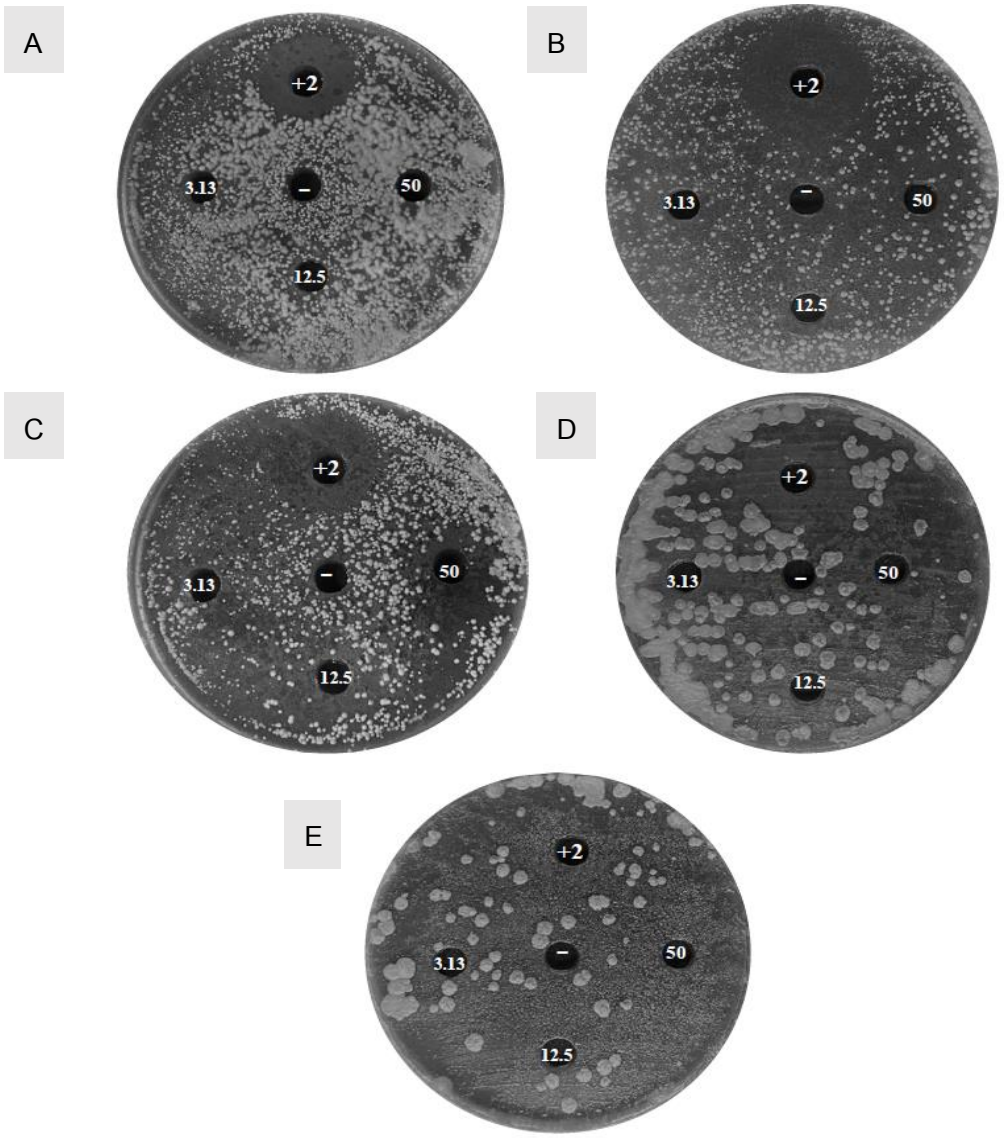


Figure 4.12 A representative of results from an agar well diffusion assay of (A) CMEP 2, (B) CMPE 3, (C) CMPE 1, (D) synthetic MG, and (E) standard MG. The symbols "+2" and "–" represent positive control (2 μg/ml KTZ) and negative control (DMSO), respectively. The numbers represent extract concentrations (mg/ml). Results of tested plates were observed and collected in 7-14 days depending on growth pattern in which could affect by the stability of extracts and some interference from the other nearly extract concentrations. Hence, size and appearance of the colonies may be different. Examination of each concentration on separately plates was also conducted, especially in highly active extracts or compounds (Appendix F; Figure F1).

4.8 Determination of MIC and MFC from broth microdilution assay

After primary screening, the extracts which had a potential inhibitory activity to *M. globosa*, including CME, CMPE, and CMPE 1, were determined for MICs and MFC. Additionally, synthetic and standard MG were also tested along. The percentage of all samples displayed in dose-dependence. MIC_{50} and MIC_{90} values of samples were mainly indicated by percentage of growth inhibition interpreted from fluorescent intensity. Moreover, visualization of color changing from resazurin assay (blue to pink) was also observed. The percentage of growth inhibition of CME and CMPE at concentrations 0.78-50 mg/ml were not significantly different ($p > 0.05$) which ranging from 33-83% and 42-91%, respectively (Figure 4.13). CME exhibited MIC_{50} at 3.13 mg/ml (51.72 ± 1.99%), but its MIC_{90} could not be observed. CMPE exhibited lower MIC_{50} at 1.56 mg/ml (51.82 ± 1.01%), and MIC_{90} value was selected at 12.5 mg/ml (73.67 ± 0.56%) because it was not significantly different to 50 mg/ml (91.49 ± 3.07%) ($p > 0.05$). The change in color from resazurin assay for both samples was not clearly distinguishable, due to the interference of color and turbidity from the extracts. In addition, reduction of colony growth after treated with CME and CMPE were obviously detected at 50 mg/ml.

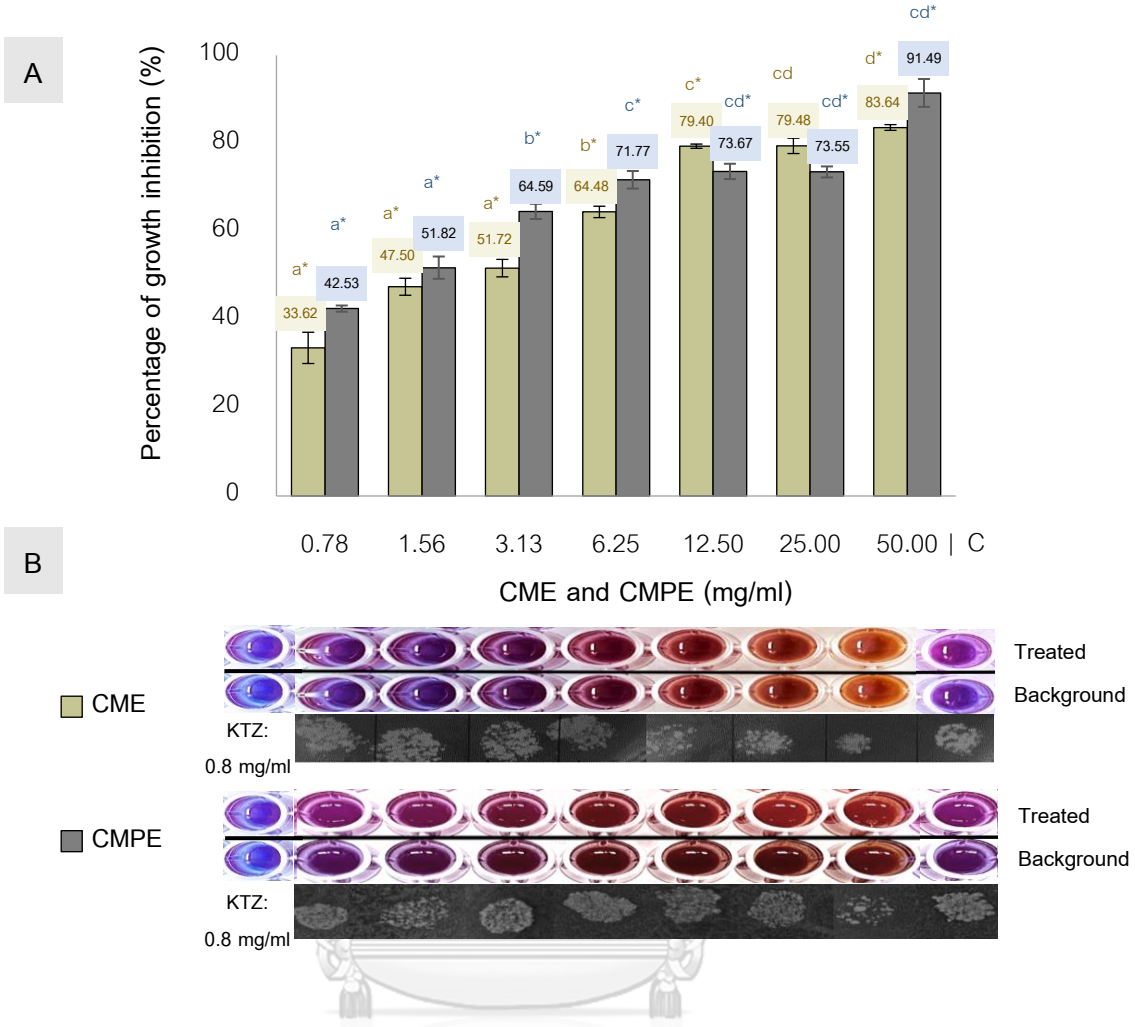


Figure 4.13 Growth inhibition of CME and CMPE (0.78-50 mg/ml). In (A), percentage of growth inhibition by using broth microdilution assay (1 repeat with 3 replicates) and, in (B), example results of resazurin assay and colony pattern on MLAN after treating with CME and CMPE at concentrations 0.78-50 mg/ml were shown. Error bars represent SEM. The data are not significantly different between extract groups ($p > 0.05$). Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 10% DMSO mixed with 5% Tween 60) and each concentration within the same extract group, respectively ($p \leq 0.05$; One-way ANOVA, Dunnett's T3).

Due to the limitation amount of obtained CMPE 1 and synthetic MG, standard MG was firstly examined to find the suitable concentration ranges for further test. The standard MG at 0.37-50 mg/ml in final 10% DMSO, which had a high concentration of solvent control condition quite similarly as CME and CMPE (10% DMSO in 5% Tween 60), were screened as displayed in Figure 4.14. Besides, KTZ at MIC_{50}, 0.8 mg/ml were used as positive control in the same solvent control conditions for all above mentioned samples. Percentage of growth inhibition of the standard MG was ranging from 77-99% at 0.37-50 mg/ml which greater than CME and CMPE. Overall results supported that more purification steps could enhance anti-*M. globosa* growth activity of the extracts as mentioned in results of agar well diffusion assay.

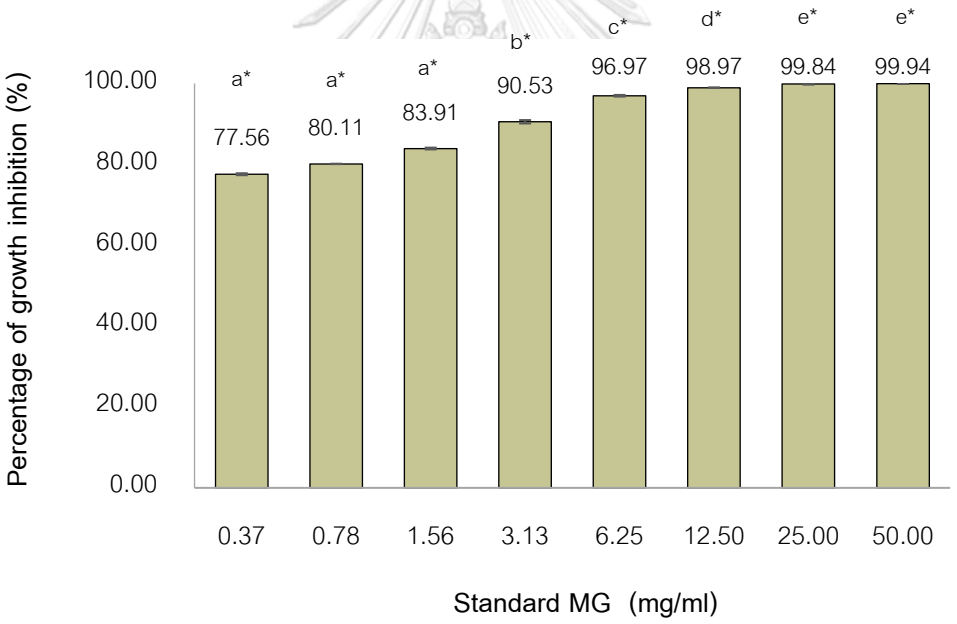


Figure 4.14 Percentage of growth inhibition from standard MG at concentrations 0.78-50 mg/ml by using broth microdilution assay (2 independent repeats with 3 replicates each). Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 10% DMSO) and each concentration within the same extract group, respectively ($p \leq 0.05$; One-way ANOVA, Dunnett's T3).

The high concentration of solvent was used for crude extract assay because of their poor solubility. Meanwhile, the solvent could interfere to the yeast growth of control treatment which could affect to actual activity of the extracts. Accordingly, it was also noticed in higher concentration of KTZ in this study, but the MIC_{50} value of KTZ (0.8 mg/ml) was still lower than both crude extracts. Therefore, standard MG in the similarly high concentration of solvent was also tested to confirm the bioactivity. Moreover, it was further examined for MICs values in 1-2% DMSO which normally was the approved concentration of solvent for testing in organisms.

Standard MG at concentrations 0.016-2 mg/ml in final 1% DMSO were examined (Figure 4.15). The percentage of growth inhibition was found ranging from 14-81%. The value of MIC_{50} was indicated at 0.063 mg/ml (57.35 ± 0.59%) with similar color of the treated concentration and its background from resazurin test. As MIC_{90} was not observed in the concentration range, standard MG at 0.5-10 mg/ml in final 2% DMSO were then evaluated (Figure 4.16 A). The overlap interval concentration at 0.5-2 mg/ml of both conditions was not significantly different ($p > 0.05$) (Appendix N; Table N21-23) which evidenced for viability and reproducibility of both data. The percentage of growth inhibition at 0.5-10 mg/ml ranged from 73-98%. The value of MIC_{90} was indicated at 4 mg/ml (90.27 ± 1.02%). The MIC_{50} value of KTZ, 0.025 mg/ml was used as positive control for both conditions in which it was 2.5x lower than MIC_{50} of standard MG.

Colony growth pattern was visually decreased after 3 mg/ml and dramatically reduced after 6 mg/ml. The MFC values were indicated at 8 mg/ml of standard MG with completely no growth of colony (Figure 4.16 B). However, there was no growth of colony at 0.025 mg/ml of KTZ and at 10x lower concentration (data not shown).

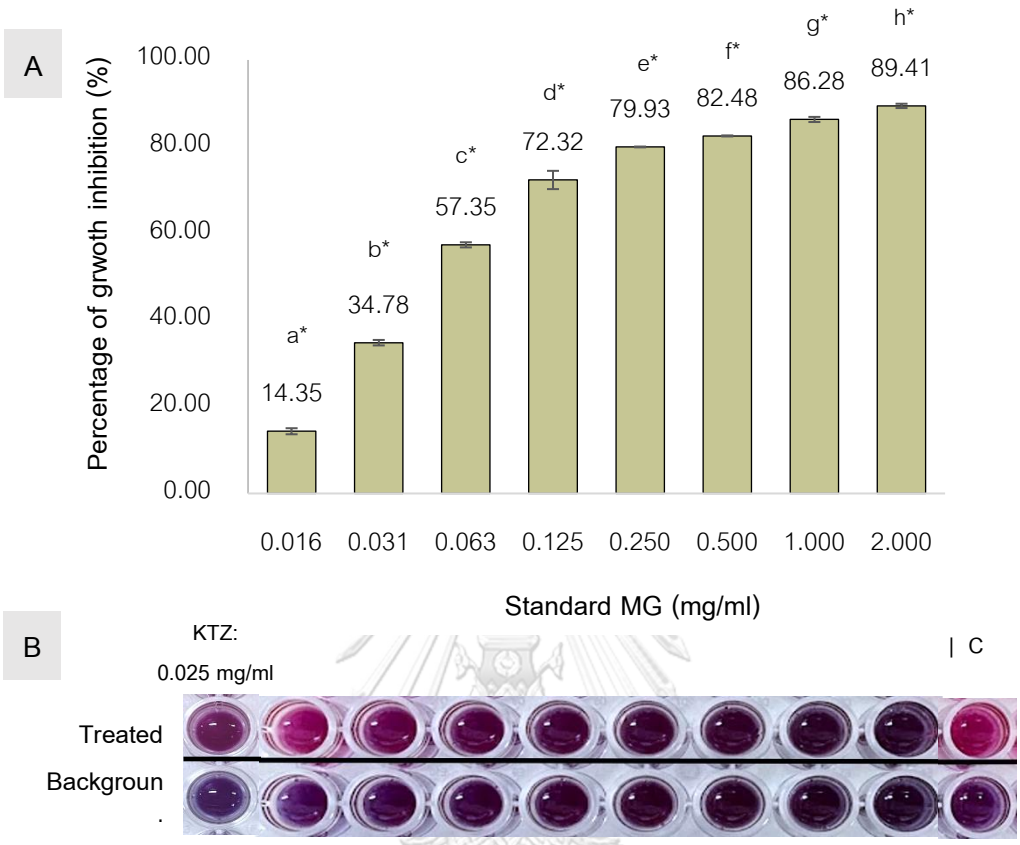


Figure 4.15 Growth inhibition of standard MG (0.016-2 mg/ml). In (A), percentage of growth inhibition by using broth microdilution assay (2 independent repeats with 3 replicates each) and, in (B), example results of resazurin assay after treating with standard MG at concentrations 0.016-2 mg/ml were shown. Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 1% DMSO) and each concentration within the same extract group, respectively ($p \leq 0.05$; One-way ANOVA, Dunnett's T3). KTZ at 0.025 mg/ml was also used as a positive control.

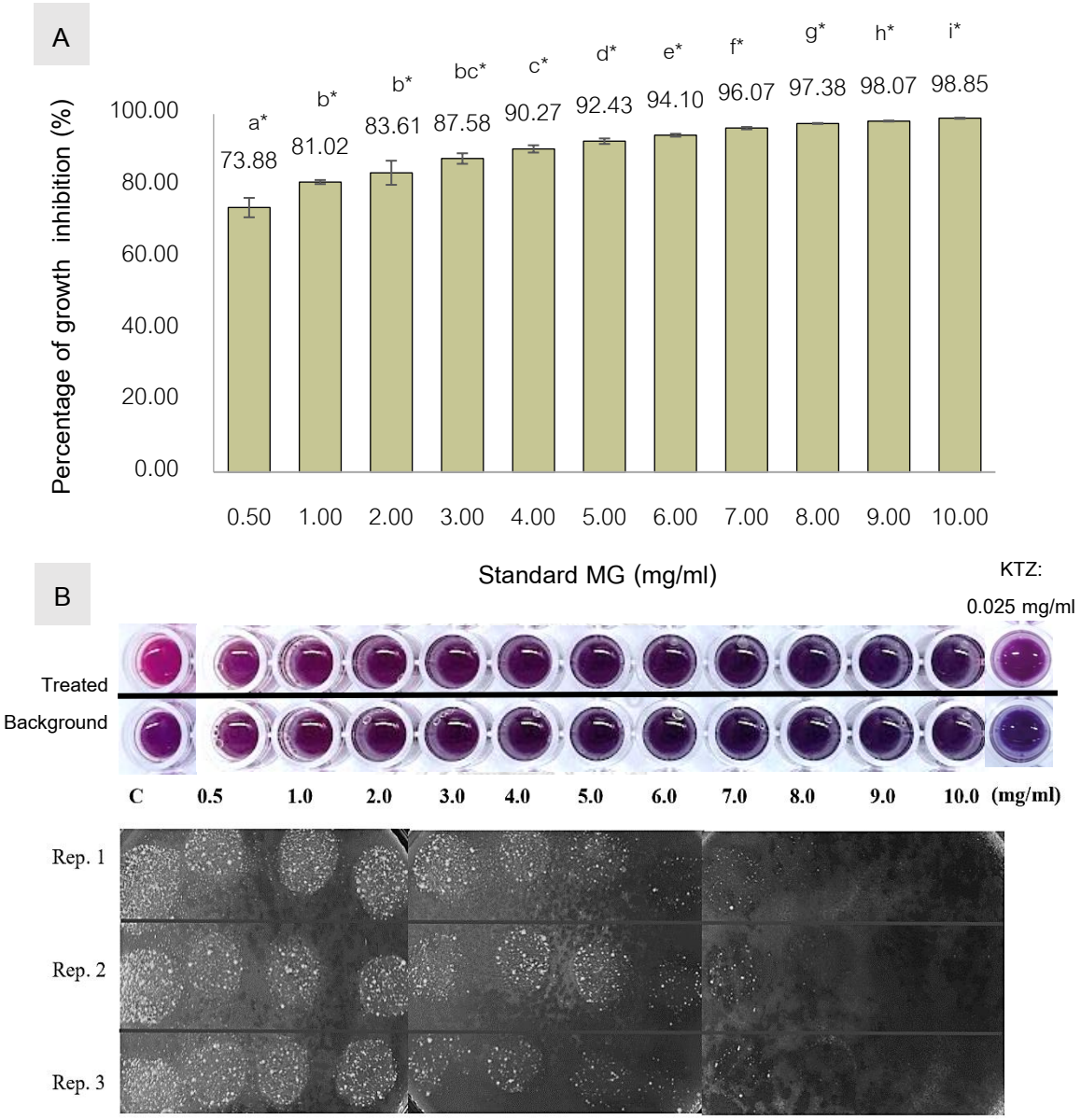


Figure 4.16 Growth inhibition of standard MG (0.5-10 mg/ml). In (A), percentage of growth inhibition by using broth microdilution assay (3 independent repeats with 3 replicates each). In (B), growth pattern of colony after treated with standard MG at concentrations 0.5-10 mg/ml. Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 2% DMSO) and each concentration within the same extract group, respectively ($p \leq 0.05$; One-way ANOVA, Games-Howell). KTZ at 0.025 mg/ml was also used as a positive control.

From previous information, concentrations 0.031-0.125 mg/ml which reaching at least 50% of growth inhibition were chosen for determining MICs values of synthetic MG and CMPE 1. The standard MG was also tested to assure the viability of the results. As results shown in Figure 4.17, percentage of growth inhibition of CMPE 1 (33-46%) was slightly higher than synthetic MG (31-44%), but they were not significantly different ($p > 0.05$). Nevertheless, percentage of growth inhibition of standard MG (39-65%) was significantly greater than both extracts at the same concentration ranges ($p \leq 0.05$). Unfortunately, MICs values of synthetic MG and CMPE 1 could not be determined because the percentage of inhibition did not reach 50% as the standard. Therefore, they were further estimated for IC_{50} and IC_{90} in the next section. The change in color of resazurin assay from all samples was not obviously different with appearing as purple. Meanwhile, colony growth pattern of standard MG was observed with slightly decreasing more than synthetic MG and CMPE 1 at 0.125 mg/ml.

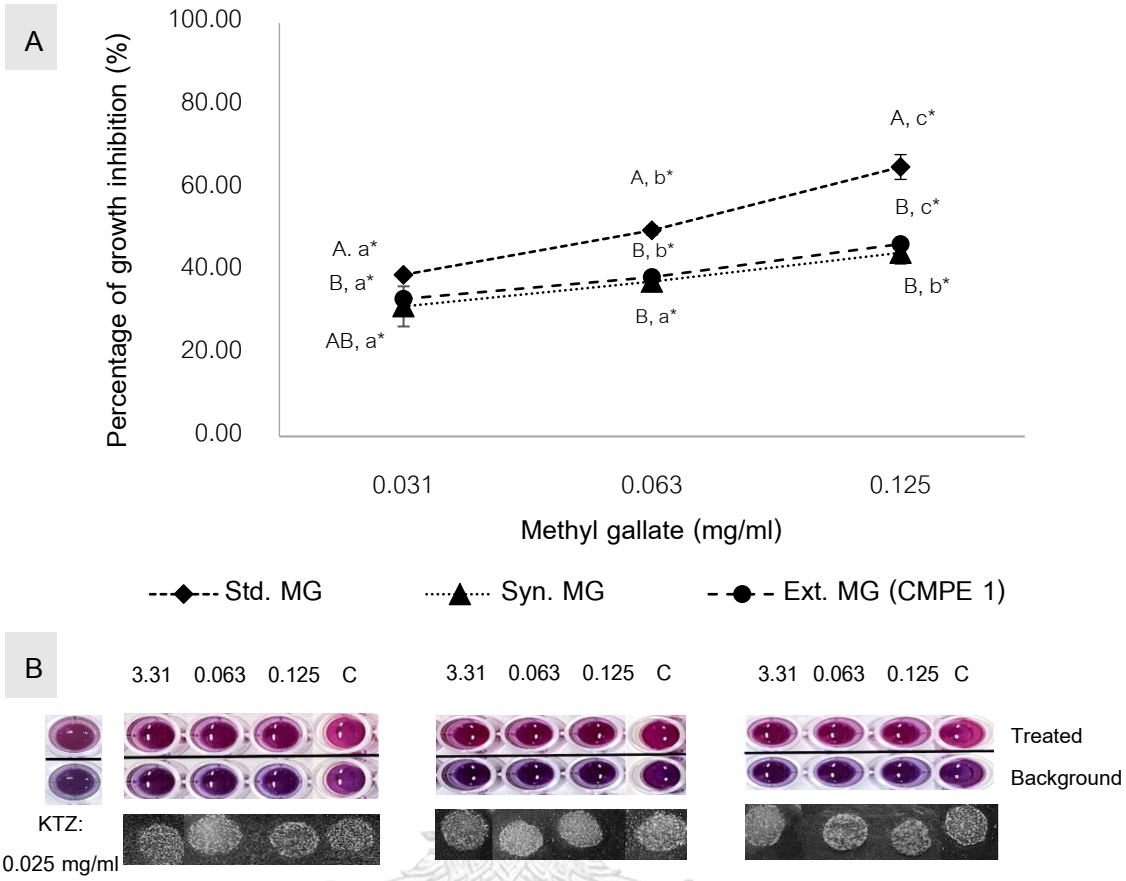


Figure 4.17 Growth inhibition of standard MG, synthetic MG, and CMPE 1 (0.031-0.125 mg/ml). It showed (A) percentage of growth inhibition by using broth microdilution assay (2 independent repeats with 3 replicates each) and (B) example results of resazurin assay and colony pattern on MLAN after treating with standard MG (Std. MG), synthetic MG (Syn. MG), and CMPE 1 (Ext. MG) at concentrations 0.031-0.125 mg/ml. Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 1% DMSO) and each concentration within the same extract group, respectively. Capital (uppercase) letters indicate that data is significantly different from each concentration between the different group of extracts ($p \leq 0.05$; One-way ANOVA, Dunnett's T3).

The percentage of inhibition of KTZ against *M. globosa* growth tested along with samples in different solvent control conditions are shown in Figure 4.18. The MIC_{50} values were determined at 0.80 mg/ml for 10% DMSO mixed with 5% Tween 60 and 10% DMSO, while 1% and 2% DMSO were 0.025 mg/ml.

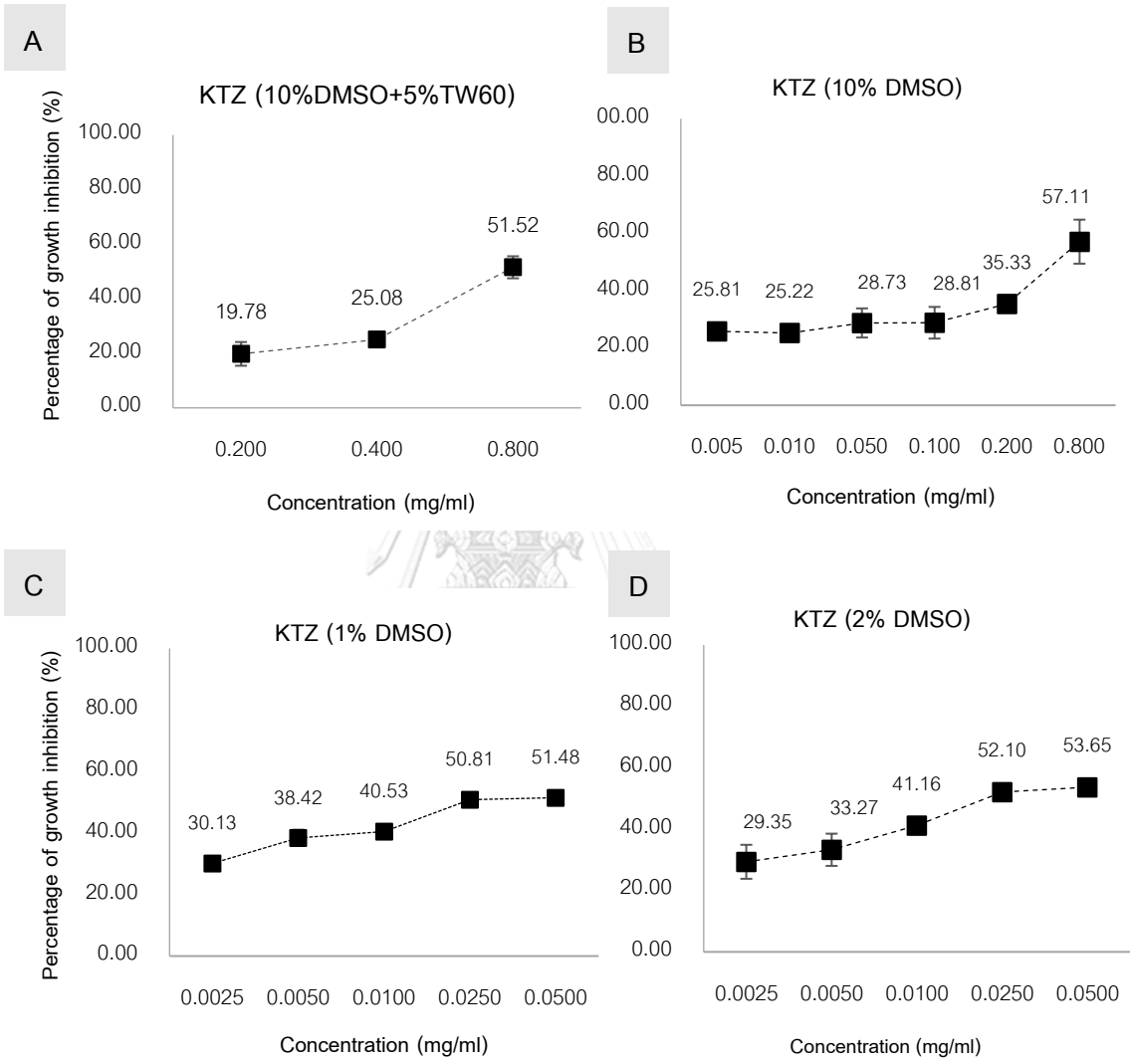


Figure 4.18 Percentage of growth inhibition of KTZ by using broth microdilution assay (≥ 2 repeats with ≥ 2 replicates each) at concentrations (A) 0.2-0.8 mg/ml in solvent 10% DMSO and 5% Tween 60, (B) 0.005-0.8 mg/ml in solvent 10% DMSO, (C) 0.0025-0.05 mg/ml in 1% DMSO, and (D) 0.0025-0.05 mg/ml in 2% DMSO. Error bars represent SEM.

4.9 Estimation of IC50 and IC90 by nonlinear regression analysis

Since all active samples revealed dose-dependent manner, the values of IC_{50} and IC_{90} were calculated by GraphPad Prism version 8 using nonlinear regression analysis of dose response-inhibition [log(inhibitor) versus normalized response]. The sigmoidal curve with variable slope of each sample data set, which obtained from broth microdilution assay, was plotted between [i] log (concentration[μg/ml]) (X-axis) and [ii] percentage of responses (Y-axis), including percentage of yeast growth (downward) and/or percentage of growth inhibition (upward). The conducted curves were set to estimate X parameter range from 0-4 or 0-6, and were fitted to the following formular: $Y = 100/(1+10^{[(LogIC50-X)*HillSlope)]})$. The ICs of both percentage responses (growth and inhibition) were the same values, only hill slope values were opposite. IC_{50} was an antilog of the X value at parameter Y reaching haft or 50% of growth or inhibition, while IC_{90} was an antilog of the X value at parameter Y reaching 10% or 90% of growth or inhibition, respectively. The results were concluded in Table 4.6 and Figure 4.19-4.20.

Overall, the calculated ICs values results showed the same trend as MICs values, but lower amounts. IC_{50} and IC_{90} values of CME were reported as 2.21 mg/ml and 89.33 mg/ml (R^2 = 0.9569, hill slope = ± 0.5941), respectively. The IC_{50} value of CMPE was lower at 1.22, but IC_{90} was slightly higher at 112.72 mg/ml (R^2 = 0.9004, hill slope = ± 0.4856). (Figure 4.19 A-B). IC_{50} and IC_{90} values of standard MG (Figure 4.18 C) were reported as 59.14 μg/ml and 648.63 μg/ml, respectively (R^2 = 0.9434, hill slope = ± 0.9175). The IC_{50} of standard MG was approximately 20-30x lower than CME and CMPE, when IC_{90} value was approximately 10x lower. The ICs values of standard MG was conducted from percentages data at 0.016-2 mg/ml in 1% DMSO because of more samples size and wider ranges of tested concentration. Despite to the amount limitation, synthetic MG and CMPE 1 data sets were conducted from less samples size and more narrow ranges of concentration 0.031-0.125 mg/ml.

Hence, R^2 values of both samples might appear lower, still IC_{50} and IC_{90} were only predicted values and the standard MG at the same concentration ranges were also considered to support the viability and continuity of the results. The IC_{50} of standard MG in the range (Figure 4.20 A) was served at 58.15 μg/ml (R^2 = 0.9536, hill slope = ± 0.7587) which quite similarly to previous data set at 0.016-2 mg/ml. The IC_{50} and IC_{90} of synthetic MG (Figure 4.20 B) were predicted as 223.7 μg/ml and 57.02 mg/ml (R^2 = 0.6784, hill slope = ± 0.3966), and CMPE 1 (Figure 4.20 C) were predicted as 185 μg/ml and 44.87 mg/ml (R^2 = 0.8949, hill slope = ± 0.4001), respectively. The lower IC_{50} and IC_{90} of standard MG comparing to both synthetic MG and CMPE 1 could occur by purity and stability of the samples. Besides, ICs values of KTZ was also reported as shown in Table 4.6, in which all IC_{50} values of KTZ were lower than the tested samples when comparing in the same solvent control conditions.

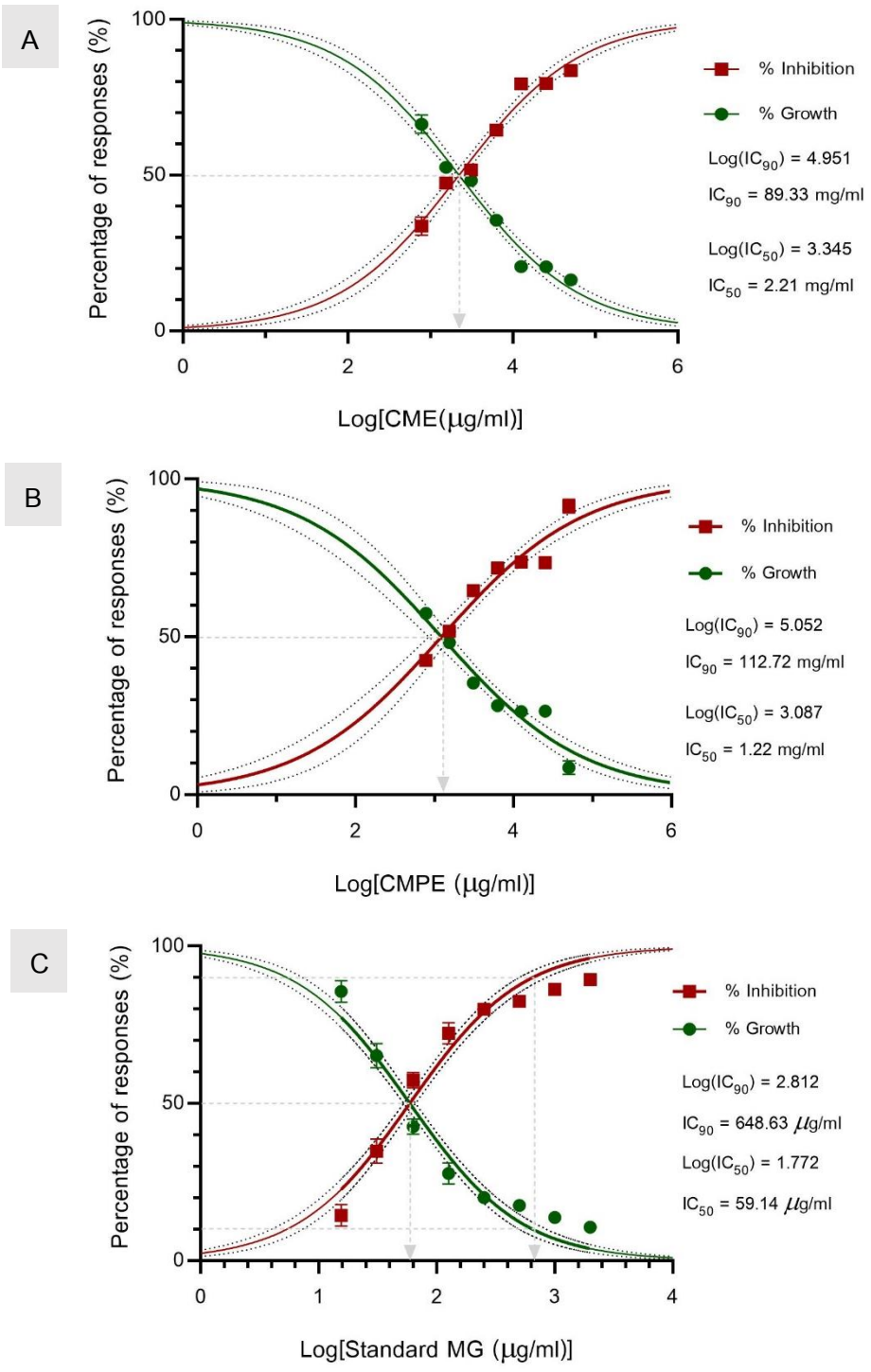


Figure 4.19 Sigmoidal curves of (A) CME, (B) CMPE, and (C) standard MG estimated by nonlinear regression analysis using dose response-inhibition [log (inhibitor) versus normalized response with variable slope] at confidence level 95%.

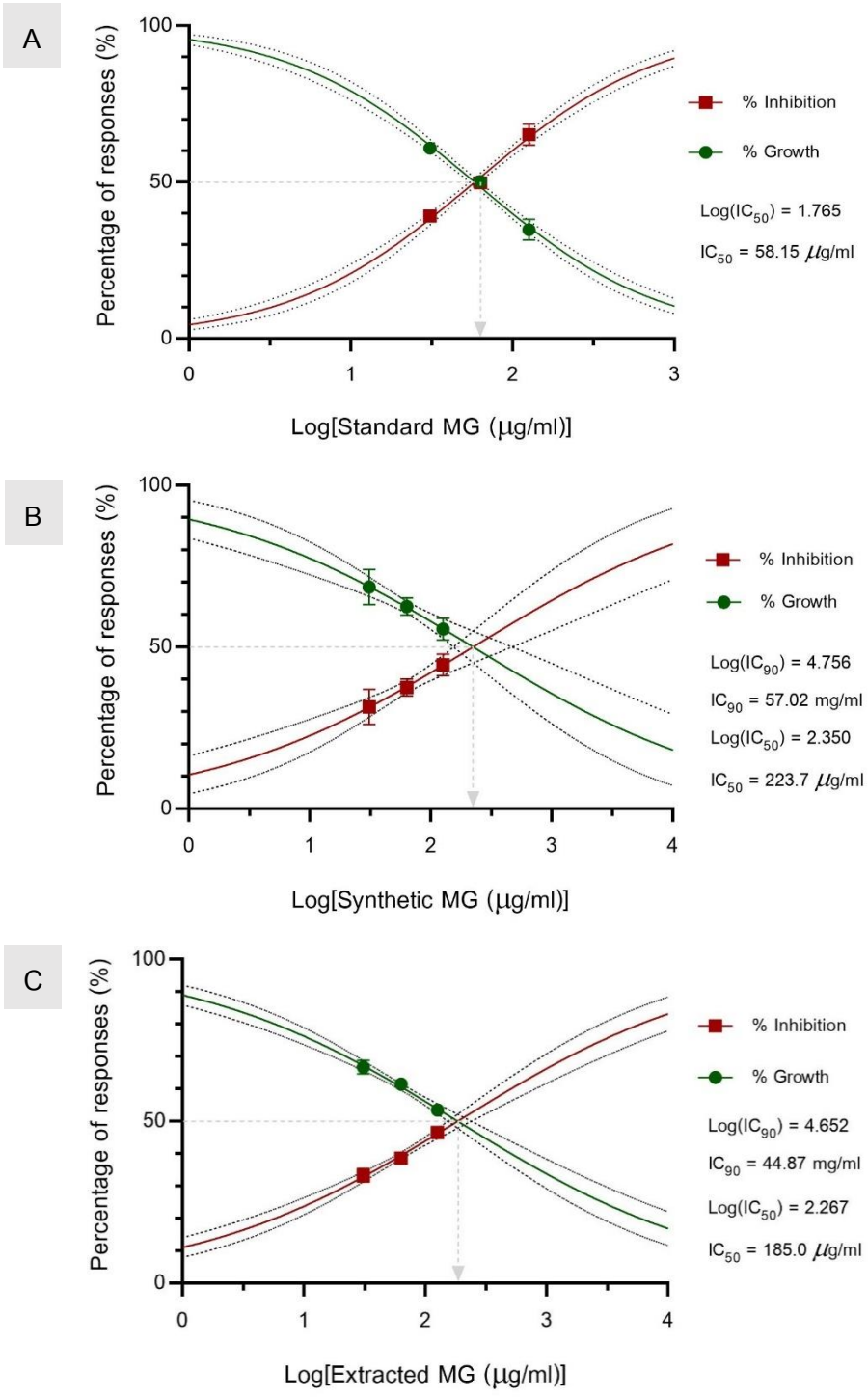


Figure 4.20 Sigmoidal curves of (A) standard MG, (B) synthetic MG, and (C) extracted MG (CMPE 1) estimated by nonlinear regression analysis using dose response-inhibition [log (inhibitor) versus normalized response with variable slope] at confidence level 95%.

Overall, the obtained results of MICs and ICs values from all samples against *M. globosa* growth are displayed in Table 4.6.

Table 4.6 Summarization of obtained MICs and ICs values against *M. globosa* growth from samples in different solvent control condition.

Solvent control	Test samples	MIC_{50} (mg/ml)	MIC_{90} (mg/ml)	IC_{50} (mg/ml)	IC_{90} (mg/ml)
10% DMSO and 5% Tween 60 (or 10% DMSO)	CME	3.13	ND[a] (> 50)	2.21	89.33
	CMPE	1.56	12.5	1.22	112.72
	KTZ[c]	0.80	N/A[b]	0.769 (or 0.797)	N/A[b]
1% DMSO (or 2% DMSO)	Standard MG	0.063	4	0.0591	0.6486
	Synthetic MG	ND[a]	ND[a]	0.2237	57.02
	CMPE 1	ND[a]	ND[a]	0.1850	44.87
	KTZ[c]	0.025	N/A[b]	0.03156 (or 0.02795)	N/A[b]

[a] ND indicates the values were not determined.

[b] N/A indicates the values were not applicational or not tested.

[c] Data of IC_{50} results for KTZ are report in Appendix J; Figure J1-4.

4.10 Evaluation of *M. globosa* lipase inhibitory activity

4.10.1 Inhibition of *M. globosa* lipase activity

Extracellular lipase activity supports growth and pathogenicity of *M. globosa.* Hence, it is one of interesting inhibitory target toward this yeast. All obtained extracts were evaluated for inhibitory effect to the lipase activity by spectrophotometry method using *p*NPP as a substrate. Percentage of lipase

inhibition at the same concentration of extracts in different solvent control condition could be varied, due to the interference of solvent to crude lipase from *M. globosa*. Moreover, the crude lipase was also not concentrated. Firstly, CME at concentrations 0.2-0.5 mg/ml in final solvent condition at 0.1% DMSO and 0.05% Tween 60 were screened. The results showed that percentage of lipase inhibition ranged from 26-70% with dose-dependent manner (Figure 4.21). The percentage reached to 50% at 0.4 mg/ml which was lower than percentage of growth inhibition that reached to 50% at 3.13 mg/ml.

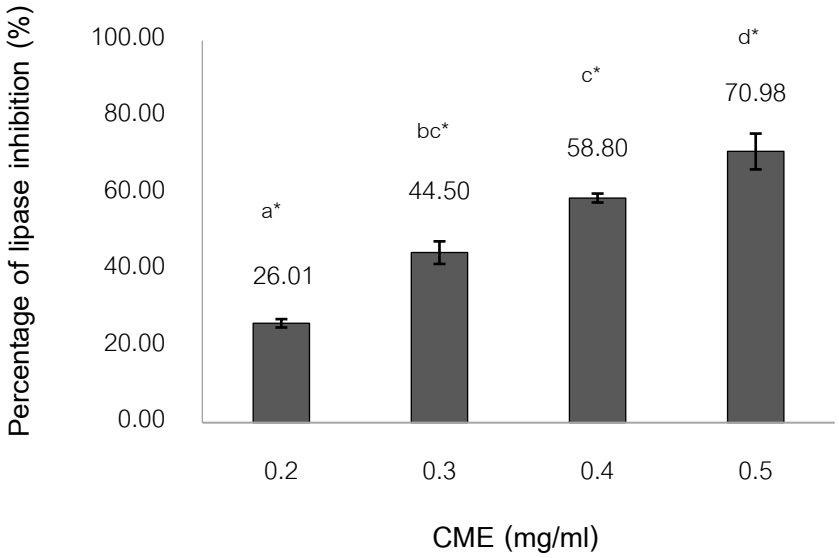


Figure 4.21 Percentage of lipase inhibition of CME at concentrations 0.2-0.5 mg/ml (3 independent repeat with ≥ 2 replicates each). Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 0.1% DMSO and 0.05% Tween 60) and each concentration within the same extract group, respectively ($p \leq 0.05$; One-way ANOVA, Dunnett's T3).

CMPE, CDPE, and CHPE were then examined comparing to CME in the same condition at concentrations 0.025-0.1 mg/ml in final solvent as 0.01% DMSO and 0.005% Tween 60 (Figure 4.22). After passed through partitioning, it revealed that all portioned crudes had lipase inhibitory activity. It appeared that percentage of lipase inhibition of CDPE (29-44%) and CHPE (26-40%) were not significantly different from CME (34-39%) ($p > 0.05$). The percentage values of the mentioned extracts were varied and might not relate to the increasing concentrations, but the values were not significantly different from each concentration within the same extract type ($p > 0.05$). Interestingly, only CMPE exhibited higher percentage of lipase inhibition in dose-dependent manner (35-81%) and was also significantly different from other crude extracts ($p \leq 0.05$).

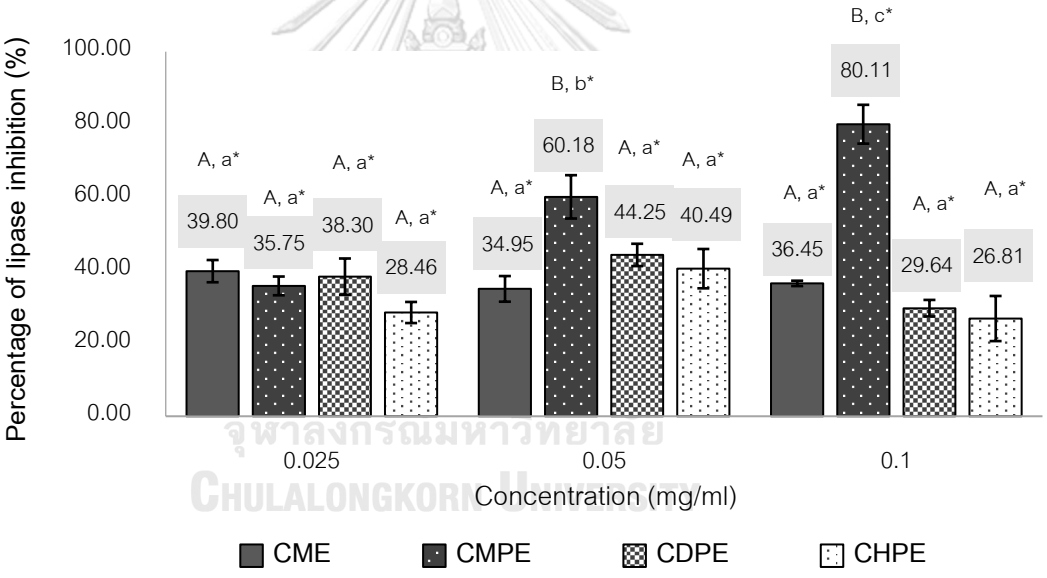


Figure 4.22 Percentage of lipase inhibition of CME, CMPE, CDPE, and CHPE at concentrations 0.025-0.1 mg/ml (≥ 2 independent repeat with ≥ 2 replicates each). Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 0.01% DMSO and 0.005% Tween 60) and each concentration within the same extract group, respectively. Capital (uppercase) letters indicate that data is significantly different from each concentration between the different group of extracts ($p \leq 0.05$; One-way ANOVA, Tukey HSD).

According to the results, both CME and CMPE could potentially inhibit lipase activity at lower concentrations which might not directly affect to the yeast growth. The fractions from CMPE were further selected to evaluate effect to lipase activity. The standard MG was firstly used for testing at high concentration as usual because of the limitation amount of CMPE 1. The percentage of lipase inhibition of CMPE 3 (0-21%) trended to be dose-dependence at 0.02-0.5 mg/ml, while standard MG and CMPE 2 did not. The activities of all samples were not significantly different from each other ($p > 0.05$) and were also lower than CME and CMPE. Therefore, standard MG exhibited slightly higher activity at low concentration, 0.2 mg/ml (Figure 4.23 A). Furthermore, extracted MG or CMPE 1 was tested and compared to CMPE 2 and CMPE 3 at the concentration and lower. CMPE 1 still displayed activity of lipase inhibition at 0.004-0.02 mg/ml (18-28%) in contrast to CMPE 2 and CMPE 3 (Figure 4.23 B). It clearly seemed that the inhibition of *M. globosa* lipase activity was decreased after passed through more purification steps, which might cause by synergistic effect of the extracts.

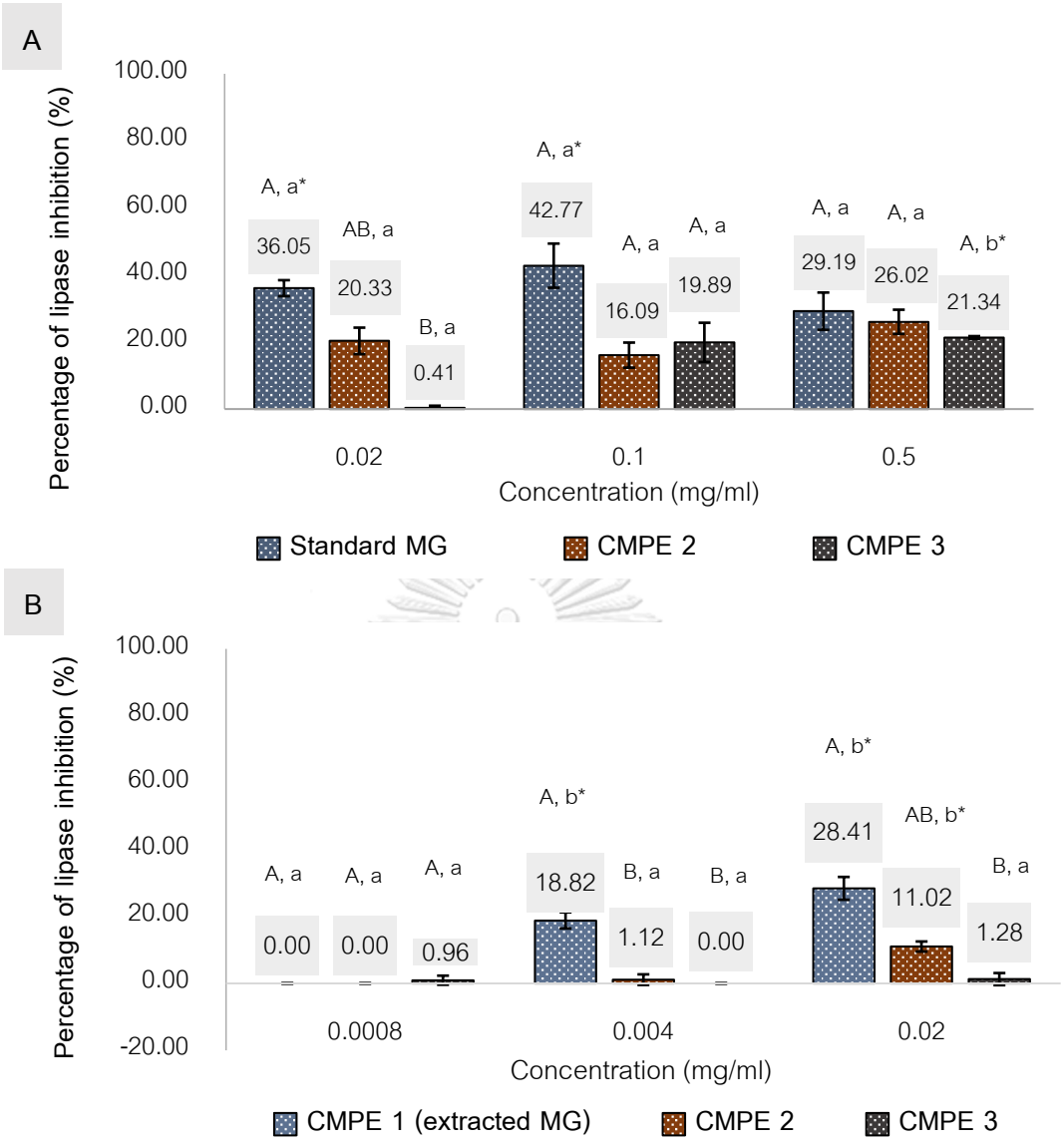


Figure 4.23 Percentage of lipase inhibition of (A) standard MG, CMPE 2, and CMPE 3 at 0.02-0.5 mg/ml and (B) extracted MG, CMPE 2, and CMPE 3 at 0.0008-0.02 mg/ml (≥ 2 independent repeats with ≥ 2 replicates each). Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control [C; 0.1% DMSO for (A), and 0.02% DMSO for (B)] and each concentration within the same extract group, respectively. Capital (uppercase) letters indicate that data is significantly different from each concentration between the different group of extracts ($p \leq 0.05$; One-way ANOVA, Tukey HSD).

4.10.2 Detection of lipase activity on agar plate

Since methyl gallate (MG) proved to have both inhibitory effect to growth and lipase activity of *M. globosa*, standard MG was further tested to confirm the inhibition of lipase activity at higher concentration by cooperating of qualitative and quantitative methods. The percentage of lipase inhibition of standard MG at concentrations 0.063-1 mg/ml was not a dose-dependence which was found in the ranges of 25-34% (Figure 4.24). In addition, lipase activity on agar medium supplemented with indicator dye, Vitoria Blue B (TW60-Vic B) was also investigated at the same concentrations. The lipase activity was clearly reduced after treated with standard MG for 24 h. The intensity of hazy or darker clearance zone was lower relating to the treated concentration (Figure 4.25 A). The results were quite similarly to the treatment with standard MG for 48 h, except for the zone at 0.5 mg/ml which was slightly more intense (Figure 4.25 B). In contrast, the results still related to the previous obtained percentage of lipase inhibition which significantly decreased at that point (Figure 4.24). It was obviously seen that the control treatment of 48 h had more intense zone than 24 h. Thus, different time of incubation might also lead to the increasing of yeast growth and enhancing lipase activity which could affect to the activity of the extract. However, interpretation of the results from both different incubation times could better ensure the activity. It appeared that the lipase inhibitory activity of MG (30.25 ± 1.91%) was lower than percentage of growth inhibition (57.35 ± 0.59%) at the same concentration at 0.063 mg/ml, and it also did not seem to increase in higher concentrations. The lipase inhibition of MG might not relate to the inhibition of *M. globosa* growth, although it could still help to reduce the pathogenicity at low concentration.

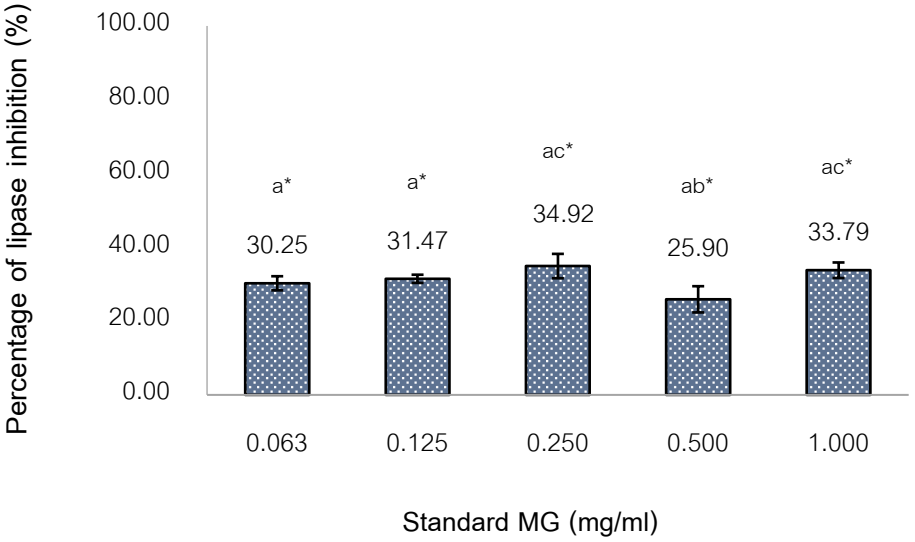


Figure 4.24 Percentage of lipase inhibition of standard MG at concentrations 0.063-1 mg/ml (3 independent repeats with ≥ 2 replicates each). Error bars represent SEM. Symbol " * " and lowercase letters indicate that data is significantly different from control (C, 0.2% DMSO) and each concentration within the same extract group, respectively ($p \leq 0.05$; One-way ANOVA, Tukey HSD).

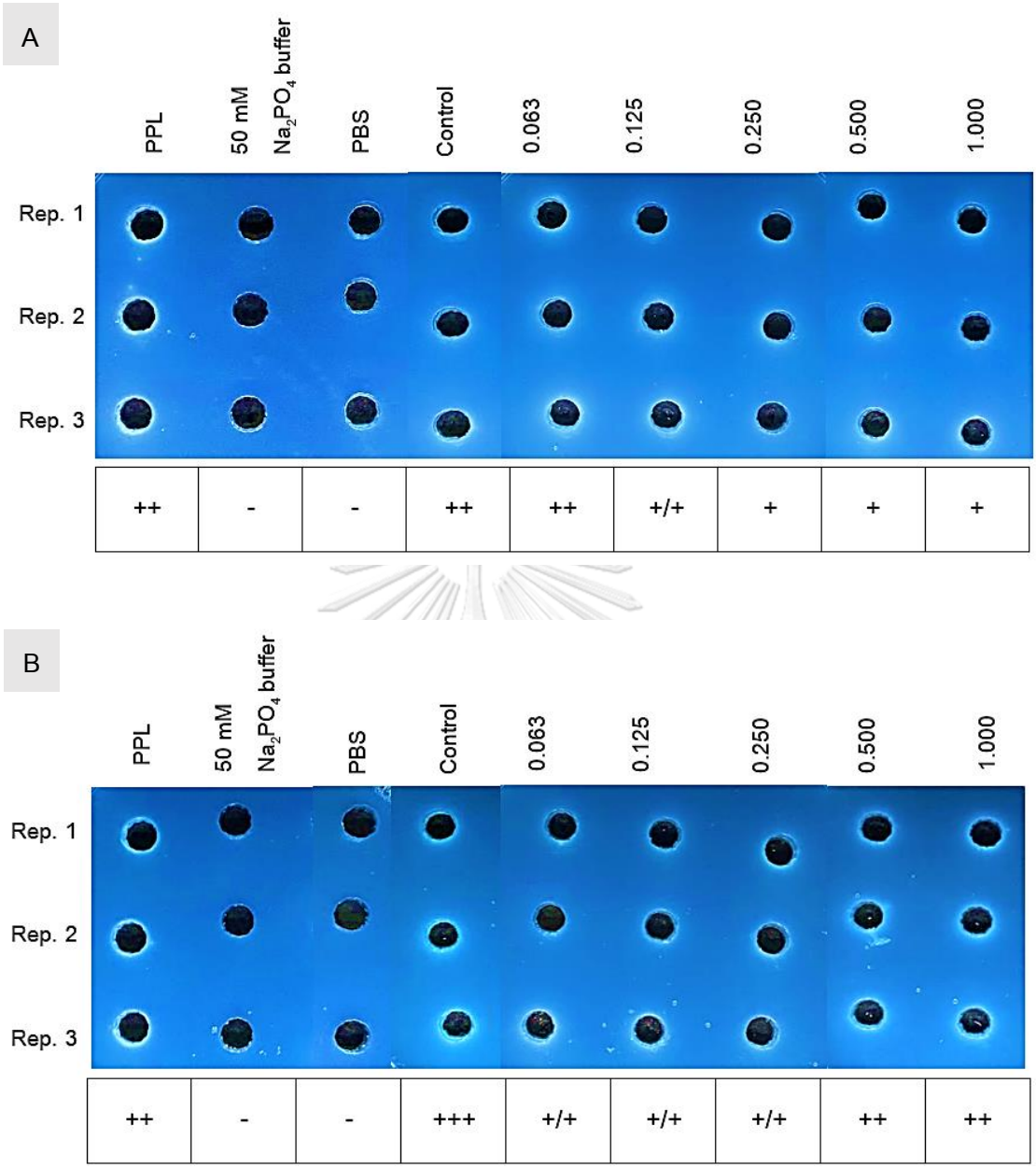


Figure 4.25 Results of lipase detection on TW60-Vic B agar plate from *M. globosa* yeast after treated with standard MG 0.063-1 mg/ml for (A) 24 h and (B) 48 h. Abbreviations; PPL and Rep. refer to porcine pancreatic lipase (2 mg/ml) and replicate, respectively. Symbols "+" and "–" represent intensity of hazy or darker clearance zone scored by visualization.

The overall extraction steps and its activities are summarized as a scheme in Figure 4.26.

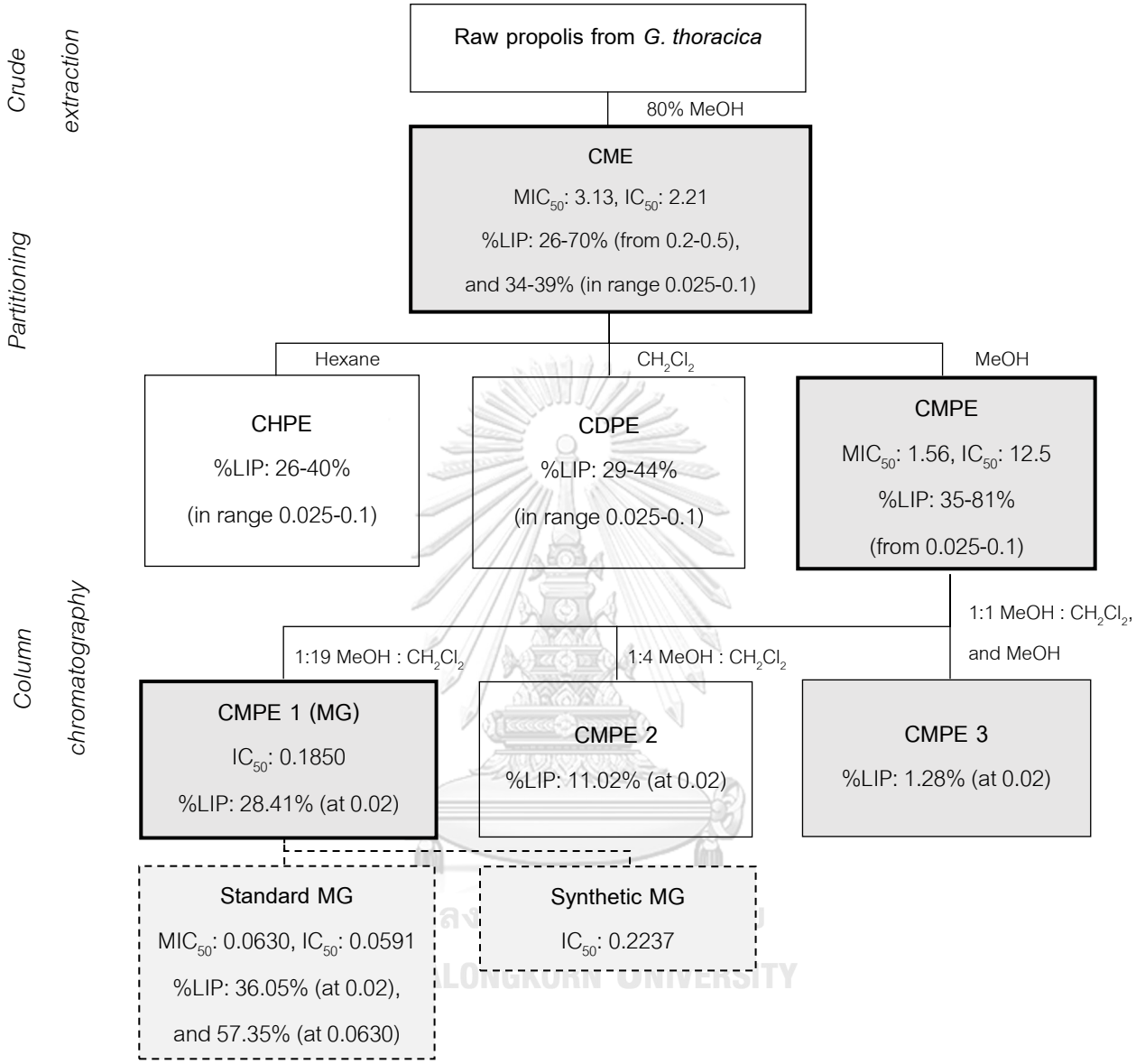


Figure 4.26 Summarization of extraction procedure and some of its inhibitory activities. The boxes with [i] color and [ii] color with bold line indicate the extracts which had [i] anti-*M. globosa* growth activity from the screening by agar well diffusion assay, and [ii] had the best activity, respectively. All concentrations unit are in mg/ml. Abbreviations: MG, MIC_{50}, IC_{50}, and %LIP refer to methyl gallate, Minimum Inhibition Concentration (MIC), Inhibitory Concentration (IC) at 50%, and approximate percentage from *M. globosa*-lipase inhibition (%), respectively.

CHAPTER V
DISCUSSION

The problems of side effects and microbial drug resistance from the commercial antibiotics have been exceedingly emerged in recent years. It is necessary to explore novel antimicrobial compounds. Thus, natural products could be used as a searching resource. Bee products have been proven several health benefits relating to many issues such as systemic disorders, cancer, allergy, and dermatology problems. They are the fascinating products that have been utilized as traditional medicines since ancient times. Various properties of their biological actions have been recognized. Propolis is one of the most important bee products displaying strong antimicrobial activities (Luo et al., 2021; Pasupuleti et al., 2017). Accordingly, the application of propolis by assessment through the activities of their bioactive components could solve the mentioned problems.

Since *Malassezia* infections have been increasingly observed in clinical diagnosis and the problems from available treatments have been reported, the anti-*Malassezia* activity of propolis was investigated in this study. Propolis from stingless bee, *Geniotrigona thoracica* which locally found in Thailand was used. Stingless bee can contribute many products like honeybee and the management cost is also lower. Moreover, stingless bees can provide more propolis (Nazir et al., 2018). The growth and lipase inhibitory activity of the propolis was performed against *Malassezia globosa* which was used as a representative species because of its common and frequent detection in causing diseases. Additionally, *M. globosa* also presents the highest lipase activity. The microscopic and macroscopic features of the yeast was examined before testing which the morphologies were similar to previous reports (Gomare & Mishra, 2018; Shams et al., 2001).

Primary screening of antimicrobial activity of crude extracts was done by agar well diffusion method using ketoconazole (KTZ) at 2 or 4 μg/ml as positive control

which zone of inhibition of both concentrations were slightly different. Since growth pattern could be affected by the stability of extracts and some interference from the other extract concentrations and range of incubation period might be differed. However, it was still in the range of 7-14 days which was mainly considered by negative and positive control. In some case, independent examinations of each extract concentrations were also conducted, especially in high concentration of active sample (Appendix F; Figure F1). CME exhibited zone of inhibition beginning at 200 mg/ml which the zone was also observed in crude ethanol extract from our preliminary study. After partitioning, CMPE exhibited zone of inhibition beginning at 12.5 mg/ml which lower than CME. It seemed that more extraction step could enhance the activity. Since alcoholic extracts including methanol and ethanol can afford antimicrobial activity against *M. globosa*, it could be implied that the active compound should be polar. The alcoholic extraction (70-80%) is the most popular. It is a simple and effective method which provides low wax extract with rich in bioactive compounds, especially polyphenolic components, including flavonoids, phenolic acids, and esters (Gómez-Caravaca et al., 2006). In addition, alcoholic solvents might be suitable for this study since *M. globosa* is lipophilic yeast. Thus, the solvents can remove low polar parts including lipids or fatty acids components. As the evidence shown in Figure 4.11 D, high growth of the yeast colony was observed around the agar wells which were added with the low polar extract part or CHPE. Nonetheless, the use of nonethanol solvents including pure water and oil had also been reported. Concentration of total phenolic contents from nonethanol solvent extractions cooperated with heating (70 ℃) did not differ significantly from ethanolic extracts and the obtained propolis extracts still had antimicrobial activity. In contrast, the extracts without heating process had lower phenolic compounds, and no antimicrobial activity (Kubiliene et al., 2015). However, the purposes in final usage of the extracts may have an impact on solvent selection. The ethanol or aqueous extracts have been commonly used in medical and food developments. Nowadays, several advance techniques for propolis extractions have

been developed, such as microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), and supercritical carbon dioxide extraction (Pobiega et al., 2019).

Later, CMPE was chosen for further purification by using silica gel 60 Å column chromatography which the optimization of mobile phase was conducted before the purification (data not shown). Considering Table 4.4., CMPE 1 displayed the most inhibitory activity, while CMPE 3 also exhibit zone of inhibition except smaller and less stable. The CMPE 1 was extracted with 1:19 (v/v) MeOH : CH_2Cl_2 which had lower polar than the extracted solvent of CMPE 3 including 1:1 (v/v) MeOH : CH_2Cl_2 and MeOH, this indicated that the active part, CMPE 1 had slightly mild polar. Thus, more purification steps could isolate and improve the activity of propolis extract components. Furthermore, this is the evidence supporting that propolis from *G. thoracica* is an interesting natural resource providing more than one potential antimicrobial compound. In this study, only CMPE 1 was analyzed for its chemical structure because of the highest activity of the extract and its TLC pattern with one intense spot. The separating pattern of spots was suggested that it could separate a single chemical composition or more with the same polar and molecular weight. Meanwhile, TLC pattern of CMPE 3 appeared as long smear bands indicated that it might contain more than one different chemical composition. Later, CMPE 1 was identified as methyl gallate comparing to previous reports (Hernández-García et al., 2019; Hisham et al., 2011; Tan et al., 2015). The chemical composition of propolis is varied depending on many factors such as bee species, harvesting period, plant source, geography, and environmental conditions. Propolis from different locations may provide different constituents. For example, Brazilian propolis commonly composed with prenylated phenylpropanoids, prenylated *p*-coumaric acids, acetophenones, diterpenic acids, caffeoylquinic acids, kaempferide, isosakuranetin, and kaempferol, while it mainly consists of flavonoids, phenolic acids, and their esters in Europe, North America, New Zealand, and Asia (Badiazaman et al., 2019).

Methyl gallate (MG) is a polyphenolic compound which is an important ester of gallic acid. It has been used in traditional Chinese medicine due to the varieties of

biological activities including antioxidant, antitumor, anti-inflammatory, anti-spasmodic, anti-atherogenic, and antimicrobial activities (Huang et al., 2021; Mazurova et al., 2015). There are many reports that MG isolated from natural sources presented antimicrobial activity against *Shigella dysenteriae, Streptococcus mutans, Streptococcus epidermidis, Streptococcus saprophyticus, Bacillus cereus, Corynebacterium, Escherichia coli, Salmonella, Enterobacter, Candida albicans, Vibrio cholera*, and herpes simplex virus type 2 (Muhammad et al., 2015; Sánchez et al., 2013; Zheng et al., 2021). Surprisingly, anti-*M. globosa* growth of MG has not been recorded. MG can derive from many plants, such as Galla rhois (*Rhus chinensis* L.), *Toona sinensis*, *Rhus glabra*, *Glochidion superbum* (Ahmed et al., 2017). In this study, propolis was collected from Samut Songkhram province, Thailand where includes many natural features and different seasons. Apart from mainland, Mae Klong River is an importantly natural heritage of the area which provides not only aquatic area, but also wetland area and riverbank. Accordingly, numerous species of mangrove forests, palms, tropical forests, and animals are distributed. Also, stingless bees have been recognized as important and economic insect (Suravanichakit, 2009; National News Bureau of Thailand, 2018). The origin of plants was not exactly determined as propolis is a mixture of resin from various plant species. Nevertheless, the isolation of MG from mango (*Mangifera indica*) twig was presented (Subramanian et al., 2016) and mango trees were found in the sample collecting area.

Due to the low aqueous solubility of propolis, the primary screening by agar well diffusion helps to determine beginning of inhibitory concentrations. The concentration could be further diluted to examine the Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) by other assay, broth microdilution which is a common method with more rapid and reproducible. Also, it requires a few amounts of test sample (The European Society of Clinical Microbiology and Infectious Diseases, 2003). Standard MG was tested along, and it was also used as a representative for extracted MG in some case because of the limitation of extract. Besides, there is still no specifically validate procedure for determining the antifungal susceptibility of *Malassezia*,

which the guidelines recommended by the CLSI and EUCAST have not been covered to slow growth yeast (Chebil et al., 2022). In this study, the optimization for the broth microdilution assay was done to find the suitable inoculum size (data not shown), some supplemented solvents, incubation period, and condition (Appendix G; Figure G1-3). Furthermore, appropriate concentrations of solvents were supplemented to increase the solubility, especially in crude extracts. Firstly, the concentration of DMSO ≤ 10% was used which might have some or less effect to microbial activity. After purification, lower (≤ 1-2%) DMSO was applied which usually approved for testing (Griebler & Slezak, 2001; Summer et al., 2022).

Moreover, Inhibitory Concentration (IC) was also calculated to estimate inhibitory concentration at the exact response point, while MIC was interpreted from experimental data by two-fold dilution and might not cover all concentrations. The formula obtained from the estimation was displayed as $y = \frac{100}{(1+10^{[(\mathrm{LogIC50}-x)*\mathrm{HillSlope})]})}$. As our data set did not provide a full sigmoidal curve, formula model was fitted by solid control data which bottom value (percentage of response when inhibitor is absent) and top value (percentage of response at high concentrations) were set as 0% and 100%, respectively according to the guideline from Prism GraphPad Software version 8. The different data sets were used for standard MG with more samples size and boarder concentration ranges comparing to synthetic MG and CMPE 1. Hence, the R^2 values of synthetic MG and CMPE 1 from the estimation might be lower. However, standard MG was also tested and estimated at the same condition of synthetic MG and CMPE 1. The obtained IC_{50} values of standard MG from both data sets in different conditions were still close which could support the viability and reproducibility of the results (Figure 4.18 C and Figure 4.19 A).

Considering MICs or ICs values from broth microdilution were lower whereas KTZ were quite higher than the beginning inhibitory concentration from agar well diffusion. In case of KTZ, the MIC value was also higher than other reports. For example, Wang et al. (2020) found MICs in range of 0.03-16 μg/ml for *Malassezia* (*M. furfur, M. sympodialis, M. pachydermatis*, and *M. globosa*). However, high MICs of

KTZ had been reported in some study such as > 4 μg/ml and > 20 μg/ml (Leong et al., 2017; Pandey et al., 2019). There also had a report about higher MIC_{50} of KTZ in skin lesions than in healthy skin (Cafarchia et al., 2012) which our study strain was isolated from patient with atopic dermatitis. The high MIC of KTZ in this study might be mainly caused by the interference of solvent to yeast growth including in solvent control treatment leading to generate the low percentage of inhibition. However, the MIC_{50} of KTZ was still lower than all extracts. Moreover, our tested strain might not verify a resistance since it was still susceptible to KTZ at 0.5-4 μg/ml by using agar well diffusion assay (data not shown). As known, there are many factors that might affect the sensitivity of the different methods such as species and strain of microorganism, inoculum size, culture medium type, interactions between the components and solvent or diluent, formation of emulsion, time, and condition of incubation (David et al., 2021). Therefore, both methods could confirm the anti-*M. globosa* growth activity of propolis extract. Also, the overall trend of results was still relevant in both assays which MICs and ICs values displayed dose-dependent manners, and more purification steps could enhance the growth inhibitory activity of components. Thus, the inactive or antagonist compounds might be removed. The standard MG significantly presented MICs or ICs ($p \leq 0.05$) comparing to synthetic and extracted (CMPE 1) MG on the strength of higher purity of the compounds. Only MFC value of (standard) MG was presented because the limitation of synthetic and extracted MG, and poor solubility of CME and CMPE (≤ 500 mg/ml in DMSO). As the chemical profile of synthetic MG was primarily checked by TLC technique, the synthetic and standard MG should be further analyzed and confirmed the chemical structures.

There are few reports of anti-*Malassezia* activity by propolis. In case of agar well diffusion method, Nadăş et al. (2007) found that alcoholic extract or propolis tincture exhibited the most activity against 14 strains of *M. pachydermatis* comparing to other bee products such as honey and royal jelly. It had zone of inhibition ranged from 19-26 mm. For broth microdilution method, Cardoso et al. (2010) reported that ethanolic extract of Brazilian propolis exhibited MIC_{50} at 2.6 mg/ml and MFC_{90} at 5.3 mg/ml for

M. pachydermatis isolated from dogs with canine otitis. Deegan et al. (2019) found that supercritical and ethanolic extracts of three types (green, red, and brown) of Brazilian propolis could inhibit *M. pachydermatis* from both normal and resistant clinical isolates. The ethanolic extract of Brazilian red propolis had the highest activity (MIC_{90} at 4 mg/ml; 84.62% of isolates), followed by supercritical extract of red propolis (MIC_{90} at 4 mg/ml; 53.85% of isolates), ethanolic extract of green (MIC_{90} at 4 mg/ml; 23.08% of isolates), and brown propolis (MIC_{90} 16 mg/ml; 76.92% of isolates). Only ethanolic extracts of red and green propolis showed the highest fungicidal activity. In addition, Khosravi et al. (2013) proved that *M. globosa*, *M. slooffiae*, and *M. pachydermatis*) isolated from onychomycosis patients were susceptible to ethanolic extract of Iranian propolis from *A. mellifera* at MIC_{80} ranged from 2-6 μg/ml.

As almost *Malassezia* species are lipid dependent yeasts, the activities of extracellular lipases are vital for their survival and pathogenesis roles. In this study, we are encouraged to investigate the efficiency of the propolis to *M. globosa* lipase. Quantitative and qualitative assay were performed including colorimetric method using *p*-nitrophenyl palmitate as a lipase substrate, and agar-based method supplemented with indicator dye (TW60-Vic B agar), respectively. Considering the results by colorimetric method, only crude extracts, CME, and CMPE showed percentages of lipase inhibition in dose-dependent pattern. They also had a potent inhibitory effect to lipase activity at concentrations (0.0025-0.5 mg/ml) lower than MIC_{50} (CME: 1.56 mg/ml and CMPE: 3.13 mg/ml). Meanwhile, the application of more purification steps in this study appeared to decrease the inhibitory activity. It could be a consequence of synergistic effect of components since natural extracts comprise with varieties of molecules leading to the complex interactions between the constituents (Caesar & Cech, 2019). Since most of extracts including MG did not exhibit dose-dependent pattern and the varieties of the activity were observed in different concentration ranges, the estimation of ICs values or others were not reported in this study. The assessment to all concentrations, especially high extract concentrations, might be not possible due to the effect of solvents, color interference from some extracts (Appendix L; Figure L1),

and low concentration of crude lipase. In different repeat, some percentages of lipase inhibition were slightly fluctuated at the same concentration through the variation of crude lipase concentration obtained from different lots. Thus, the experiment was performed at least 2 separated repeats to certify the activity. Furthermore, the optimization of suitable incubation time, condition, inoculum size in assay, and production of lipase were conducted (Appendix K; Figure K1 and Figure K4). The positive control for *Malassezia* lipase inhibition was excluded because commercial drugs were not available or less well-known. In this work, crude lipase was still used for the screening, while a few inhibitors had been reported to some specific lipases (Ali et al., 2019; Guo et al., 2015). However, negative control or background (without lipase supplement) was assayed along in each treatment. Also, porcine pancreatic lipase (0.02 mg/ml) was additionally performed to assure the availability of the assay which the visual change in color to yellow and change in absorbance was observed in all experiments (Appendix K; Figure K2-3). Standard MG was used as a representative, even so extracted MG was tested at lower concentration. At the overlap concentration 0.02 mg/ml (Figure 4.23 A-B), both MG were proved as the most active compound comparing to other components. In addition, the influence of MG to lipolytic activity was mainly detected in TW60-Vic B agar due to more rapid change than without indicator dye supplement (TW60 agar). In TW60-Vic B agar, the change of indicator (Victoria blue) to darker or turbid blue zones was observed within 1-2 days which was caused by free fatty acids released from lipolysis (Wang et al., 2009). In contrast, TW60 agar was observed for opaque halo zone around the colonies after 7-10 days which was formed by crystals of the calcium salt after lipolysis (Sierra, 1957) (Appendix K; Figure K4). The different zone appearances from time of incubation, 24 h and 48 h might be different due to the increasing of yeast growth leading to stronger lipase activity (Figure 25 A-B). Nevertheless, the intensification zone with darker or hazy was appeared and some were reduced by MG which was related to the colorimetric method (Figure 24-25). The interpretation by using both methods could ensure the activity of lipase inhibition by MG.

Together, the anti-*M. globosa* activity by MG did not relate to lipase inhibition because percentage of lipase inhibition was lower than percentage of growth inhibition at the same concentration including at MIC_{50} 0.063 mg/ml. Also, it did not trend to increase in higher concentrations (Figure 4.15A and Figure 4.24). Considering at low concentration, percentage of growth inhibition by (standard) MG was found 14% at 0.016 mg/ml and might trend to reduce by dose dependence, while (extracted) MG still exhibited percentage of lipase inhibition up to 18% at concentration 0.004 mg/ml or 4x lower. Interestingly, anti-*M. globosa* lipase activity by MG has not been reported yet. It was previously reported that MG from methanolic extract of Galla Rhois (IC_{50} > 300 μM), and other natural extracts which containing MG including ethyl extract of *Phyllanthus emblica* (IC_{50} 2.45 ± 0.003 mg/ml), and aqueous extracts of *Rhus coriaria* (IC_{50} 19.95 μg/ml) had inhibitory activity to pancreatic lipase (Jaradat et al., 2017; Kwon et al., 2013; Sapkota et al., 2022).

In this work, a major mechanism of MG on anti-*M. globosa* activity is still unveiled. There are many action pathways of compounds affecting microbial inhibition. Flavonoid and phenolic compounds have been revealed the influence on cell wall and membrane which is important for microbial survival as the first protection barrier, exchanging gateway of matter, energy, and information. Some of alkyl esters of gallic acid including MG were reported for antimicrobial activity through membrane damaging and causing membrane hyperpolarization which could affect cell homeostasis. Furthermore, MG had been proved to affect the tricarboxylic acid (TCA) cycle via inhibiting activities of succinate dehydrogenase and malate de hydrogenase. As MG has lipophilic properties, other action mechanisms for antimicrobial activity had been proposed such as inhibition of microbial extracellular enzymes, and microbial metabolism through inhibition of oxidative phosphorylation. For example, *in vitro* inhibitory effect of MG to adherence of *Streptococcus mutans* was demonstrated. Mechanisms of MG action against *Salmonella* via DNA gyrase or ATPase inhibition were reported (Choi et al., 2014; Kang et al., 2008; Wang et al., 2022). Thus, MG could exhibit many antimicrobial mechanisms and different pathogen species

might be affected by different mechanisms. Hence, a mechanism of MG on anti-*M. globosa* activity should be further investigated.

Therefore, this research revealed that anti-*M. globosa* activities of MG through growth and lipase inhibition could be cooperative by supporting each other for directly inhibiting or killing the pathogenic yeast and reducing the pathogenicity. Less potential may be due to the stability loss of compound. Also, IC_{50} values of (standard) MG 59.14 μg/ml was higher, but still closer to KTZ (31.56 μg/ml) in this research condition. Thus, MG could be developed as a new or combination for anti-*Malassezia* drug in the future. Further evaluation such as toxicity, other mechanisms, and activities, mode of action, standardization in case of crude extract usage, and *ex vivo* or *in vivo* examination are encouraged for drug development.

CHAPTER VI
CONCLUSION

From all crude extracts, crude methanol extracts of Thai propolis from *G. thoracica* including CME and CMPE exhibited the potent anti-*M. globosa* growth activity in dose-dependent manner, which CMPE showed the best activity. More enrichment and purification steps could isolate higher active compound, CMPE 1 (IC_{50} 59.14 μg/ml) which exhibited more anti-*M. globosa* growth activity than CME (IC_{50} 2.21 mg/ml) and CMPE (IC_{50} 1.22 mg/ml). Apart from CMPE 1, CMPE 3 also exhibited anti-*M. globosa* growth activity. Thus, Thai propolis from *G. thoracica* could be a good candidate of natural resources providing more than one anti-*Malassezia* compound. From NMR analysis, it revealed that the main bioactive compound or CMPE 1 was methyl gallate (MG). The IC_{50} values of standard MG with purity > 98.0% (59.14 μg/ml) was lower than the values of both synthetic MG (223.7 μg/ml) and extracted MG (185 μg/ml) due to the purity and stability of the compounds. In this research condition, IC_{50} value of (standard) MG was approximately 2x higher than KTZ (31.56 μg/ml). Therefore, MG could be developed as a new or combination for anti-*Malassezia* drug in the future. Moreover, the MFC value of (standard) MG was indicated at 8 mg/ml with completely no growth of colony while the colonies began to decrease at 3 mg/ml.

The constituents in extracts of Thai propolis from *G. thoracica* displayed synergistic mode against *M. globosa* lipase activity. Only CME and CMPE presented anti-*M. globosa* lipase activity with dose-dependent manners. CMPE had the highest percentage of lipase inhibition (81.11%) at concentration 0.1 mg/ml comparing to other samples including CME (36.45%), CDPE (29.64%), CHPE (26.81%), CMPE 2 (16.09%), CMPE 3 (19.89%), and (standard) MG (42.77%). Anti-*M. globosa* lipase activity of (standard) MG (30.25 ± 1.912%) was lower than percentage of growth inhibition (57.35 ± 0.59%) at 0.063 mg/ml (MIC_{50}), and it also did not increase in higher concentrations. Percentage of growth inhibition by (standard) MG was found 14% at 0.016 mg/ml and had to be decreased as a dose-dependent pattern, while percentage

of lipase inhibition was still presented up to 18% at (extracted) MG concentration 4x lower. Anti-*M. globosa* activities by MG might not relate to lipase inhibition. Thus, both activities might be cooperated by directly inhibiting or killing *M. globosa* and reducing the pathogenicity through lipase inhibition, especially when the stability loss of the compound occurred.

This is the first report revealed MG as potential anti-*M. globosa* compound which is investigated from the natural resources. Lipase inhibitions might not a direct target mechanism of action. Propolis from *G. thoracica* is proved to be an effective source for searching new antifungal agents. This information could be used to develop alternative drugs, supplements, and additive of cosmetic and other products.

REFERENCES

Abdullah, N. A., Zullkiflee, N., Zaini, S. N. Z., Taha, H., Hashim, F., & Usman, A. (2020). Phytochemicals, mineral contents, antioxidants, and antimicrobial activities of propolis produced by Brunei stingless bees *Geniotrigona thoracica*, *Heterotrigona itama*, and *Tetrigona binghami*. *Saudi Journal of Biological Sciences*, *27*(11), 2902–2911.

Ahmed, M. D., Taher, M., Maimusa, A. H., Rezali, M. F., & Mahmud, M. I. A.-d. M. (2017). Antimicrobial activity of methyl gallate isolated from the leaves of *Glochidion superbum* against hospital isolates of methicillin resistant *Staphylococcus aureus*. *Natural Product Sciences*, *23*(1), 5–8.

Ali, S., Khan, F. I., Mohammad, T., Lan, D., Hassan, M. I., & Wang, Y. (2019). Identification and evaluation of inhibitors of lipase from *Malassezia restricta* using virtual high-throughput screening and molecular dynamics studies. *International Journal of Molecular Sciences*, *20*(4), 884–902.

American Type Culture Collection. (2003). Reference strains: how many passages are too many. *Tech Bulletin*, *23*(2), 6–7.

Angileri, M., Pasquetti, M., De Lucia, M., & Peano, A. (2019). Azole resistance of *Malassezia pachydermatis* causing treatment failure in a dog. *Medical Mycology Case Reports*, *23*, 58–61.

Ashbee, H. (2007). Update on the genus *Malassezia*. *Medical Mycology*, *45*(4), 287–303.

Ashley, E. D., & Perfect, J. R. (2022, September 14). *Pharmacology of azoles* https://www.medilib.ir/uptodate/show/495

Athikomkulchai, S. (2008). Propolis: a gift from nature. *Thai Pharmaceutical and Health Science Journal*, *3*(2), 286–295.

Badiazaman, A. A. M., Zin, N. B. M., Annisava, A. R., Nafi, N. E. M., & Mohd, K. S. (2019). Phytochemical screening and antioxidant properties of stingless bee *Geniotrigona thoracica* propolis. *Malaysian Journal of Fundamental and Applied Sciences*, *15*(2-1), 330–335.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79.

Batac, M. C. R., Sison, M. A. C., Cervancia, C. R., & Nicolas, M. E. O. (2020). Honey and Propolis have Antifungal Property against Select Dermatophytes and *Candida albicans*. *Acta Medica Philippina*, *54*(1), 11–14.

Batra, R., Boekhout, T., Guého, E., Cabanes, F. J., Dawson Jr, T. L., & Gupta, A. K. (2005). *Malassezia* Baillon, emerging clinical yeasts. *FEMS Yeast Research*, *5*(12), 1101–1113.

Bebout, D., & Pagola, S. (2009). Methyl gallate. *Acta Crystallographica Section E: Structure Reports Online*, *65*(2), o317–o318.

Benitez, L. L., & Carver, P. L. (2019). Adverse effects associated with long-term administration of azole antifungal agents. *Drugs*, *79*(8), 833–853.

Boekhout, T., Guého-Kellermann, E., Mayser, P., & Velegraki, A. (2010). *Malassezia and the skin: science and clinical practice*. Springer Science & Business Media.

Boonsai, P., Phuwapraisirisan, P., & Chanchao, C. (2014). Antibacterial activity of a cardanol from Thai *Apis mellifera* propolis. *International Journal of Medical Sciences*, *11*(4), 327–366.

Boukraâ, L., Abdellah, F., & Ait-Abderrahim, L. (2013). Antimicrobial properties of bee products and medicinal plants. In A. Méndez-Vilas (Ed.), *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education* (pp. 960–970). Formatex Research Center.

BYJU'S. (n.d.). *NMR spectroscopy* https://byjus.com/chemistry/nmr-spectroscopy

Cabañes, F. J. (2014). *Malassezia* yeasts: how many species infect humans and animals. *PLoS Pathogens*, *10*(2), Article e1003892.

Caesar, L. K., & Cech, N. B. (2019). Synergy and antagonism in natural product extracts: when 1+ 1 does not equal 2. *Natural Product Reports*, *36*(6), 869–888.

Cafarchia, C., Figueredo, L. A., Iatta, R., Colao, V., Montagna, M. T., & Otranto, D. (2012). *In vitro* evaluation of *Malassezia pachydermatis* susceptibility to azole compounds using E-test and CLSI microdilution methods. *Medical Mycology*, *50*(8), 795–801.

Campos, J. F., Santos, U. P. d., Rocha, P. d. S. d., Damião, M. J., Balestieri, J. B. P., Cardoso, C. A. L., Paredes-Gamero, E. J., Estevinho, L. M., de Picoli Souza, K., & Santos, E. L. d. (2015). Antimicrobial, antioxidant, anti-inflammatory, and cytotoxic activities of propolis from the stingless bee *Tetragonisca fiebrigi* (Jataí). *Evidence-Based Complementary and Alternative Medicine*, *2015*, 296186.

Cania, A., Oetari, A., & Sjamsuridzal, W. (2020). Detection of olive oil and Tween 80 utilization by *Rhizopus azygosporus* UICC 539 at various temperatures. *AIP Conference Proceedings*, *2242*(1), 050009.

Cardoso, R. L., Maboni, F., Machado, G., Alves, S. H., & de Vargas, A. C. (2010). Antimicrobial activity of propolis extract against *Staphylococcus coagulase* positive and *Malassezia pachydermatis* of canine otitis. *Veterinary Microbiology*, *142*(3-4), 432–434.

Chayakulkeeree, M., & Denning, D. (2017). Serious fungal infections in Thailand. *European Journal of Clinical Microbiology & Infectious Diseases*, *36*(6), 931–935.

Chebil, W., Haouas, N., Eskes, E., Vandecruys, P., Belgacem, S., Belhadj Ali, H., Babba, H., & Van Dijck, P. (2022). *In vitro* assessment of azole and amphotericin B susceptibilities of *Malassezia* spp. isolated from healthy and lesioned skin. *Journal of Fungi*, *8*(9), 959–968.

Choi, J.-G., Mun, S.-H., Chahar, H. S., Bharaj, P., Kang, O.-H., Kim, S.-G., Shin, D.-W., & Kwon, D.-Y. (2014). Methyl gallate from Galla rhois successfully controls clinical isolates of *Salmonella* infection in both *in vitro* and *in vivo* systems. *PLoS One*, *9*(7), Article e102697.

Chuttong, B., Chanbang, Y., & Burgett, M. (2014). Meliponiculture: Stingless bee beekeeping in Thailand. *Bee World*, *91*(2), 41–45.

Coskun, O. (2016). Separation techniques: chromatography. *Northern Clinics of Istanbul*, *3*(2), 156–160.

David, V., Andrea, A.-N., Aleksandr, K., Lourdes, J.-A., Eugenia, P., Nancy, C., Isabel, W., Jessica, C., & León-Tamariz, F. (2021). Validation of a method of broth microdilution for the determination of antibacterial activity of essential oils. *BMC Research Notes*, *14*(1), 1–7.

Deegan, K. R., Fonseca, M. S., Oliveira, D. C. P., Santos, L. M., Fernandez, C. C., Hanna, S. A., Machado, B. A. S., Umsza-Guez, M. A., Meyer, R., & Portela, R. W. (2019). Susceptibility of *Malassezia pachydermatis* clinical isolates to allopathic antifungals and Brazilian red, green, and brown propolis extracts. *Frontiers in Veterinary Science*, *6*, Article 460.

Desamero, M. J., Kakuta, S., Tang, Y., Chambers, J. K., Uchida, K., Estacio, M. A., Cervancia, C., Kominami, Y., Ushio, H., & Nakayama, J. (2019). Tumor-suppressing potential of stingless bee propolis in *in vitro* and *in vivo* models of differentiated-type gastric adenocarcinoma. *Scientific Reports*, *9*(1), 1–13.

Dutra, R. P., Bezerra, J. L., Silva, M. C. P. d., Batista, M. C. A., Patrício, F. J. B., Nascimento, F. R. F., Ribeiro, M. N. S., & Guerra, R. N. M. (2019). Antileishmanial activity and chemical composition from Brazilian geopropolis produced by stingless bee *Melipona fasciculata*. *Revista Brasileira de Farmacognosia*, *29*, 287–293.

Far, F., Al-Obaidi, M., & Desa, M. (2018). Efficacy of modified Leeming-Notman media in a resazurin microtiter assay in the evaluation of *in-vitro* activity of fluconazole against *Malassezia furfur* ATCC 14521. *Journal de Mycologie Medicale*, *28*(3), 486–491.

Findley, K., Oh, J., Yang, J., Conlan, S., Deming, C., Meyer, J. A., Schoenfeld, D., Nomicos, E., Park, M., & Kong, H. H. (2013). Human skin fungal diversity. *Nature*, *498*(7454), 367–370.

Gharib, A. A., & Taha, M. (2013). Antimicrobial activity of propolis against some bacteria and fungi. *Zagazig Veterinary Journal*, *41*(1), 81–97.

Gomare, K. S., & Mishra, D. (2018). Characterized identification and low cost preservation of *Malassezia* spp. - enabling future possibility for control. *International Journal of Pharmacy and Biological Sciences*, *8*(1), 1–10.

Gómez-Caravaca, A., Gómez-Romero, M., Arráez-Román, D., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2006). Advances in the analysis of phenolic compounds in products derived from bees. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(4), 1220–1234.

GraphPad Software. (2022). *Equation: log(inhibitor) vs. normalized response - variable slope* https://www.graphpad.com/guides/prism/8/curve-fitting/REG_DR_inhibit_normalized_variable.htm

Griebler, C., & Slezak, D. (2001). Microbial activity in aquatic environments measured by dimethyl sulfoxide reduction and intercomparison with commonly used methods. *Applied and Environmental Microbiology*, *67*(1), 100–109.

Gucwa, K., Kusznierewicz, B., Milewski, S., Van Dijck, P., & Szweda, P. (2018). Antifungal activity and synergism with azoles of polish propolis. *Pathogens*, *7*(2), 56–82.

Guo, S., Huang, W., Zhang, J., & Wang, Y. (2015). Novel inhibitor against *Malassezia globosa* LIP1 (SMG1), a potential anti-dandruff target. *Bioorganic & Medicinal Chemistry Letters*, *25*(17), 3464–3467.

Gur, N., Bayrak, N., & Topdemir, A. (2020). Determination of antimicrobial activity and some biochemical properties of honey and propolis in Turkish markets. *Progress in Nutrition*, *22*(3), Article e2020040.

Hay, R. J., Johns, N. E., Williams, H. C., Bolliger, I. W., Dellavalle, R. P., Margolis, D. J., Marks, R., Naldi, L., Weinstock, M. A., & Wulf, S. K. (2014). The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions. *Journal of Investigative Dermatology*, *134*(6), 1527-1534.

Hendi, N. K., Naher, H. S., & Al-Charrakh, A. H. (2011). *In vitro* antibacterial and antifungal activity of Iraqi propolis. *Journal of Medicinal Plants Research*, *5*(20), 5058–5066.

Hernández-García, E., García, A., Avalos-Alanís, F. G., Rivas-Galindo, V. M., Delgadillo-Puga, C., & del Rayo Camacho-Corona, M. (2019). Nuclear magnetic resonance spectroscopy data of isolated compounds from *Acacia farnesiana* (L) Willd fruits and two esterified derivatives. *Data in Brief*, *22*, 255-268.

Hisham, D. M. N., Lip, J. M., Noh, J. M., Normah, A., & Nabilah, M. N. (2011). Identification and isolation of methyl gallate as a polar chemical marker for *Labisia pumila* Benth. *Journal of Tropical Agriculture and Food Science*, *39*(2), 279-284.

Honnavar, P., Chakrabarti, A., Prasad, G. S., Joseph, J., Dogra, S., Handa, S., & Rudramurthy, S. M. (2018). The Lipase Activities of *Malassezia* Species Isolated from Seborrhoeic Dermatitis/Dandruff Patients. *Journal of Clinical & Diagnostic Research*, *12*(5), DC17–DC19.

Hrncir, M., Jarau, S., & Barth, F. G. (2016). Stingless bees (Meliponini): senses and behavior. *Journal of Comparative Physiology A*, *202*(9), 597-601.

Huang, C.-Y., Chang, Y.-J., Wei, P.-L., Hung, C.-S., & Wang, W. (2021). Methyl gallate, gallic acid-derived compound, inhibit cell proliferation through increasing ROS production and apoptosis in hepatocellular carcinoma cells. *PLoS One*, *16*(3), Article e0248521.

Ibrahim, N., Niza, N., Rodi, M. M., Zakaria, A. J., Ismail, Z., & Mohd, K. S. (2016). Chemical and biological analyses of Malaysian stingless bee propolis extracts. *Malaysian Journal of Analytical Sciences*, *20*(2), 413–422.

Integrated Taxonomic Information System. (2022). *Geniotrigona thoracica (Smith, 1857)* https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=763754#null

Jaradat, N., Zaid, A. N., Hussein, F., Zaqzouq, M., Aljammal, H., & Ayesh, O. (2017). Anti-lipase potential of the organic and aqueous extracts of ten traditional edible and medicinal plants in Palestine; a comparison study with orlistat. *Medicines*, *4*(4), 89–101.

Jongjitaree, S., Koontongkaew, S., Niyomtham, N., Yingyongnarongkul, B.-e., & Utispan, K. (2022). The oral wound healing potential of Thai propolis based on its antioxidant activity and stimulation of oral fibroblast migration and proliferation. *Evidence-Based Complementary and Alternative Medicine*, *2022*, Article 3503164.

Jongjitvimol, T. (2014). Biology and species diversity of stingless bees (Apidae: Meliponinae) in Thailand. *Life Sciences and Environment Journal*, *10*(1–2), 12–21.

Kang, M.-S., Oh, J.-S., Kang, I.-C., Hong, S.-J., & Choi, C.-H. (2008). Inhibitory effect of methyl gallate and gallic acid on oral bacteria. *The Journal of Microbiology*, *46*(6), 744–750.

Khalid, A., Ullah, H., Ul-Islam, M., Khan, R., Khan, S., Ahmad, F., Khan, T., & Wahid, F. (2017). Bacterial cellulose-TiO_2 nanocomposites promote healing and tissue regeneration in burn mice model. *RSC Advances*, *7*(75), 47662–47668.

Khongkarat, P., Ramadhan, R., Phuwapraisirisan, P., & Chanchao, C. (2020). Safflospermidines from the bee pollen of *Helianthus annuus* L. exhibit a higher *in vitro* antityrosinase activity than kojic acid. *Heliyon*, *6*(3), Article e03638.

Khosravi, A. R., Shokri, H., Nikaein, D., Mansouri, P., Erfanmanesh, A., Chalangari, R., & Katalin, M. (2013). Yeasts as important agents of onychomycosis: *in vitro* activity of propolis against yeasts isolated from patients with nail infection. *The Journal of Alternative and Complementary Medicine*, *19*(1), 57–62.

King, T. C., & Krogstad, D. J. (1983). Spectrophotometric assessment of dose-response curves for single antimicrobial agents and antimicrobial combinations. *Journal of Infectious Diseases*, *147*(4), 758–764.

Kubiliene, L., Laugaliene, V., Pavilonis, A., Maruska, A., Majiene, D., Barcauskaite, K., Kubilius, R., Kasparaviciene, G., & Savickas, A. (2015). Alternative preparation of propolis extracts: comparison of their composition and biological activities. *BMC Complementary and Alternative Medicine*, *15*(1), 1–7.

Kuiper, K., Devlin, P., P., K., Sharpe, P., Boomfield, C., & Silvester, H. (2019). Chromatography techniques In *Chemistry for Queensland unit 1 and 2* (pp. 3–27). Oxford University Press.

Kwapong, P., Aidoo, K., Combey, R., & Karikari, A. (2010). Stingless bees. In *Importance, management and utilization: a training manual for stingless beekeeping* (1 ed., pp. 1–70). Macmillan Publishers Limited.

Kwon, O. J., Bae, J.-S., Lee, H. Y., Hwang, J.-Y., Lee, E.-W., Ito, H., & Kim, T. H. (2013). Pancreatic lipase inhibitory gallotannins from Galla Rhois with inhibitory effects on adipocyte differentiation in 3T3-L1 cells. *Molecules*, *18*(9), 10629–10638.

Lee, Y., & Goldman, M. (1997). The role of azole antifungal agents for systemic antifungal therapy. *Cleveland Clinic journal of medicine*, *64*(2), 99–106.

Leong, C., Buttafuoco, A., Glatz, M., & Bosshard, P. P. (2017). Antifungal susceptibility testing of *Malassezia* spp. with an optimized colorimetric broth microdilution method. *Journal of Clinical Microbiology*, *55*(6), 1883–1893.

Leong, C., Chan, J. W. K., Lee, S. M., Lam, Y. I., Goh, J. P., Ianiri, G., & Dawson Jr, T. L. (2021). Azole resistance mechanisms in pathogenic *Malassezia furfur*. *Antimicrobial Agents and Chemotherapy*, *65*(5), Article e01975–01920.

LibreTexts libraries. (2022, October 12). *Nuclear magnetic resonance* https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Supplemental_Modules_(Physical_and_Theoretical_Chemistry)/Spectroscopy/Magnetic_Resonance_Spectroscopies/Nuclear_Magnetic_Resonance

Lorch, J., Palmer, J., Vanderwolf, K., Schmidt, K., Verant, M., Weller, T., & Blehert, D. (2018). *Malassezia vespertilionis* sp. nov.: a new cold-tolerant species of yeast isolated from bats. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, *41*(1), 56–70.

Lumsa-ed, J., Muangtip, K., & Sutthaso, W. (2019). Diversity of the stingless bees (Apidae: Meliponini) in Surat Thani Province. *KKU Science Journal*, *47*(1), 93–100.

Luo, X., Dong, Y., Gu, C., Zhang, X., & Ma, H. (2021). Processing technologies for bee products: an overview of recent developments and perspectives. *Frontiers in Nutrition*, *8*, Article 727181.

Lyles, R. H., Poindexter, C., Evans, A., Brown, M., & Cooper, C. R. (2008). Nonlinear model-based estimates of IC_{50} for studies involving continuous therapeutic dose-response data. *Contemporary Clinical Trials*, *29*(6), 878–886.

Makvandi, P., Josic, U., Delfi, M., Pinelli, F., Jahed, V., Kaya, E., Ashrafizadeh, M., Zarepour, A., Rossi, F., & Zarrabi, A. (2021). Drug delivery (nano) platforms for oral and dental applications: tissue regeneration, infection control, and cancer management. *Advanced Science*, *8*(8), Article 2004014.

Maximo da Silva, M., Comin, M., Santos Duarte, T., Foglio, M. A., De Carvalho, J. E., do Carmo Vieira, M., & Nazari Formagio, A. S. (2015). Synthesis, antiproliferative activity and molecular properties predictions of galloyl derivatives. *Molecules*, *20*(4), 5360–5373.

Mazurova, J., Kukla, R., Rozkot, M., Lustykova, A., Slehova, E., Sleha, R., Lipensky, J., & Opletal, L. (2015). Use of natural substances for boar semen decontamination. *Veterinarni Medicina*, *60*(5), 235–247.

Muhammad, M. T., Fayyaz, N., Tauseef, S., Razaq, U., Versiani, M. A., Ahmad, A., Faizi, S., & Rasheed, M. (2015). Antibacterial activity of flower of *Melia azedarach* Linn. and identification of its metabolites. *Journal of the Korean Society for Applied Biological Chemistry*, *58*(2), 219–227.

Nadăş, G., Răpuntean, G., Fiń, N., & Cuc, C. (2007). The antimycotic effect of some bee products and vegetal extracts on genus *Malassezia* fungi. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca*, *64*, 198–201.

National News Bureau of Thailand. (2018, July 13). *PRD leads foreign media on tour of secondary cities* http://nwnt.prd.go.th/centerweb/newsen/NewsDetail?NT01_NewsID=WNECO6107140010004

Nazir, H., Shahidan, W. N. S., Ibrahim, H. A., & Tuan Ismail, T. N. N. (2018). Chemical constituents of Malaysian *Geniotrigona thoracica* propolis. *Pertanika Journal of Tropical Agricultural Science*, *41*(3), 955–962.

Nolkemper, S., Reichling, J., Sensch, K. H., & Schnitzler, P. (2010). Mechanism of herpes simplex virus type 2 suppression by propolis extracts. *Phytomedicine*, *17*(2), 132–138.

Oliveira, A. C. P., Shinobu, C. S., Longhini, R., Franco, S. L., & Svidzinski, T. I. E. (2006). Antifungal activity of propolis extract against yeasts isolated from onychomycosis lesions. *Memórias do Instituto Oswaldo Cruz*, *101*, 493–497.

Oryan, A., Alemzadeh, E., & Moshiri, A. (2018). Potential role of propolis in wound healing: biological properties and therapeutic activities. *Biomedicine & Pharmacotherapy*, *98*, 469–483.

Pandey, P., Upadhya, R. K., & Singh, V. R. (2019). Screening of antifungal activity of essential oils and its chemical composition against *Malassezia furfur*. *International Journal of Bio-Technology and Research*, *9*(2), 31–36.

Park, M., Park, S., & Jung, W. H. (2021). Skin commensal fungus *Malassezia* and its lipases. *Journal of Microbiology and Biotechnology*, *31*(5), 637–644.

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity*, *2017*, Article 1259510.

Pobiega, K., Kraśniewska, K., Derewiaka, D., & Gniewosz, M. (2019). Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods. *Journal of Food Science and Technology*, *56*(12), 5386–5395.

Pohanka, M. (2019). Biosensors and bioassays based on lipases, principles and applications, a review. *Molecules*, *24*(3), 616.

Pumirat, P., Tunyong, W., & Luplertlop, N. (2013). Medical mycology. *Journal of Medicine and Health Sciences*, *20*(2), 31–44.

Ramnath, L., Sithole, B., & Govinden, R. (2017). Identification of lipolytic enzymes isolated from bacteria indigenous to *Eucalyptus* wood species for application in the pulping industry. *Biotechnology Reports*, *15*, 114–124.

Rasmussen, C., & Cameron, S. A. (2010). Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biological Journal of the Linnean Society*, *99*(1), 206–232.

Rattanawannee, A., & Duangphakdee, O. (2019). Southeast Asian meliponiculture for sustainable livelihood. In *Modern beekeeping-bases for sustainable production* (pp. 173–189). IntechOpen.

Rautenbach, M., Gerstner, G. D., Vlok, N. M., Kulenkampff, J., & Westerhoff, H. V. (2006). Analyses of dose–response curves to compare the antimicrobial activity of model cationic α-helical peptides highlights the necessity for a minimum of two activity parameters. *Analytical Biochemistry*, *350*(1), 81–90.

Reller, L. B., Weinstein, M., Jorgensen, J. H., & Ferraro, M. J. (2009). Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases*, *49*(11), 1749–1755.

Rhimi, W., Theelen, B., Boekhout, T., Otranto, D., & Cafarchia, C. (2020). Malassezia spp. yeasts of emerging concern in fungemia. *Frontiers in Cellular and Infection Microbiology*, *10*, Article 370.

Rojas, F. D., Sosa, M. d. l. A., Fernandez, M. S., Cattana, M. E., Cordoba, S. B., & Giusiano, G. E. (2014). Antifungal susceptibility of *Malassezia furfur*, *Malassezia sympodialis*, and *Malassezia globosa* to azole drugs and amphotericin B evaluated using a broth microdilution method. *Sabouraudia*, *52*(6), 641–646.

Roubik, D. W. (2022). Stingless Bee (Apidae: Apinae: Meliponini) Ecology. *Annual Review of Entomology*, *68*, 231–256.

Rozman, A. S., Hashim, N., Maringgal, B., & Abdan, K. (2022). A comprehensive review of stingless bee products: phytochemical composition and beneficial properties of honey, propolis, and pollen. *Applied Sciences*, *12*(13), 6370.

Samad, M. Y. A., Razak, C. N. A., Salleh, A. B., Yunus, W. Z. W., Ampon, K., & Basri, M. (1989). A plate assay for primary screening of lipase activity. *Journal of Microbiological Methods*, *9*(1), 51–56.

Sánchez, E., Heredia, N., Camacho-Corona, M. d. R., & García, S. (2013). Isolation, characterization and mode of antimicrobial action against *Vibrio cholerae* of methyl gallate isolated from *A cacia farnesiana*. *Journal of Applied Microbiology*, *115*(6), 1307–1316.

Sapkota, B. K., Khadayat, K., Aryal, B., Bashyal, J., Jaisi, S., & Parajuli, N. (2022). LC-HRMS-based profiling: antibacterial and lipase inhibitory activities of some medicinal plants for the remedy of obesity. *Scientia Pharmaceutica*, *90*(3), 55–71.

Sarker, S. D., Nahar, L., & Kumarasamy, Y. (2007). Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods*, *42*(4), 321–324.

Seifert, K. A. (2013). The human microbiome project: fungi on human skin. *IMA Fungus*, *4*, 16–18.

Seth, D., Cheldize, K., Brown, D., & Freeman, E. E. (2017). Global burden of skin disease: inequities and innovations. *Current Dermatology Reports*, *6*(3), 204–210.

Sforcin, J. M. (2016). Biological properties and therapeutic applications of propolis. *Phytotherapy Research*, *30*(6), 894–905.

Shams, M., Rasaee, M. J., Moosavi, M., & Razzaghi, M. (2001). Identification of *Malassezia* species in patients with pityriasis versicolor submitted to the Razi Hospital in Tehran. *Iranian Biomedical Journal*, *5*(4), 121–126.

Shanahan, M., & Spivak, M. (2021). Resin use by stingless bees: a review. *Insects*, *12*(8), 719–738.

Shehu, A., Ismail, S., Rohin, M. A. K., Harun, A., Abd Aziz, A., & Haque, M. (2016). Antifungal properties of Malaysian Tualang honey and stingless bee propolis against *Candida albicans* and *Cryptococcus neoformans*. *Journal of Applied Pharmaceutical Science*, *6*(2), 44–50.

Sierra, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek*, *23*(1), 15–22.

Sivasankar, C., Gayathri, S., Bhaskar, J. P., Krishnan, V., & Pandian, S. K. (2017). Evaluation of selected Indian medicinal plants for antagonistic potential against *Malassezia* spp. and the synergistic effect of embelin in combination with ketoconazole. *Microbial Pathogenesis*, *110*, 66–72.

Sparber, F., & LeibundGut-Landmann, S. (2017). Host responses to *Malassezia* spp. in the mammalian skin. *Frontiers in Immunology*, *8*, Article 1614.

Stoytcheva, M., Montero, G., Zlatev, R., A Leon, J., & Gochev, V. (2012). Analytical methods for lipases activity determination: a review. *Current Analytical Chemistry*, *8*(3), 400–407.

Subramanian, R., Chandra, M., Yogapriya, S., Aravindh, S., & Ponmurugan, K. (2016). Isolation of methyl gallate from mango twigs and its anti-biofilm activity. *Journal of Biologically Active Products from Nature*, *6*(5–6), 383–392.

Summer, K., Browne, J., Hollanders, M., & Benkendorff, K. (2022). Out of control: The need for standardised solvent approaches and data reporting in antibiofilm assays incorporating dimethyl-sulfoxide (DMSO). *Biofilm*, *4*, 100081.

Suravanichakit, N. (2009). *Natural heritage value and sustainable use of the lower segment of The Mae Klong River, Samut Songkhram Province* [Doctoral dissertation, Silpakorn University].

Tan, Y. P., Chan, E. W. C., & Lim, C. S. Y. (2015). Potent quorum sensing inhibition by methyl gallate isolated from leaves of *Anacardium occidentale* L. (cashew). *Chiang Mai Journal of Science*, *42*(3), 650–656.

The European Society of Clinical Microbiology and Infectious Diseases. (2003). Determination of minimum inhibitory concentrations (MICs) of antibaterial agents by broth dilution. *Clinical Microbiology and Infection*, *9*, 1–7.

Thomson, C. A., Delaquis, P. J., & Mazza, G. (1999). Detection and measurement of microbial lipase activity: a review. *Critical Reviews in Food Science and Nutrition*, *39*(2), 165–187.

Tuckman, A. (2017). The potential psychological impact of skin conditions. *Dermatology and Therapy*, *7*(1), 53–57.

Umthong, S., Phuwapraisirisan, P., Puthong, S., & Chanchao, C. (2011). *In vitro* antiproliferative activity of partially purified *Trigona laeviceps* propolis from Thailand on human cancer cell lines. *BMC Complementary and Alternative Medicine*, *11*(1), 1–8.

Veiga, F. F., Gadelha, M. C., Da Silva, M. R., Costa, M. I., Kischkel, B., de Castro-Hoshino, L. V., Sato, F., Baesso, M. L., Voidaleski, M. F., & Vasconcellos-Pontello, V. (2018). Propolis extract for onychomycosis topical treatment: from bench to clinic. *Frontiers in Microbiology*, *9*, Article 779.

Wang, H., Liu, R., Lu, F., Qi, W., Shao, J., & Ma, H. (2009). A novel alkaline and low-temperature lipase of *Burkholderia cepacia* isolated from Bohai in China for detergent formulation. *Annals of Microbiology*, *59*(1), 105–110.

Wang, K., Cheng, L., Li, W., Jiang, H., Zhang, X., Liu, S., Huang, Y., Qiang, M., Dong, T., & Li, Y. (2020). Susceptibilities of *Malassezia* strains from pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis to antifungal drugs. *Heliyon*, *6*(6), Article e04203.

Wang, Y., Xu, Y., & Liu, Z. (2022). A review of plant antipathogenic constituents: Source, activity and mechanism. *Pesticide Biochemistry and Physiology*, *188*, 105225.

Weebly. (2009). *Spectroscopy: principles, theory, techniques and applications* https://mazams.weebly.com/uploads/4/8/2/6/48260335/spectroscopyovrview.pdf

White, T. C., Findley, K., Dawson, T. L., Scheynius, A., Boekhout, T., Cuomo, C. A., Xu, J., & Saunders, C. W. (2014). Fungi on the skin: dermatophytes and *Malassezia*. *Cold Spring Harbor Perspectives in Medicine*, *4*(8), Article a019802.

Wijaya, W. H., Timotius, K. H., & Wijaya, J. K. (2020). Extracellular lipase of *Malassezia* as anti dandruff drug target: a review. *Systematic Reviews in Pharmacy*, *11*(8), 446–451.

Xool-Tamayo, J., Chan-Zapata, I., Arana-Argaez, V. E., Villa-de la Torre, F., Torres-Romero, J. C., Araujo-Leon, J. A., Aguilar-Ayala, F. J., Rejón-Peraza, M. E., Castro-Linares, N. C., & Vargas-Coronado, R. F. (2020). *In vitro* and i*n vivo* anti-inflammatory properties of Mayan propolis. *European Journal of Inflammation*, *18*, 1–11.

Yang, S., Peng, L., Su, X., Chen, F., Cheng, Y., Fan, G., & Pan, S. (2011). Bioassay-guided isolation and identification of antifungal components from propolis aga inst *Penicillium italicum*. *Food Chemistry*, *127*(1), 210–215.

Zheng, D., Xu, Y., Yuan, G., Wu, X., & Li, Q. (2021). Bacterial ClpP protease is a potential target for methyl gallate. *Frontiers in Microbiology*, *11*, Article 598692.

APPENDICES

Appendix A: Chemicals and equipments

1. Chemicals

- Calcium chloride dehydrate, $CaCl_2$, M.W. = 110.99, Fluka, Switzerland
- Dimethyl sulfoxide, $(CH_3)_2SO$, M.W. = 78.13, Honeywell Riedel-de Haen, Germany
- Di sodium hydrogen phosphate, Na_2HPO_4, M.W. 141.96, VWR International, LLC., USA
- Gummi arabicum, Merck, Germany
- Hexane, C_6H_{14}, M.W. = 86.18, TSL chemical, Thailand
- Immersion oil for microscopy, Olympus, Japan
- Ketoconazole, Sigma-Aldrich, USA
- Methanol, CH_3OH, M.W. = 32.04, TSL chemical, Thailand
- Methanol-*d*4, CD_3OD, M.W. = 36.07, Eurisotop, Cambridge Isotope Laboratories, Inc., USA
- Methylene chloride, CH_2Cl_2, M.W. = 84.93, TSL chemical, Thailand
- Methylene blue hydrate, Sigma-Aldrich, USA
- Methyl gallate, $C_8H_8O_5$, M.W. = 184.15, purity: >98.0% (GC)(T), TCI, Japan
- Modified Leeming-Notman (MLN) media components (Appendix B), HiMedia, India and Sigma-Aldrich, USA
- Molecular sieves, 4 Å 1.6-2.6 mm, Sigma-Aldrich, USA
- Porcine pancreatic lipase Type II, 30-90 units/mg protein, Sigma-Aldrich, USA
- Potassium dihydrogen phosphate, KH_2PO_4, M.W. = 136.08, Merck, Germany
- *p*-nitrophenyl palmitate, Sigma-Aldrich, USA
- Resazurin sodium salt, Sigma-Aldrich, USA
- Silica gel 60 for column chromatography (0.063-0.200 mm), SiO_2, M.W. = 60.08, Merck, Germany
- Sodium chloride, NaCl, Merck, Germany
- Sodium desoxycholate, Sigma-Aldrich, USA
- Sodium dihydrogen phosphate dihydrate, M.W. = 156.02, Merck, Germany
- Sodium hydroxide, NaOH, Merck, Germany
- Sodium sulfate anhydrous, Supelco, Merck, Germany
- TLC silica gel 60 F_{254}, Merck, Germany
- Tris base, Sigma-Aldrich, USA

- Tri-sodium citrate dihydrate, Merck, Germany
- Victoria blue, BHD chemical, Ltd., England

2. Equipments

- 96-well microplate, Thermo Scientific, Germany
- Autoclave, Model: SX-500E, TOMY, Japan
- Automatic micropipette, P10, P20, P100, P200, and P1,000 μl, Eppendorf, Germany
- Beaker, model: 50, 250, 600, and 1,000 ml, Pyrex, Germany
- Biological safety cabinet Class II, Biobase Biodusty (Shandong), Co., Ltd., China
- Biological safety cabinet Class II, Model: V6-T Microtech, LabMicrotech, Thailand
- Barnstead™ Smart2Pure™ Water Purification System, Thermo Scientific, USA
- Centrifuge 5804R, Eppendorf, Germany
- Centrifuge, MIKRO 200/200R centrifuge, Hettich, Merck, Germany
- Column chromatography, model: 250 and 500 ml, NK Laboratory, Thailand
- Conical Polypropylene Centrifuge Tubes, Nunc™ 15 ml and 50 ml, Thermo Scientific, USA
- Digital dry bath, Accublock, Labnet International, Inc., Germany
- Drying oven, Model: SOV140B, KWF, China
- Flask, model: 50, 250, 500, and 1,000 mL, Schott Duran, Germany
- Freezer (-20 ℃), model: MDF-U332, Sanyo, Japan
- Glass bottle, Screw Cap Bottle, Duran, Germany
- Hemocytometer, BOECO, Germany
- Laminar flow, Model: Clean H2, LAB service Ltd., Part, Thailand
- Light microscope, Olympus, Germany
- Light microscope series and Olympus digital imaging software, Olympus, Germany
- Measuring cylinder, model: 10, 100, 500, and 1,000 ml, Witex, Germany
- Microcentrifuge tube, polypropylene 1.5 ml, Biologix, China
- Microplate reader, EnSight Multimode Plate Reader, PerkinElmer, USA
- Microwave, Sharp, Japan
- Multichannel pipette, Omnipette 8 channel, Cleaver scientific, UK
- NMR spectrometer, JNM-ECZ500R, Jeol, USA

- pH meter, pH100 Ionix, Nexbio, Thailand
- Pipette tip, volume 10, 100, and 1,000 ml, QSP, Molecular BioProduct, Inc., USA
- Refrigerator (4 ℃), model: NR-BT262, Panasonic, Thailand
- Refrigerator (4 ℃), LG, Thailand
- Rotary evaporator, model: Basis Hei-VAP Value, Heidolph, Germany
- Round bottom flask, model: 50, 100, 500, and 1,000 mL, NK Laboratory, Schott Duran, Germany
- Separatory funnel, model: 500 and 1,000 ml, Bucher, Germany
- Shanking incubator, New Brunswick Innova 44 incubator Shaker, Eppendorf, Germany
- Shaking incubator, LSI-3016R, LabTech, Thailand
- Shaking incubator, SI-23MC, Bioer Technology, China
- Spectrophotometer, Thermo Scientific, USA
- Sterile disposable syringe, 10 ml, Nipro, Japan
- Syringe membrane filter, 0.22 μm polyvinylidene fluoride, Wintech, Japan
- Static incubator, Incucell incubator, MMM Medcenter, Germany
- Sterile cotton swab stick, size: s, length: 6 ich, MEGA-D, Soqins Co., Ltd. Thailand
- Superspeed centrifuge, Sorvall RC 6 Plus Centrifuge, Thermo Scientific, USA
- Ultraviolet light, model: AB-409U electronic money detector, China
- Vortex mixer, model: KMC-1300V, Vision Scientific Co., Ltd., South Korea
- Weighing machine, model: AG285, Mettler Toledo Ltd., USA
- Weighing machine, model: 240A , Precisa instrument Ltd., Switzerland

Appendix B: Medium and chemical preparations

1. Preparation of modified Leeming-Notman medium

The yeast was grown in modified Leeming-Notman broth (MLNB) or modified Leeming-Notman agar (MLNA) composing of bacteriological peptone 10 g, D-glucose 10 g, yeast extract 2 g, ox bile 8 g, glycerol 10 ml, glycerol monostearate 0.5 mg, Tween 60 5 ml, olive oil 20 ml, and/or agar 15 g (HiMedia, India) in total volume 1,000 ml.

The media were sterilized by using autoclave (TOMY, Japan) at 121 ℃, 15 psi pressure for 20 min.

2. Preparation of TW60-Vic B agar

Agar medium plates supplemented with indicator dye (TW60-Vic B agar) were prepared composing of 2.5% agar, 2% Tween 60 (lipase substrate), and 0.01% Vitoria Blue B (indicator).

The agar dissolving in deionized (DI) water and Tween 60 were separately sterilized at 121 ℃, 15 psi pressure for 15 min and were cooled down to 50-60 ℃ before combining. The solution of Vic B (in DI water) was filtrated by 0.22 μm PVDF syringe membrane filter and was prewarmed at 50-60 ℃ before used. The agar solution supplemented with Tween 60 was then aseptically mixed with Vic B solution. TW60-Vic B agar was poured at 20 ml per plate.

3. Preparation of TW60 agar

Agar medium plates without indicator dye (TW60 agar) were prepared. The composition of the medium was [i] basal medium including 10 g peptone, 5 g $NaCl_2$, 0.1 g $CaCl_2{\cdot}2H_2O$ [or 0.0755 g $CaCl_2$ dehydrated (MW 110.99)] and 20 g agar, and [ii] 10 ml (v/v) Tween 60. The basal medium without agar was firstly mixed with DI and was adjusted to pH 7.4 by using 1 M NaOH or conc. HCl. Then, the agar was added. The basal medium and Tween 60 were separately sterilized at 121 ℃, 15 psi pressure for 15 min. They were cooled down to 50-60 ℃ before aseptically mixing. TW60 agar were poured at 20 ml per plate.

4. Preparation of 10x phosphate buffered saline (PBS), pH 7.2-7.4

10x phosphate buffered saline (PBS), pH 7.2-7.4 was used as a stock solution to prepare 1x PBS which its compositions are shown as following:

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
DI	1,000 ml

The mixtures were dissolved in 800 ml DI and were adjusted pH to 7.2-7.4 by using 1 M NaOH. The final volume was adjusted to 1,000 ml and was sterilized by autoclaving at 121 ℃, 15 psi pressure for 15 min. The stock solution was kept at room temperature for further used.

The buffer stock was then diluted to 1x PBS by sterile DI and 1x PBS was sterilized by autoclaving at 121 ℃, 15 psi pressure for 15 min.

5. Preparation of 100 mM Na_2PO_4 buffer, pH 8.0

100 mM Na_2PO_4 buffer, pH 8.0 was used as a stock solution to prepare 50 mM Na_2PO_4 buffer. The buffer stock was freshly prepared which its compositions are shown as following:

NaH_2PO_4 anhydrous (MW 156.02)	0.87 g
Na_2HPO_4 (MW 141.96)	4.87 g
DI	400 ml

The mixtures were firstly dissolved in 200 ml DI and were adjusted pH to 8 by using 1 M NaOH. The final volume was adjusted to 400 ml and was sterilized by autoclaving at 121 ℃, 15 psi pressure for 15 min. The stock solution was kept at room temperature or 4 ℃ and was used within 2 weeks.

The buffer stock was then diluted to 50 mM Na_2PO_4 buffer by sterile DI.

6. Preparation of 1 M Tris-HCl, pH 8.0

1 M Tris-HCl, pH 8.0 was freshly prepared by dissolving Tris base 121.1 g with 800 ml DI. The buffer was adjusted pH to 8 by using HCl. After, DI was added to make final volume to be 1,000 ml. The buffer was sterilized by autoclaving at 121 ℃, 15 psi pressure for 15 min. The buffer was kept at room temperature or 4 ℃ and was used within 2 weeks.

7. Preparation of anisaldehyde dripping reagent

Anisaldehyde dripping reagent compositions are shown as following:

Anisaldehyde	3 ml
H_2SO_4	3 ml
Methanol	95 ml

The testing or dripping process was performed in chemical fume hood.

8. Preparation of test samples

Each sample stock solution (w/v) was prepared by weighting and dissolving in DMSO to desired final volume. Stock solution was well mixed by using vortex. In some cases, heating process, 40-50 ℃ for 5-10 min was used. The stock solution was kept in dark at 4 ℃ or -20 ℃ for short and long-term usage, respectively.

Later, the stock solution was diluted to receive desired final concentrations for testing by the formula described below:

$$C_1V_1 = C_2V_2$$

where: C_1 is indicated as initial concentration of solution.

V_1 is indicated as initial volume of solution.

C_2 is indicated as final concentration of solution.

V_2 is indicated as final volume of solution.

Appendix C: Data of $O.D._{600}$ and number of yeast cells.

Table C1 Raw data of number of alive and dead yeast cells in different $O.D._{600}$.

$O.D._{600}$		Number of yeast cells (cells)					
		Alive			Dead		
Measured $O.D._{600}$	Adjusted $O.D._{600}$ (1/10 dilution factor)	1	2	3	1	2	3
0.019	0.19	7	7	6	6	5	5
0.044	0.44	23	26	22	11	10	14
0.111	1.11	45	45	41	15	16	11

Table C2 Data of alive yeast cells density (cells/ml) in different $O.D._{600}$.

$O.D._{600}$	Alive yeast cells density (cells/ml)			
	1	2	3	Mean ± SD
0.19	0.4	0.4	0.3	0.3 ± 0.024
0.44	1.2	1.3	1.1	1.2 ± 0.085
1.11	2.3	2.3	2.1	2.2 ± 0.094

* Total volume is 4×10^{-6} cm^3 per square.

Appendix D: Raw data of diameter of zone of inhibition from primary screening of antimicrobial activity by using agar well diffusion assay.

Table D1 Diameter of zone of inhibition and percentage of growth inhibition from primary screening of antimicrobial activity of CME at concentrations 50-400 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)			
			Concentrations (mg/ml)			
			50	100	200	400
CME	1	1	0.0	0.0	12.0	11.0
		2	0.0	0.0	12.0	11.0
		3	0.0	0.0	13.0	12.0
	2	1	0.0	0.0	12.0	14.0
		2	0.0	0.0	11.0	11.0
		3	0.0	0.0	11.0	13.0
Mean ± SEM			0.00 ± 0.00	0.00 ± 0.00	11.83 ± 0.50	12.00 ± 0.67
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	0.00 ± 0.00	56.00 ± 3.69	56.61 ± 1.76

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E1).

Table D2 Diameter of zone of inhibition from primary screening of antimicrobial activity of CMPE at concentrations 50-200 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			50	100	200
CMPE	1	1	11.0	10.0	14.0
		2	12.0	12.0	16.0
		3	10.0	11.0	13.0
	2	1	11.0	12.0	15.0
		2	9.0	12.0	14.0
		3	11.0	14.0	16.0
		4	9.0	13.0	14.0
	3	1	9.0	9.0	10.0
		2	10.0	9.0	12.0
		3	11.0	11.0	13.0
Mean ± SEM			10.33 ± 0.47	11.14 ± 1.26	13.58 ± 1.37
Percentage of growth inhibition[a] (%)			46.88 ± 2.39	50.43 ± 3.68	61.04 ± 2.67

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E2).

Table D3 Diameter of zone of inhibition from primary screening of antimicrobial activity of CMPE at concentrations 6.25-25 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			6.25	12.5	25
CMPE	1	1	0.0	9.0	10.0
		2	0.0	10.0	11.0
		3	0.0	10.0	12.0
	2	1	0.0	9.0	10.0
		2	0.0	9.0	11.0
		3	0.0	9.0	9.0
Mean ± SEM			0.00 ± 0.00	9.00 ± 0.00	10.00 ± 0.82
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	40.70 ± 0.98	45.69 ± 0.47

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E3).

Table D4 Diameter of zone of inhibition from primary screening of antimicrobial activity of CHPE at concentrations 50-200 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			50	100	200
CHPE	1	1	0.0	0.0	0.0
		2	0.0	0.0	0.0
		3	0.0	0.0	0.0
	2	1	0.0	0.0	0.0
		2	0.0	0.0	0.0
		3	0.0	0.0	0.0
Mean ± SEM			0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E4).

Table D5 Diameter of zone of inhibition from primary screening of antimicrobial activity of CDPE at concentrations 50-200 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			50	100	200
CDPE	1	1	0.0	0.0	0.0
		2	0.0	0.0	0.0
		3	0.0	0.0	0.0
	2	1	0.0	0.0	0.0
		2	0.0	0.0	0.0
		3	0.0	0.0	0.0
Mean ± SEM			0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 4 μg/ml (Appendix E; Table E5).

Table D6 Diameter of zone of inhibition from primary screening of antimicrobial activity of CMPE 1 at concentrations 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			3.13	12.5	50
CMPE 1	1	1	0.0	12.0	23.0
		2	0.0	15.0	24.0
		3	0.0	11.0	21.0
	2	1	0.0	12.0	21.0
		2	0.0	10.0	20.0
		3	0.0	11.0	21.0
Mean ± SEM			0.00 ± 0.00	11.83 ± 0.83	21.67 ± 1.00
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	46.36 ± 3.54	84.97 ± 4.46

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E6).

Table D7 Diameter of zone of inhibition from primary screening of antimicrobial activity of CMPE 2 at concentrations 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			3.13	12.5	50
CMPE 2	1	1	0.0	0.0	0.0
		2	0.0	0.0	0.0
		3	0.0	0.0	0.0
	2	1	0.0	0.0	0.0
		2	0.0	0.0	0.0
		3	0.0	0.0	0.0
Mean ± SEM			0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E7).

Table D8 Diameter of zone of inhibition from primary screening of antimicrobial activity of CMPE 3 at concentrations 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			3.13	12.5	50
CMPE 3	1	1	0.0	11.0	11.0
		2	0.0	13.0	15.0
	2	1	0.0	10.0	12.0
		2	0.0	9.0	11.0
		3	0.0	9.0	11.0
	3	1	0.0	9.0	11.0
		2	0.0	10.0	10.0
Mean ± SEM			0.00 ± 0.00	10.28 ± 1.22	11.61 ± 1.04
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	40.57 ± 5.26	45.85 ± 4.53

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E8).

Table D9 Diameter of zone of inhibition from primary screening of antimicrobial activity of synthetic MG at concentrations 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			3.13	12.5	50
Synthetic MG	1	1	0.0	14.0	21.0
		2	0.0	16.0	23.0
		3	0.0	12.0	20.0
Mean ± SEM			0.00 ± 0.00	14.00 ± 1.63	21.33 ± 1.25
Percentage of growth inhibition[a] (%)			0.00 ± 0.00	52.33 ± 3.75	79.97 ± 1.78

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E9).

Table D10 Diameter of zone of inhibition from primary screening of antimicrobial activity of standard MG at concentrations 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameter of zone of inhibition[a] (mm)		
			Concentration (mg/ml)		
			3.13	12.5	50
Standard MG	1	1	12.0	18.0	29.0
		2	11.0	17.0	28.0
		3	10.0	15.0	26.0
Mean ± SEM			11.00 ± 0.82	16.67 ± 1.25	27.67 ± 1.25
Percentage of growth inhibition[a] (%)			39.73 ± 2.40	60.19 ± 3.58	99.96 ± 2.97

[a] The percentage of growth inhibition values were calculated by comparing to diameter of zone of inhibition from KTZ at 2 μg/ml (Appendix E; Table E10).

Appendix E: Raw data of diameter of zone of inhibition from KTZ in primary screening of antimicrobial activity by using agar well diffusion assay.

Table E1 Diameter of zone of inhibition by 4 μg/ml KTZ obtained from agar well diffusion assay for CME at concentration 50-400 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 4 μg/ml KTZ (mm)
CME	1	1	20.0
		2	20.0
		3	22.0
	2	1	22.0
		2	21.0
		3	22.0
	Mean ± SEM		21.17 ± 0.50

Table E2 Diameter of zone of inhibition by 4 μg/ml KTZ obtained from agar well diffusion assay for CMPE at concentration 50-200 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 4 μg/ml KTZ (mm)
CMPE	1	1	24.0
		2	25.0
		3	20.0
	2	1	24.0
		2	22.0
		3	25.0
		4	21.0
	3	1	20.0
		2	20.0
		3	21.0
	Mean ± SEM		22.11 ± 1.26

Table E3 Diameter of zone of inhibition by 4 μg/ml KTZ obtained from agar well diffusion assay for CMPE at concentration 6.25-25 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 4 μg/ml KTZ (mm)
CMPE	1	1	24
		2	25
		3	24
	2	1	23
		2	22
		3	20
	Mean ± SEM		23.00 ± 1.33

Table E4 Diameter of zone of inhibition by 4 μg/ml KTZ obtained from agar well diffusion assay for CHPE at concentration 50-200 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 4 μg/ml KTZ (mm)
CHPE	1	1	24.0
		2	24.0
		3	24.0
	2	1	21.0
		2	22.0
		3	23.0
	Mean ± SEM		23.00 ± 1.00

Table E5 Diameter of zone of inhibition by 4 μg/ml KTZ obtained from agar well diffusion assay for CDPE at concentration 50-200 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 4 μg/ml KTZ (mm)
CDPE	1	1	23.0
		2	24.0
		3	25.0
	2	1	21.0
		2	23.0
		3	23.0
	Mean ± SEM		23.17 ± 0.83

Table E6 Diameter of zone of inhibition by 2 μg/ml KTZ obtained from agar well diffusion assay for CMPE 1 at concentration 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 2 μg/ml KTZ (mm)
CMPE 1	1	1	25.0
		2	26.0
		3	25.0
	2	1	26.0
		2	25.0
		3	26.0
	Mean ± SEM		25.50 ± 0.17

Table E7 Diameter of zone of inhibition by 2 μg/ml KTZ obtained from agar well diffusion assay for CMPE 2 at concentration 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 2 μg/ml KTZ (mm)
CMPE 2	1	1	25.0
		2	26.0
		3	25.0
	2	1	24.0
		2	26.0
		3	23.0
	Mean ± SEM		24.83 ± 0.50

Table E8 Diameter of zone of inhibition by 2 μg/ml KTZ obtained from agar well diffusion assay for CMPE 3 at concentration 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 2 μg/ml KTZ (mm)
CMPE 3	1	1	25.0
		2	25.0
	2	1	28.0
		2	26.0
		3	23.0
	3	1	25.0
		2	26.0
	Mean ± SEM		25.39 ± 0.28

Table E9 Diameter of zone of inhibition by 2 μg/ml KTZ obtained from agar well diffusion assay for synthetic MG at concentration 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 2 μg/ml KTZ (mm)
Synthetic MG	1	1	27.0
		2	28.0
		3	25.0
	Mean ± SEM		26.67 ± 1.25

Table E10 Diameter of zone of inhibition by 4 μg/ml KTZ obtained from agar well diffusion assay for standard MG at concentration 3.13-50 mg/ml.

Extracts	Number of repeats	Number of replicates	Diameters of zone of inhibition of 2 μg/ml KTZ (mm)
Standard MG	1	1	28.0
		2	28.0
		3	27.0
	Mean ± SEM		27.67 ± 0.47

Appendix F: Supplementary data of agar well diffusion assay.

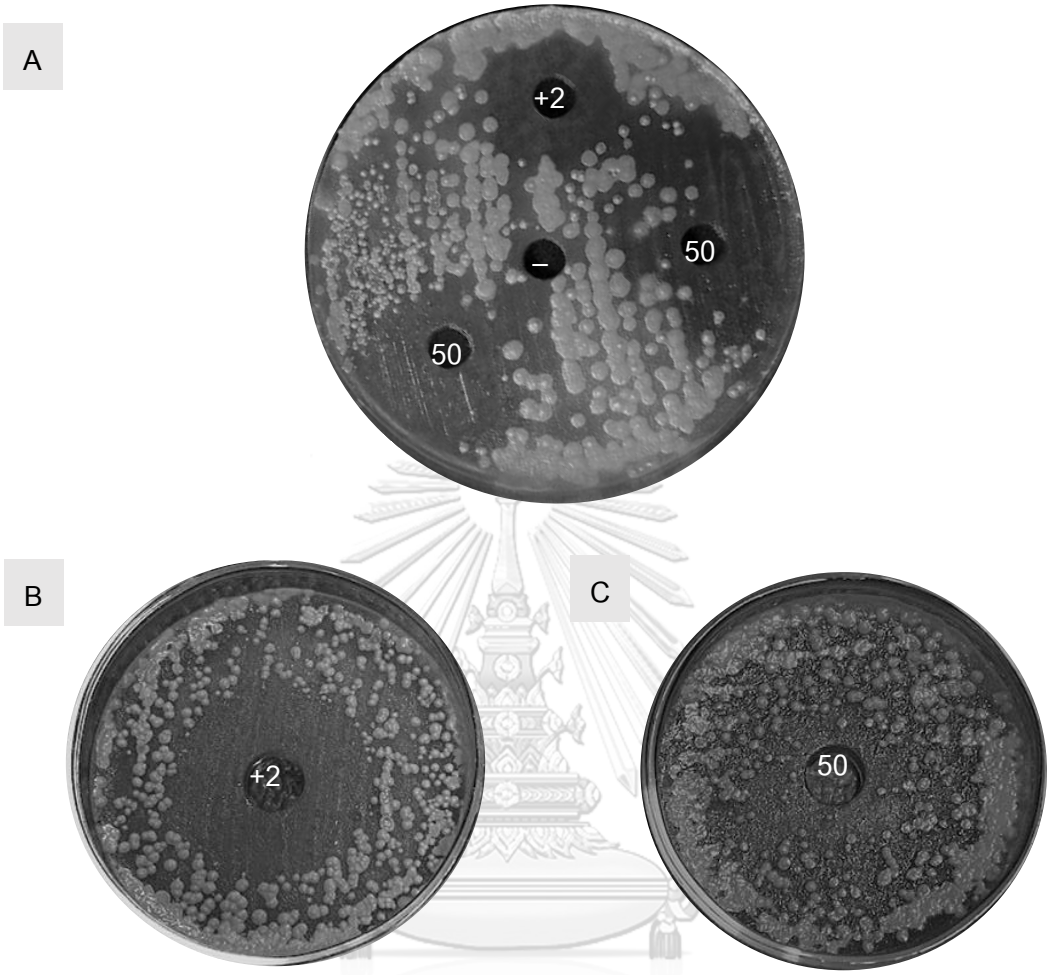


Figure F1 A representative of results from an agar well diffusion assay of standard MG at 50 mg/ml. The examination on (A) the same plate or (B-C) small separating plates were conducted to observe the effect of extract interference or incubation period to different zones of inhibition, especially in highly active extracts or compounds. In (A), results of tested plates were observed and collected in 14 days. In (B-C), results of the tested plates were observed and collected in 7 days. The symbols "+2" and "–" represent positive control (2 μg/ml KTZ) and negative control (DMSO), respectively. The numbers represent extract concentrations (mg/ml).

According to the results (Figure F1), tested plates in the same normal plate or small separating plate were not different. It was found that the colonies which were not inhibited by the sample only expanded within 14 days and new growth of colonies were not observed. Thus, the results were not (or less) influenced by extract interference, incubation period, size, and appearance

of the colonies. The examination of highly active extracts or compounds on the same plates were particularly conducted to reduce the effect of external factors.

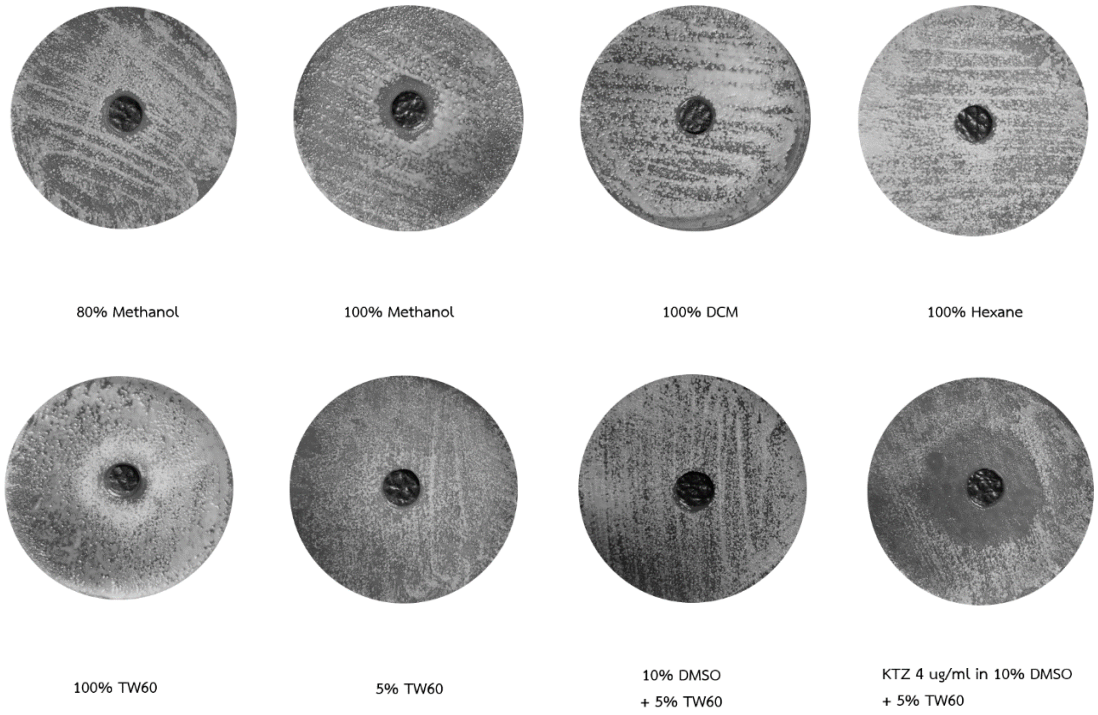


Figure F2 A representative of some results from an agar well diffusion assay treated with different solvents.

Appendix G: Supplementary data from the optimizations of broth microdilution assay.

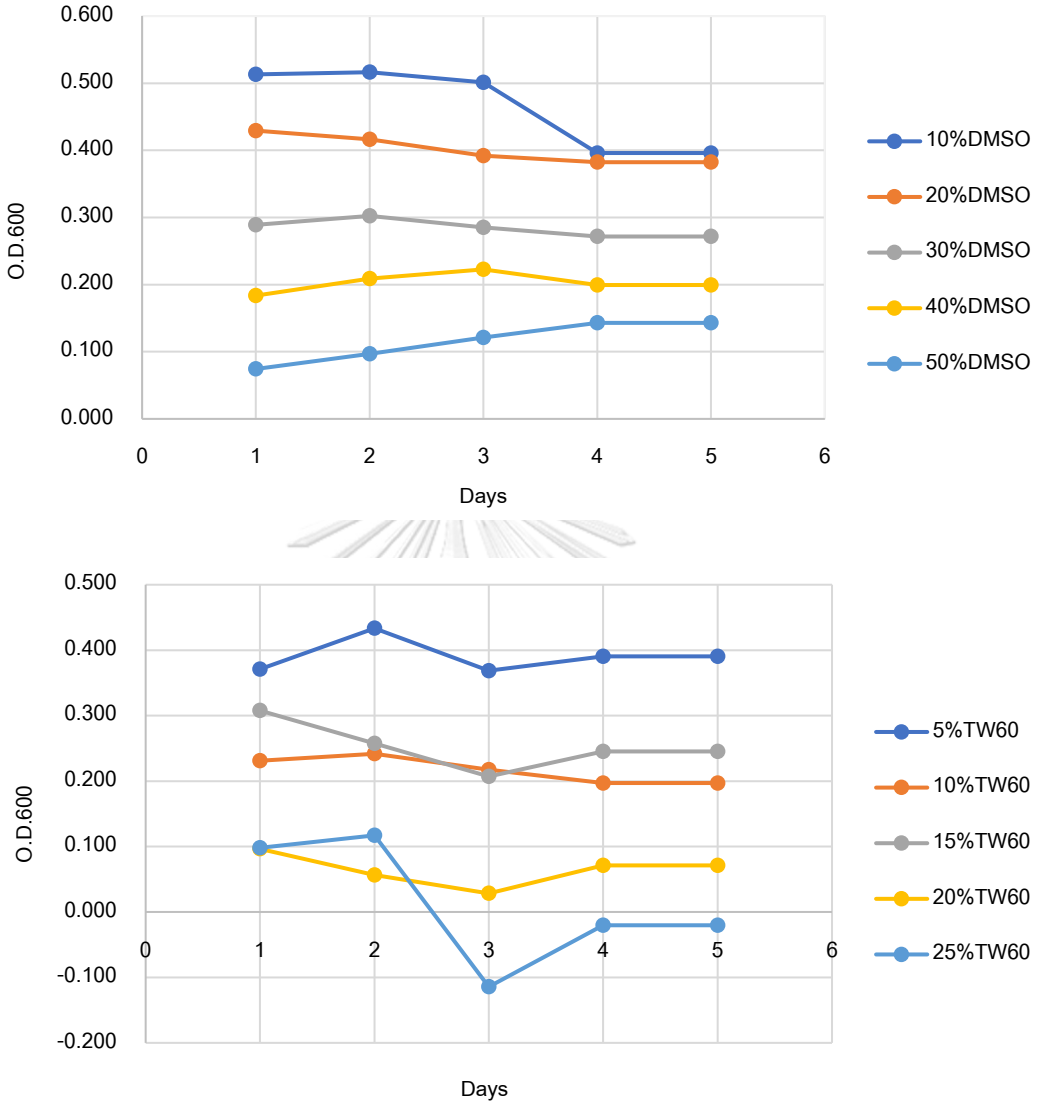


Figure G1 A result from optimization of solvent concentrations (%) for broth microdilution assay. The inoculums (final concentration: 1-2 x 10^5 cells/ml) were treated with different solvents including (A) DMSO and (B) Tween 60. An inoculum without solvent (replaced by 1x PBS) was served as a control. The results are displayed as optical density at 600 nm ($O.D._{600}$) in different days of incubation which lower $O.D._{600}$ value indicates the growth inhibition affected by solvents.

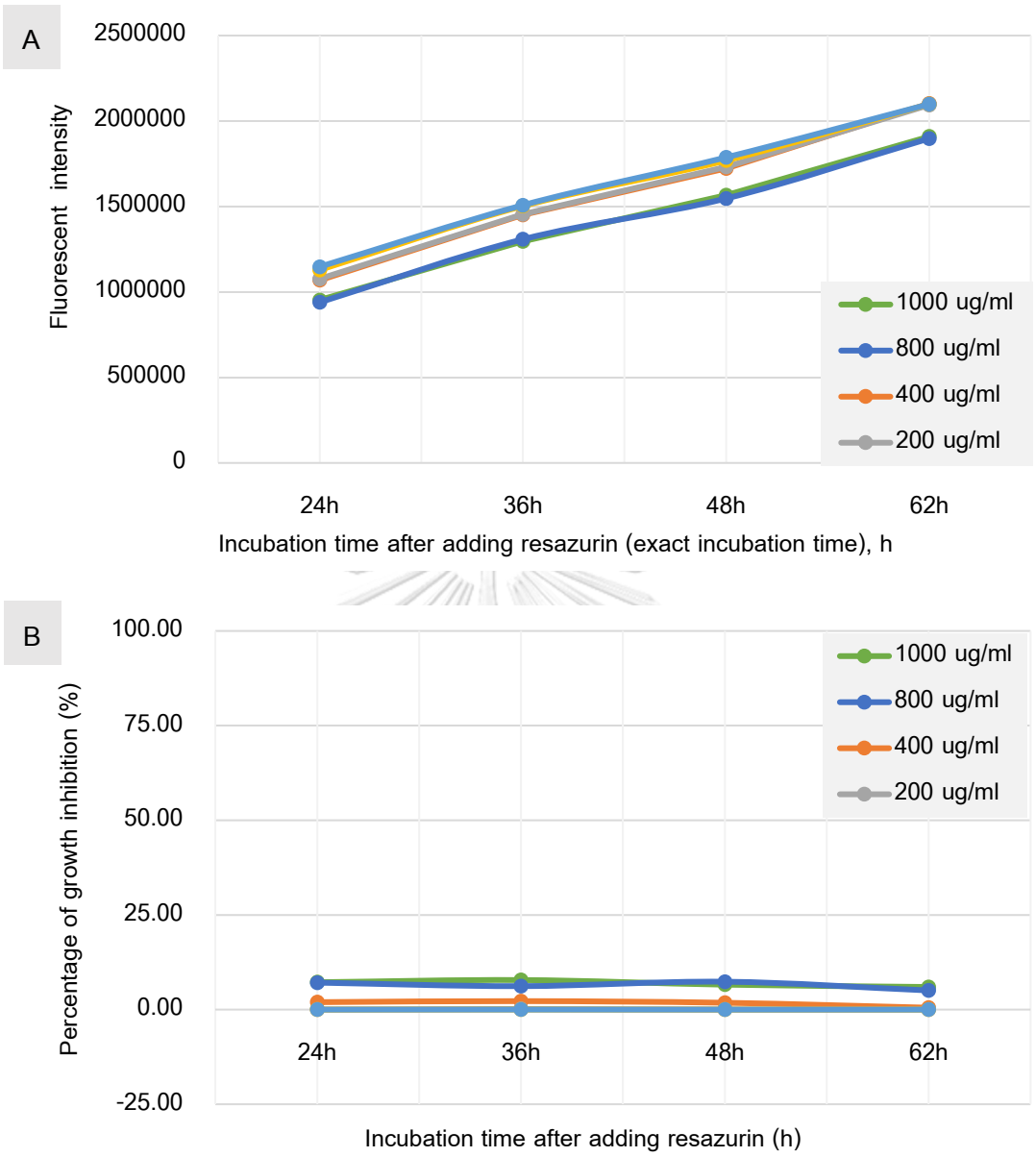


Figure G2 A representative result from optimization of incubation period for broth microdilution assay. The inoculums (final concentration: 1-2 x 10^5 cells/ml) were treated with different concentrations of ketoconazole and 10% DMSO mixed with 5% Tween 60 was served as a solvent control. Later, 0.02% (v/w) resazurin solution was added before incubation (0 h). The results are displayed as (A) fluorescent intensity and (B) percentage of inhibitions in different times of incubation.

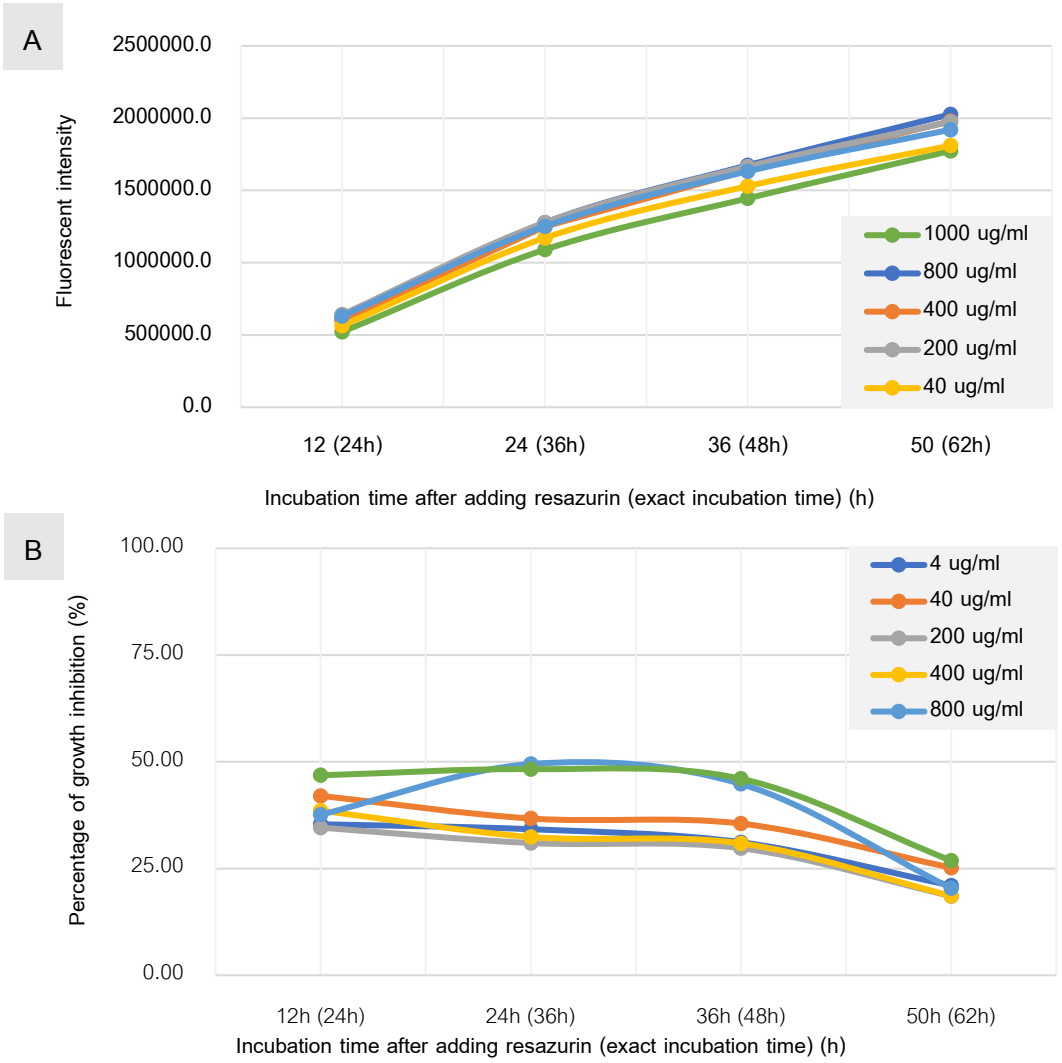


Figure G3 A representative result from optimization of incubation period for broth microdilution assay. The inoculums (final concentration: 1-2 x 10^5 cells/ml) were treated with different concentrations of ketoconazole and 10% DMSO mixed with 5% Tween 60 was served as a solvent control. Later, 0.02% (v/w) resazurin solution was added after 12 h of incubation. The results are displayed as (A) fluorescent intensity and (B) percentage of inhibitions in different times of incubation.

From the results, more change in fluorescent intensity and percentage of inhibitions were observed in the experiment which was added resazurin after 12 h of incubation (Figure G3) comparing to adding before incubation (Figure G2). Moreover, at 24 h (36 h) to 36 h (48 h) of incubations, it exhibited a suitable period for results measurement. Since most of broth microdilution assay are commonly performed in total incubation time at 48 h and the results from both time points are slightly different, the time point at 36 h (48 h) which resazurin was added after 12 h of incubation was used as a mensuration time in this study.

Appendix H: Data of percentage of growth inhibition from by microdilution assay.

Table H1 Percentage of growth inhibition of CME from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.78	29.57	39.24	32.05	33.62	3.55
1.56	44.37	49.42	48.70	47.50	1.93
3.13	48.48	53.51	53.16	51.72	1.99
6.25	62.50	66.11	64.84	64.48	1.30
12.50	78.88	79.13	80.18	79.40	0.49
25.00	82.31	78.43	77.71	79.48	1.75
50.00	84.06	84.28	82.59	83.64	0.65

Table H2 Percentage of growth inhibition of CME from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.78	57.33	58.06	–	57.69	0.36
1.56	72.31	70.13	–	71.22	1.09
3.13	75.69	75.31	75.40	75.47	0.16
6.25	79.21	62.78	57.52	66.50	9.24
12.50	79.70	72.19	70.47	74.12	4.01
25.00	66.53	72.39	–	69.46	2.93
50.00	89.56	99.24	–	94.40	4.84

*Symbol "–" represents as the excluded or outlier data.

Remark: Since the percentage of growth inhibition of CME from the second repeat is more varied, only the first repeat of CME results is presented.

Table H3 Percentage of growth inhibition of CMPE from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.78	–	41.67	43.39	42.53	0.86
1.56	53.02	50.55	51.90	51.82	1.01
3.13	65.61	64.24	63.93	64.59	0.73
6.25	72.58	72.80	69.92	71.77	1.31
12.50	74.10	74.03	72.88	73.67	0.56
25.00	74.97	73.76	71.93	73.55	1.25
50.00	90.15	88.59	95.73	91.49	3.07

*Symbol "–" represents as the excluded or outlier data.

Table H4 Percentage of growth inhibition of CMPE from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.78	65.35	73.78	–	69.57	4.21
1.56	80.09	79.02	77.71	78.94	0.98
3.13	87.27	78.85	80.82	82.31	3.60
6.25	87.85	87.25	82.23	85.78	2.52
12.50	93.47	86.73	–	90.10	3.37
25.00	99.40	96.16	–	97.78	1.62
50.00	98.04	98.76	–	98.40	0.36

*Symbol "–" represents as the excluded or outlier data.

Remark: Since the percentage of growth inhibition of CME1 from the second repeat is more varied, only the first repeat of CME1 result is presented.

Table H5 Percentage of growth inhibition of standard MG (10% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.37	80.84	69.64	81.48	77.32	5.44
0.78	79.70	76.55	83.93	80.06	3.02
1.56	83.47	83.52	84.05	83.68	0.26
3.13	90.53	89.22	93.11	90.95	1.62
6.25	97.48	96.75	97.28	97.17	0.31
12.50	99.35	98.58	98.75	98.89	0.33
25.00	100.04	100.36	99.52	99.97	0.35
50.00	100.01	99.77	99.92	99.90	0.10

Table H6 Percentage of growth inhibition of standard MG (10% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.37	78.78	76.72	77.91	77.80	0.84
0.78	79.70	79.32	81.46	80.16	0.93
1.56	84.29	83.05	85.08	84.14	0.84
3.13	90.87	90.35	89.11	90.11	0.74
6.25	97.13	96.73	96.42	96.76	0.29
12.50	99.28	99.15	98.69	99.04	0.25
25.00	99.73	99.87	99.54	99.72	0.14
50.00	99.98	99.99	99.98	99.98	0.01

Table H7 Average percentage of growth inhibition of standard MG (10% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.37	77.56	0.24
0.78	80.11	0.05
1.56	83.91	0.23
3.13	90.53	0.42
6.25	96.97	0.20
12.50	98.97	0.07
25.00	99.84	0.13
50.00	99.94	0.04

* Results from 2 separated repeats with 3 replicates each

Table H8 Percentage of growth inhibition of standard MG (1% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.016	19.18	13.77	12.21	15.05	2.99
0.031	39.65	27.88	34.92	34.15	4.83
0.063	56.40	56.60	60.84	57.94	2.05
0.125	74.50	74.93	73.85	74.42	0.44
0.250	79.68	79.46	80.94	80.03	0.65
0.500	83.39	83.14	81.26	82.60	0.95
1.000	86.25	87.12	87.23	86.87	0.44
2.000	90.02	89.20	90.50	89.91	0.54

Table H9 Percentage of growth inhibition of standard MG (1% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.016	18.10	11.87	11.00	13.66	3.16
0.031	35.10	35.26	35.89	35.42	0.34
0.063	56.26	54.36	59.66	56.76	2.19
0.125	67.97	68.03	74.63	70.21	3.13
0.250	81.22	77.88	80.42	79.84	1.42
0.500	82.70	81.34	83.02	82.35	0.73
1.000	85.64	84.78	86.64	85.69	0.76
2.000	89.06	88.45	89.22	88.91	0.33

Table H10 Average percentage of growth inhibition of standard MG (1% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.016	14.35	0.70
0.031	34.78	0.63
0.063	57.35	0.59
0.125	72.32	2.11
0.250	79.93	0.09
0.500	82.48	0.12
1.000	86.28	0.59
2.000	89.41	0.50

* Results from 2 separated repeats with 3 replicates each.

Table H11 Percentage of growth inhibition of standard MG (2% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.50	76.30	77.68	73.04	75.67	1.95
1.00	81.13	80.75	79.42	80.43	0.74
2.00	85.46	86.33	85.25	85.68	0.47
3.00	88.71	88.34	87.20	88.08	0.64
4.00	91.58	90.67	89.89	90.71	0.69
5.00	92.79	92.86	92.67	92.77	0.08
6.00	94.20	94.37	93.83	94.13	0.23
7.00	96.35	96.44	96.69	96.49	0.14
8.00	96.98	97.27	97.49	97.25	0.21
9.00	97.98	98.27	97.51	97.92	0.31
10.00	98.97	98.70	98.57	98.75	0.17

Table H12 Percentage of growth inhibition of standard MG (2% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.50	71.70	72.88	65.56	70.05	3.21
1.00	81.69	81.49	79.32	80.83	1.07
2.00	79.94	80.65	76.00	78.86	2.05
3.00	86.16	86.60	84.03	85.59	1.12
4.00	90.09	89.22	87.28	88.87	1.17
5.00	91.28	91.38	91.42	91.36	0.06
6.00	93.82	94.19	92.76	93.59	0.60
7.00	95.64	96.21	95.20	95.68	0.41
8.00	97.82	97.54	97.33	97.56	0.20
9.00	98.20	98.46	98.09	98.25	0.16
10.00	98.99	98.86	98.34	98.73	0.28

Table H13 Percentage of growth inhibition of standard MG (2% DMSO) from the third repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.50	75.24	77.45	75.07	75.92	1.09
1.00	81.12	82.28	81.97	81.79	0.49
2.00	85.83	86.82	86.23	86.29	0.41
3.00	88.22	89.65	89.36	89.08	0.62
4.00	90.70	91.47	91.54	91.24	0.38
5.00	93.43	93.25	92.78	93.15	0.27
6.00	94.60	94.79	94.33	94.57	0.19
7.00	95.98	96.04	96.10	96.04	0.05
8.00	97.29	97.30	97.43	97.34	0.06
9.00	98.31	97.85	97.91	98.02	0.20
10.00	99.20	98.99	99.03	99.07	0.09

Table H14 Average percentage of growth inhibition of standard MG (2% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.50	73.88	2.71
1.00	81.02	0.57
2.00	83.61	3.37
3.00	87.58	1.46
4.00	90.27	1.02
5.00	92.43	0.77
6.00	94.10	0.40
7.00	96.07	0.33
8.00	97.38	0.13
9.00	98.07	0.14
10.00	98.85	0.16

* Results from 3 separated repeats with 3 replicates each.

Table H15 Percentage of growth inhibition of standard MG (1% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.031	37.95	39.52	40.73	39.40	1.14
0.063	49.26	48.43	48.67	48.78	0.35
0.125	62.47	61.83	62.28	62.19	0.26

Table H16 Percentage of growth inhibition of standard MG (1% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.031	38.29	40.34	37.94	38.86	1.06
0.063	50.36	51.05	51.21	50.87	0.37
0.125	68.86	67.13	68.60	68.20	0.76

Table H17 Average percentage of growth inhibition of standard MG (1% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.031	39.13	0.27
0.063	49.83	1.04
0.125	65.19	3.00

* Results from 2 separated repeats with 3 replicates each.

Table H18 Percentage of growth inhibition of synthetic MG (1% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.031	25.33	27.09	27.39	26.61	0.91
0.063	36.06	33.99	35.37	35.14	0.86
0.125	44.18	40.82	40.25	41.75	1.73

Table H19 Percentage of growth inhibition of synthetic MG (1% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.031	35.86	37.35	35.79	36.33	0.72
0.063	38.37	40.49	40.36	39.74	0.97
0.125	48.46	47.24	45.78	47.16	1.10

Table H20 Average percentage of growth inhibition of synthetic MG (1% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.031	31.47	4.86
0.063	37.44	2.30
0.125	44.46	2.71

* Results from 2 separated repeats with 3 replicates each.

Table H21 Percentage of growth inhibition of CMPE 1 or extracted MG (1% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.031	34.09	32.88	30.24	32.40	1.61
0.063	38.91	36.71	38.45	38.02	0.95
0.125	45.08	48.25	48.33	47.22	1.52

Table H22 Percentage of growth inhibition of CMPE 1 or extracted MG (1% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.031	32.06	33.99	36.38	34.14	1.77
0.063	36.85	38.92	41.55	39.11	1.93
0.125	44.37	44.99	48.38	45.91	1.76

Table H23 Average percentage of growth inhibition of CMPE 1 or extracted MG (1% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.031	33.27	0.87
0.063	38.56	0.54
0.125	46.57	0.65

* Results from 2 separated repeats with 3 replicates each.

Appendix I: Data of percentage of growth inhibition of KTZ by broth microdilution assay.

Table I1 Percentage of growth inhibition of KTZ (10% DMSO mixed with 5% Tween 60) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.200	20.05	12.85	–	16.45	3.597
0.400	29.49	22.76	–	26.13	3.367
0.800	59.74	54.55	–	57.14	2.596

*Symbol "–" represents the excluded or outlier data.

Table I2 Percentage of growth inhibition of KTZ (10% DMSO mixed with 5% Tween 60) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.200	13.88	37.96	–	25.92	12.043
0.400	18.13	33.68	–	25.90	7.778
0.800	48.32	50.76	–	49.54	1.218

*Symbol "–" represents the excluded or outlier data.

Table I3 Percentage of growth inhibition of KTZ (10% DMSO mixed with 5% Tween 60) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.200	13.25	9.33	28.32	16.97	8.184
0.400	14.79	24.93	29.87	23.20	6.276
0.800	52.43	48.77	42.41	47.87	4.142

Table I4 Average percentage of growth inhibition of KTZ (10% DMSO mixed with 5% Tween 60) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.200	19.78	4.35
0.400	25.08	1.33
0.800	51.52	4.04

* Results from 3 separated repeats with ≥ 2 replicates each.

Table I5 Percentage of growth inhibition of KTZ (10% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.005	17.84	33.78	31.38	27.67	7.015
0.010	19.61	33.93	29.61	27.72	5.995
0.050	35.99	31.63	–	33.81	2.181
0.100	39.41	29.26	–	34.33	5.074
0.200	34.60	31.09	–	32.85	1.753
0.800	41.46	54.64	52.17	49.42	5.721

*Symbol "–" represents the excluded or outlier data.

Table I6 Percentage of growth inhibition of KTZ (10% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.005	23.23	24.68	–	23.96	0.72
0.010	24.55	21.36	22.27	22.73	1.34
0.050	23.99	20.19	26.74	23.64	2.69
0.100	25.98	21.77	22.09	23.28	1.91
0.200	39.15	36.49	–	37.82	1.33
0.800	64.95	62.53	66.91	64.79	1.79

*Symbol "–" represents the excluded or outlier data.

Table I7 Average percentage of growth inhibition of KTZ (10% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.005	25.81	1.85
0.010	25.22	2.49
0.050	28.73	5.08
0.100	28.81	5.53
0.200	35.33	2.49
0.800	57.11	7.69

* Results from 2 separated repeats with ≥ 2 replicates each.

Table I8 Percentage of growth inhibition of KTZ (1% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.0025	36.15	27.18		31.66	4.48
0.0050	43.47	38.67		41.07	2.40
0.0100	43.54	40.01		41.77	1.76
0.0250	53.28	49.97		51.62	1.65
0.0500	51.98	48.30		50.14	1.84

*Symbol "–" represents the excluded or outlier data.

Table I9 Percentage of growth inhibition of KTZ (1% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.0025	29.01	21.44	35.32	28.59	5.67
0.0050	37.47	29.74	40.13	35.78	4.40
0.0100	39.88	34.29	43.67	39.28	3.85
0.0250	49.39	46.23	54.38	50.00	3.35
0.0500	49.48	55.24	53.73	52.82	2.44

*Symbol "–" represents the excluded or outlier data.

Table I10 Average percentage of growth inhibition of KTZ (1% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.0025	30.13	1.54
0.0050	38.42	2.64
0.0100	40.53	1.25
0.0250	50.81	0.81
0.0500	51.48	1.34

* Results from 2 separated repeats with ≥ 2 replicates each

Table I11 Percentage of growth inhibition of KTZ (2% DMSO) from the first repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.0025	31.70	35.79	–	34.89	2.33
0.0050	36.55	36.18	–	38.51	3.04
0.0100	37.61	39.29	–	40.41	2.85
0.0250	52.76	48.07	–	51.69	2.62
0.0500	52.60	56.43	–	54.01	1.72

*Symbol "–" represents the excluded or outlier data.

Table I12 Percentage of growth inhibition of KTZ (2% DMSO) from the second repeat by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)				
	Replicate			Mean	SEM
	1	2	3		
0.0025	28.18	22.36	20.91	23.82	3.15
0.0050	31.62	25.39	27.07	28.03	2.63
0.0100	40.58	43.34	41.81	41.91	1.13
0.0250	50.34	53.12	54.10	52.52	1.59
0.0500	57.11	53.67	49.11	53.30	3.28

*Symbol "–" represents the excluded or outlier data.

Table I13 Average percentage of growth inhibition of KTZ (2% DMSO) by using broth microdilution assay.

Concentration (mg/ml)	Percentage of growth inhibition (%)	
	Mean	SEM
0.0025	29.35	5.54
0.0050	33.27	5.24
0.0100	41.16	0.75
0.0250	52.10	0.42
0.0500	53.65	0.36

* Results from 2 separated repeats with ≥ 2 replicates each

Appendix J: Estimation of IC_{50} of KTZ by nonlinear regression analysis.

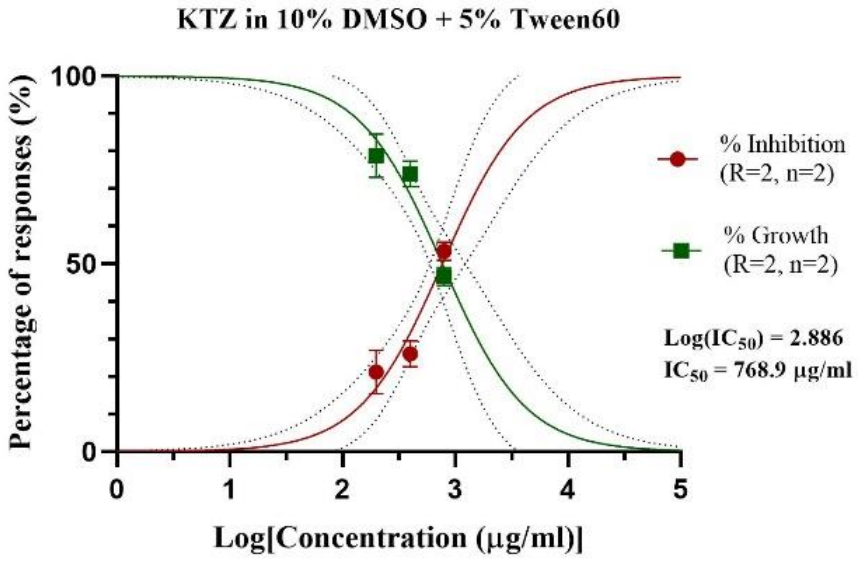


Figure J1 Sigmoidal curves estimated by nonlinear regression analysis using dose response-inhibition [log (inhibitor) versus normalized response with variable slope] at confidence level 95% for KTZ in 10% DMSO mixed with 5% Tween 60.

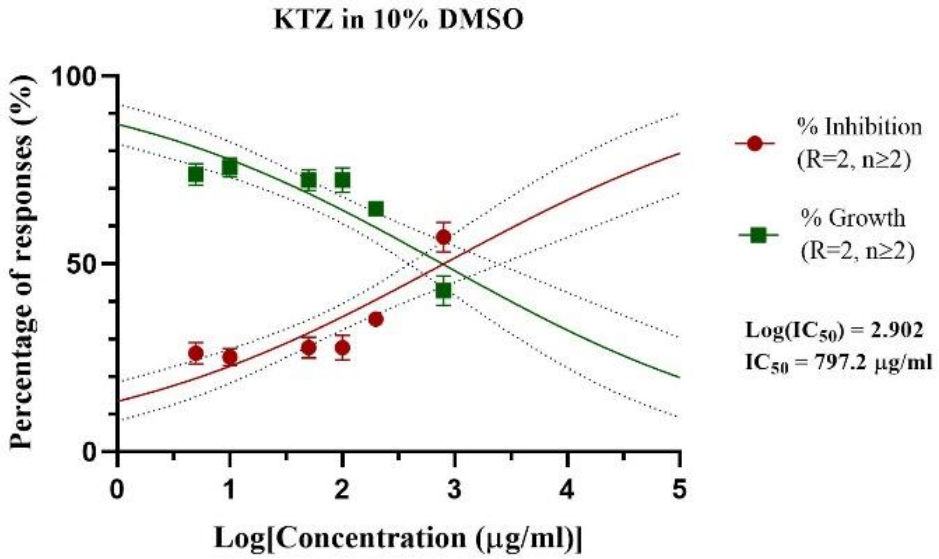


Figure J2 Sigmoidal curves estimated by nonlinear regression analysis using dose response-inhibition [log (inhibitor) versus normalized response with variable slope] at confidence level 95% for KTZ in 10% DMSO.

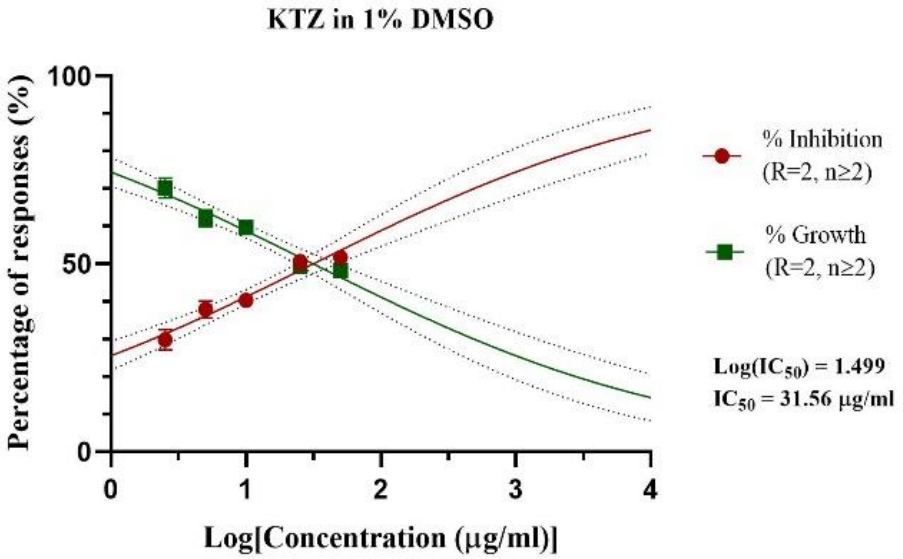


Figure J3 Sigmoidal curves estimated by nonlinear regression analysis using dose response-inhibition [log (inhibitor) versus normalized response with variable slope] at confidence level 95% for KTZ in 1% DMSO.

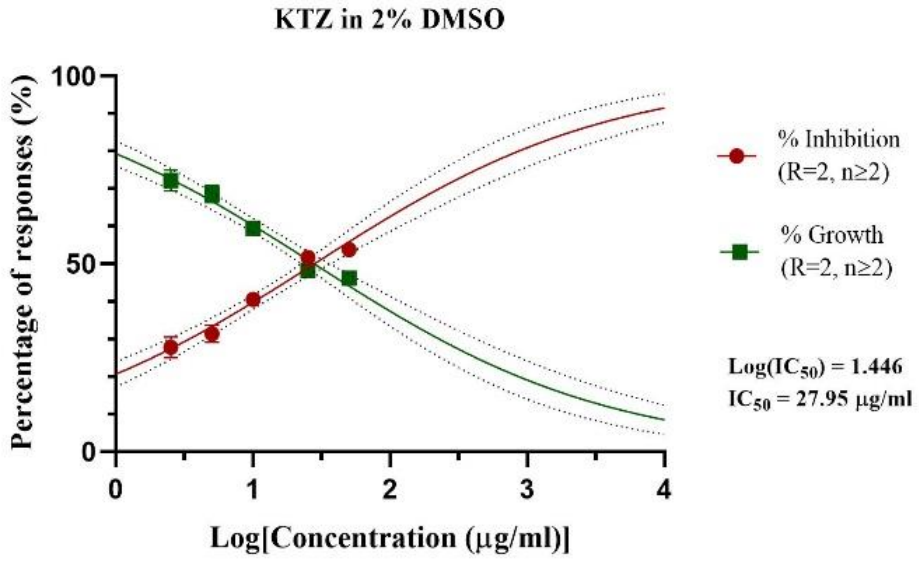
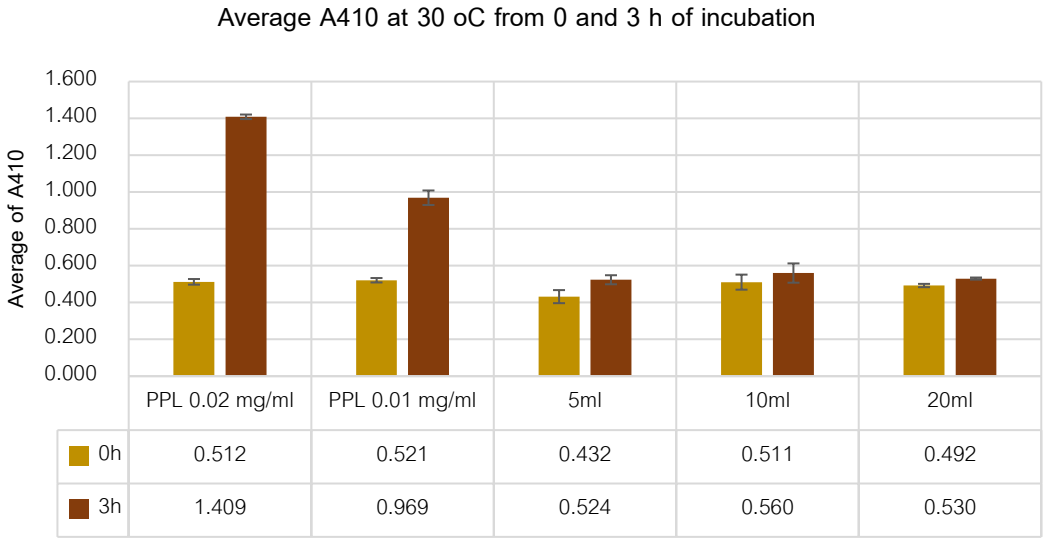


Figure J4 Sigmoidal curves estimated by nonlinear regression analysis using dose response-inhibition [log (inhibitor) versus normalized response with variable slope] at confidence level 95% for KTZ in 2% DMSO.

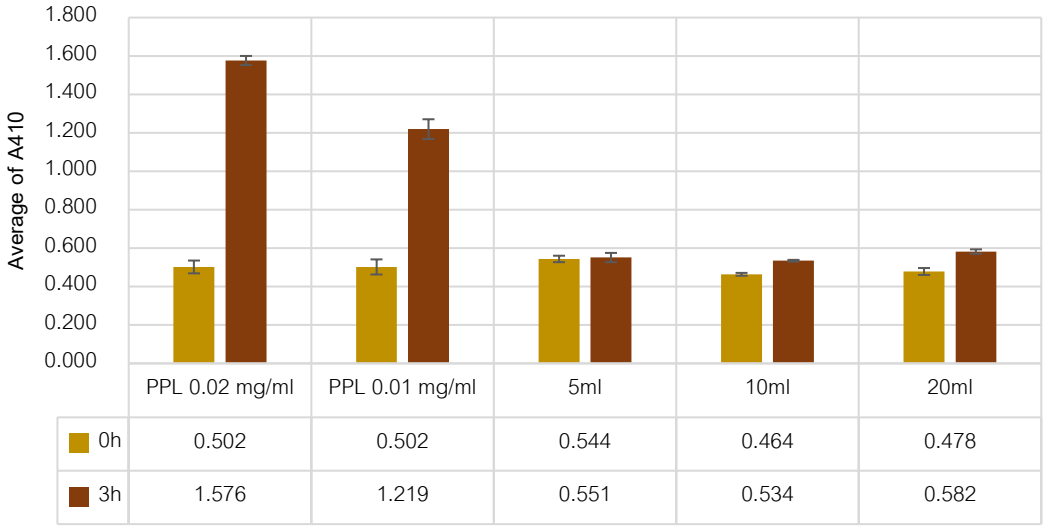
Appendix K: Supplementary data from the optimizations of lipase inhibition.

A



	PPL 0.02 mg/ml	PPL 0.01 mg/ml	5ml	10ml	20ml
0h	0.512	0.521	0.432	0.511	0.492
3h	1.409	0.969	0.524	0.560	0.530

B



	PPL 0.02 mg/ml	PPL 0.01 mg/ml	5ml	10ml	20ml
0h	0.502	0.502	0.544	0.464	0.478
3h	1.576	1.219	0.551	0.534	0.582

Figure K1 Optimization for colorimetric assay for lipase inhibition activity. The change of absorbance at 410 nm (A_{410}) from 0-3 h of incubation was determined in different temperatures including (A) 30 ℃ and (B) 37 ℃. Crude lipase from *M. globosa* obtained from different volume (5-20 ml) of culture cultivation were used. Besides, porcine pancreatic lipase (PPL) at 0.01-0.02 mg/ml were tested along.

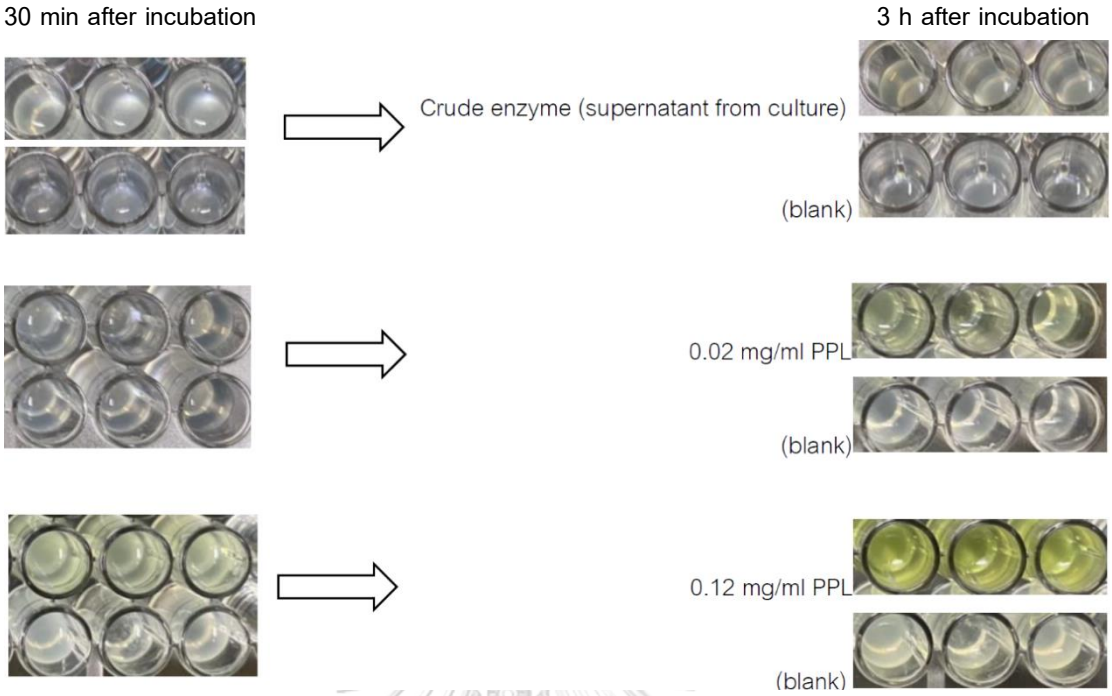


Figure K2 A representative result of the visual change in color after incubation for 30 min and 3 h of porcine pancreatic lipase (PPL) at 0.02 and 0.1 mg/ml comparing to crude lipase from *M. globosa* obtained from 5 ml cultivated culture.

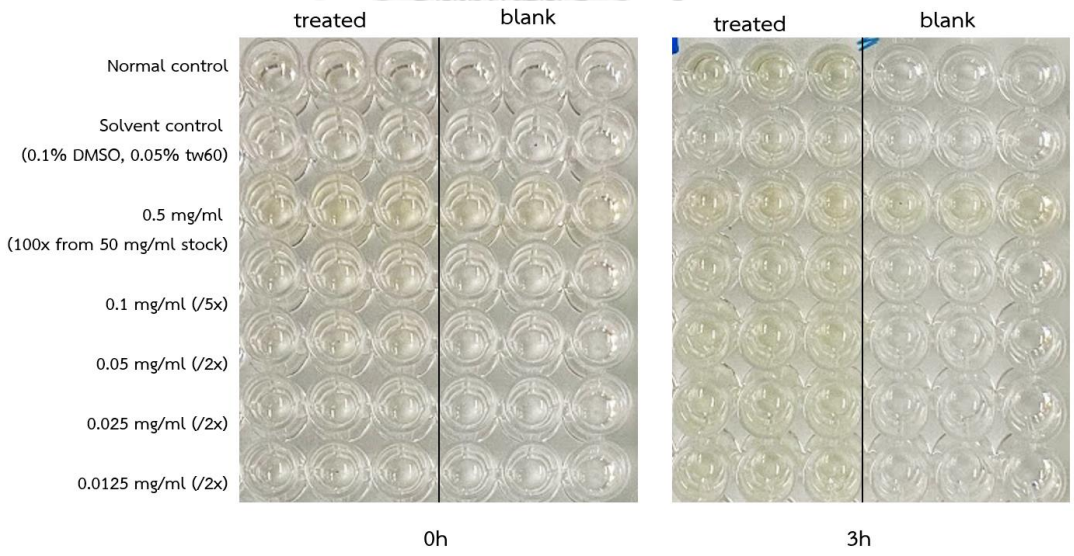
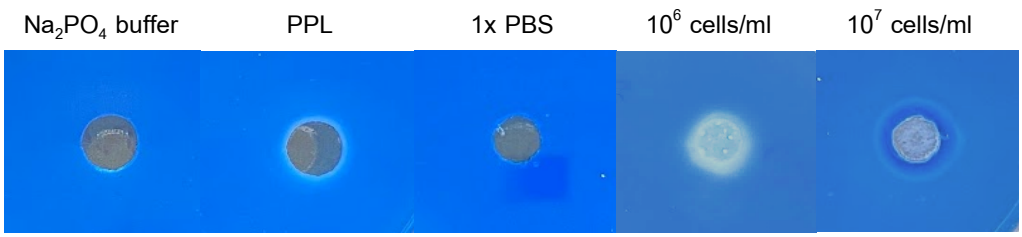


Figure K3 A representative result of the change in color from 0-3 h of incubation of crude lipase from *M. globosa* treated with different concentrations of CME (mg/ml).

A



B

Figure K4 A representative result from (A) agar medium plates supplemented with indicator dye (TW60-Vic B agar) and without indicator dye (TW60 agar). The results of zone appeared around the wells were shown, which were added with different negative control solution (Na_2PO_4 buffer and 1x PBS), and test samples including the porcine pancreatic lipase (PPL), and *M. globosa* at cell density 10^6-10^7 cells/ml.

Appendix L: Raw data of percentage of lipase inhibition.

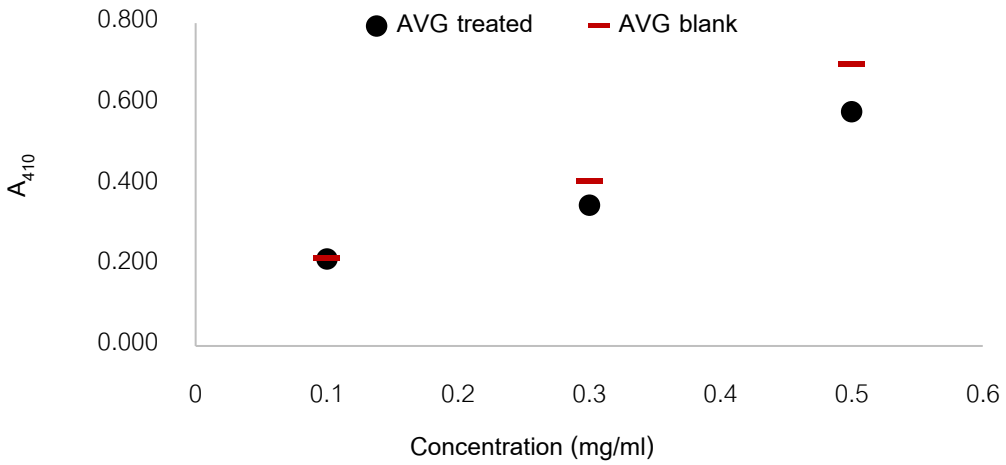


Figure L1 A representative result of the effect from CMPE color interference which treated absorbances at 410 nm (A_{410}) were lower than its blanks.

Table L1 Average percentage of lipase inhibition from CME by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.2	26.70	29.23	22.09	26.01	2.96
0.3	43.75	46.15	43.60	44.50	1.17
0.4	52.27	63.08	61.05	58.80	4.69
0.5	68.75	73.85	70.35	70.98	2.13

* Results from ≥ 2 replicates for each separated repeat.

Table L2 Average percentage of lipase inhibition from CME by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.025	42.86	36.75	–	39.80	3.05
0.05	31.43	38.46	–	34.95	3.52
0.1	35.71	37.18	–	36.45	0.73

* Results are from ≥ 2 replicates for each separated repeat. Symbol "–" represents the excluded or outlier data.

Table L3 Average percentage of lipase inhibition from CMPE by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.025	39.32	33.33	34.62	35.75	2.57
0.05	56.41	68.57	55.56	60.18	5.95
0.1	82.91	84.76	72.65	80.11	5.33

* Results from ≥ 2 replicates for each separated repeat.

Table L4 Average percentage of lipase inhibition from CDPE by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.025	37.18	32.86	44.87	38.30	4.97
0.05	42.31	48.57	41.88	44.25	3.06
0.1	26.92	29.52	32.48	29.64	2.27

* Results from ≥ 2 replicates for each separated repeat.

Table L5 Average percentage of lipase inhibition from CHPE by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.025	30.77	20.00	34.62	28.46	6.19
0.05	47.44	34.29	39.74	40.49	5.39
0.1	30.77	25.71	23.93	26.81	2.90

* Results from ≥ 2 replicates for each separated repeat.

Table L6 Average percentage of lipase inhibition from CMPE 1 by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.0008	0.00	0.00	0.00	0.00	0.00
0.004	17.95	16.40	22.12	18.82	2.41
0.02	23.93	29.10	32.21	28.41	3.41

* Results from ≥ 2 replicates for each separated repeat.

Table L7 Average percentage of lipase inhibition from CMPE 2 by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.0008	0.00	0.00	0.00	0.00	0.00
0.004	0.00	0.00	3.37	1.12	1.59
0.02	12.82	11.11	9.13	11.02	1.51

* Results from ≥ 2 replicates for each separated repeat.

Table L8 Average percentage of lipase inhibition from CMPE 3 by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.0008	0.00	0.00	2.88	0.96	1.36
0.004	0.00	0.00	0.00	0.00	0.00
0.02	0.00	0.00	3.85	1.28	1.81

* Results from ≥ 2 replicates for each separated repeat.

Table L9 Average percentage of lipase inhibition from standard MG by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.02	38.36	32.76	37.04	36.05	2.39
0.1	52.05	38.79	37.45	42.77	6.59
0.5	24.66	25.86	37.04	29.19	5.57

* Results from ≥ 2 replicates for each separated repeat.

Table L10 Average percentage of lipase inhibition from CMPE 2 by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.02	16.38	24.28	–	20.33	3.95
0.1	19.83	12.35	–	16.09	3.74
0.5	22.41	29.63	–	26.02	3.61

* Results are from ≥ 2 replicates for each separated repeat. Symbol "–" represents the excluded or outlier data.

Table L11 Average percentage of lipase inhibition from CMPE 3 by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.02	0.00	0.00	1.23	0.41	0.58
0.1	18.72	13.36	27.57	19.89	5.86
0.5	21.92	21.12	20.99	21.34	0.41

* Results from ≥ 2 replicates for each separated repeat.

Table L12 Average percentage of lipase inhibition from standard MG by colorimetric method.

Concentration (mg/ml)	Percentage of lipase inhibition (%)				
	Number of repeats			Mean	SEM
	1	2	3		
0.063	28.44	29.41	32.89	30.25	1.91
0.125	31.19	32.94	30.26	31.47	1.11
0.250	30.28	37.65	36.84	34.92	3.30
0.500	21.56	25.88	30.26	25.90	3.55
1.000	36.70	31.76	32.89	33.79	2.11

* Results from ≥ 2 replicates for each separated repeat.

Appendix M: Statistical analysis of agar well diffusion results.

Table M1 Results of Homogeneity of Variances test of percentage of growth inhibition (%) from CME, CMPE, CMPE 1, and CMPE 3 at concentration 50 mg/ml.

Percentage of growth inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	**16.585**	**4**	**53**	**0.000**
Based on Median	7.919	4	53	0.000
Based on Median and with adjusted df	7.919	4	13.194	0.002

*The data is a normal distribution. From the Based on Mean value, Sig. 0.000 < 0.05 which indicates that variances of the mean are unequal or equal variance is not assumed.

Table M2 Results of ANOVA test of percentage of growth inhibition (%) from CME, CMPE, CMPE 1, and CMPE 3 at concentration 50 mg/ml.

Percentage of growth inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	50632.321	4	12658.080	1184.670	0.000
Within Groups	566.300	53	10.685		
Total	51198.621	57			

*Sig. 0.000 < 0.05 indicates that mean of the data at least 2 groups are significantly different at 95% ($p \leq 0.05$).

Table M3 Results of Post Hoc test, Games-Howell of percentage of growth inhibition (%) from CME, CMPE, CMPE 1, and CMPE 3 at concentration 50 mg/ml.

Groups	Extracts		Mean Difference (I-J)	Std. Error	Sig.
a	Control	CME	0.00000	0.00000	
		CMPE	-46.48000*	1.13214	0.000
		CMPE1	-84.97436*	2.33950	0.000
		CMPE3	-45.63607*	2.61291	0.000
a	CME	CMPE	-46.48000*	1.13214	0.000
		CMPE1	-84.97436*	2.33950	0.000
		CMPE3	-45.63607*	2.61291	0.000
		Control	0.00000	0.00000	
b	CMPE	CME	46.48000*	1.13214	0.000
		CMPE1	-38.49436*	2.59903	0.000
		CMPE3	0.84393	2.84764	0.998
		Control	46.48000*	1.13214	0.000
c	CMPE1	CME	84.97436*	2.33950	0.000
		CMPE	38.49436*	2.59903	0.000
		CMPE3	39.33829*	3.50721	0.000
		Control	84.97436*	2.33950	0.000
b	CMPE3	CME	45.63607*	2.61291	0.000
		CMPE	-0.84393	2.84764	0.998
		CMPE1	-39.33829*	3.50721	0.000
		Control	45.63607*	2.61291	0.000

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. From a group column, different letters are significantly different. Games-Howell was used due to a big population size with highly different number of samples and some treatments have number of samples more than 5.

Table M4 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) from CMPE 1, synthetic MG, and standard MG at concentration 3.13-50 mg/ml.

Percentage of growth inhibition (%)	Concentrations (mg/ml)	Levene's Statistic	df1	df2	Sig.
Based on Mean	3.13	18.070	3	20	0.000
	12.5	5.679	3	20	0.006
	50	16.234	3	20	0.000

*The data is a normal distribution. From the Based on Mean value, all Sig. values < 0.05 which indicates that variances of the mean are unequal or equal variance are not assumed.

Table M5 Results of ANOVA test of percentage of growth inhibition (%) from CMPE 1, synthetic MG, and standard MG at concentration 3.13-50 mg/ml.

Concentrations (mg/ml)		Sum of Squares	df	Mean Square	F	Sig.
3.13	Between Groups	4144.187	3	1381.396	1562.076	0.000
	Within Groups	17.687	20	0.884		
	Total	4161.873	23			
12.5	Between Groups	16185.347	3	5395.116	394.523	0.000
	Within Groups	273.500	20	13.675		
	Total	16458.847	23			
50	Between Groups	46579.179	3	15526.393	1551.151	0.000
	Within Groups	200.192	20	10.010		
	Total	46779.371	23			

*All Sig. values < 0.05 which indicates that all means of the data at least 2 groups are significantly different at 95% ($p \leq 0.05$).

Table M6 Results of Post Hoc test, Dunnett's T3 of percentage of growth inhibition (%) from CMPE 1, synthetic MG, and standard MG at concentration 3.13 mg/ml.

Groups	Extracts		Mean Difference (I-J)	Std. Error	Sig.
a	Control	Extracted MG	0.00000	0.00000	
		Synthetic MG	0.00000	0.00000	
		Standard MG	-39.73333*	1.71691	0.006
a	Extracted MG	control	0.00000	0.00000	
		Synthetic MG	0.00000	0.00000	
		Standard MG	-39.73333*	1.71691	0.006
a	Synthetic MG	control	0.00000	0.00000	
		Extracted MG	0.00000	0.00000	
		Standard MG	-39.73333*	1.71691	0.006
b	Standard MG	control	39.73333*	1.71691	0.006
		Extracted MG	39.73333*	1.71691	0.006
		Synthetic MG	39.73333*	1.71691	0.006

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. From a group column, different letters are significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table M7 Results of Post Hoc test, Dunnett's T3 of percentage of growth inhibition (%) from CMPE 1, synthetic MG, and standard MG at concentration 12.5 mg/ml.

Groups	Extracts		Mean Difference (I-J)	Std. Error	Sig.
a	Control	Extracted MG	-46.35897*	2.54030	0.000
		Synthetic MG	-52.33333*	2.63586	0.008
		Standard MG	-60.20000*	2.52389	0.005
b	Extracted MG	control	46.35897*	2.54030	0.000
		Synthetic MG	-5.97436	3.66072	0.543
		Standard MG	-13.84103*	3.58094	0.043
bc	Synthetic MG	control	52.33333*	2.63586	0.008
		Extracted MG	5.97436	3.66072	0.543
		Standard MG	-7.86667	3.64935	0.347
c	Standard MG	control	60.20000*	2.52389	0.005
		Extracted MG	13.84103*	3.58094	0.043
		Synthetic MG	7.86667	3.64935	0.347

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. From a group column, different letter is significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table M8 Results of Post Hoc test, Dunnett's T3 of percentage of growth inhibition (%) from CMPE 1, synthetic MG, and standard MG at concentration 50 mg/ml.

Groups	Extracts		Mean Difference (I-J)	Std. Error	Sig.
a	Control	Extracted MG	-84.97436*	2.33950	0.000
		Synthetic MG	-79.97354*	1.26016	0.001
		Standard MG	-99.95591*	2.10027	0.001
b	Extracted MG	control	84.97436*	2.33950	0.000
		Synthetic MG	5.00081	2.65730	0.404
		Standard MG	-14.98155*	3.14394	0.014
b	Synthetic MG	control	79.97354*	1.26016	0.001
		Extracted MG	-5.00081	2.65730	0.404
		Standard MG	-19.98236*	2.44931	0.011
c	Standard MG	control	99.95591*	2.10027	0.001
		Extracted MG	14.98155*	3.14394	0.014
		Synthetic MG	19.98236*	2.44931	0.011

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. From a group column, different letter is significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Appendix N: Statistical analysis of broth microdilution assay results.

Table N1 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing CME and CMPE, concentrations 0.78-50 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	7.8388E+29	13	14	0.000

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.000 < 0.05 which indicates that variances of the mean are unequal or equal variance is not assumed.

Table N2 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing CME and CMPE, concentrations 0.78-50 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4883.987	13	375.691	2.175	0.081
Within Groups	2417.962	14	172.712		
Total	7301.949	27			

*Sig. 0.081 > 0.05 indicates that all means of the data are not significantly different at 95% ($p \leq 0.05$).

Table N3 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing CME, its control and within group concentrations 0.78-50 mg/ml.

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	4.365	7	16	0.007

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.007 < 0.05 which indicate that variances of the mean are unequal or equal variance is not assumed.

Table N4 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing CME, its control and within group concentrations 0.78-50 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16962.560	7	2423.223	377.370	0.000
Within Groups	102.742	16	6.421		
Total	17065.301	23			

*Sig. 0.000 < 0.05 indicates that all means of the data at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N5 Results of Post Hoc test, Dunnett's T3 of growth inhibition (%) by using broth microdilution assay by comparing CME, its control and within group concentrations 0.78-50 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.78	-33.62000*	2.89976	0.040
	1.56	-47.49667*	1.57709	0.006
	3.13	-51.71667*	1.62148	0.005
	6.25	-64.48333*	1.05727	0.001
	12.5	-79.39667*	0.39826	0.000
	25	-79.48333*	1.42853	0.002
	50	-83.64333*	0.53048	0.000
0.78	control	33.62000*	2.89976	0.040
	1.56	-13.87667	3.30089	0.177
	3.13	-18.09667	3.32233	0.088
	6.25	-30.86333*	3.08649	0.030
	12.5	-45.77667*	2.92699	0.020
	25	-45.86333*	3.23254	0.007
	50	-50.02333*	2.94789	0.015

1.56	control	47.49667*	1.57709	0.006
	0.78	13.87667	3.30089	0.177
	3.13	-4.22000	2.26195	0.761
	6.25	-16.98667*	1.89869	0.015
	12.5	-31.90000*	1.62660	0.009
	25	-31.98667*	2.12789	0.001
	50	-36.14667*	1.66392	0.005
3.13	control	51.71667*	1.62148	0.005
	0.78	18.09667	3.32233	0.088
	1.56	4.22000	2.26195	0.761
	6.25	-12.76667*	1.93572	0.042
	12.5	-27.68000*	1.66968	0.013
	25	-27.76667*	2.16100	0.003
	50	-31.92667*	1.70605	0.007
6.25	control	64.48333*	1.05727	0.001
	0.78	30.86333*	3.08649	0.030
	1.56	16.98667*	1.89869	0.015
	3.13	12.76667*	1.93572	0.042
	12.5	-14.91333*	1.12979	0.014
	25	-15.00000*	1.77722	0.016
	50	-19.16000*	1.18289	0.005
12.5	control	79.39667*	0.39826	0.000
	0.78	45.77667*	2.92699	0.020
	1.56	31.90000*	1.62660	0.009
	3.13	27.68000*	1.66968	0.013
	6.25	14.91333*	1.12979	0.014
	25	-0.08667	1.48301	1.000
	50	-4.24667*	0.66334	0.039

25	control	79.48333*	1.42853	0.002
	0.78	45.86333*	3.23254	0.007
	1.56	31.98667*	2.12789	0.001
	3.13	27.76667*	2.16100	0.003
	6.25	15.00000*	1.77722	0.016
	12.5	0.08667	1.48301	1.000
	50	-4.16000	1.52385	0.470
50	control	83.64333*	0.53048	0.000
	0.78	50.02333*	2.94789	0.015
	1.56	36.14667*	1.66392	0.005
	3.13	31.92667*	1.70605	0.007
	6.25	19.16000*	1.18289	0.005
	12.5	4.24667*	0.66334	0.039
	25	4.16000	1.52385	0.470

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Different letter is significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table N6 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing CMPE, its control and within group concentrations 0.78-50 mg/ml.

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	4.249	7	15	0.009

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.009 < 0.05 which indicates that variances of the mean are unequal or equal variance are not assumed.

Table N7 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing CMPE, its control and within group concentrations 0.78-50 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16167.352	7	2309.622	768.455	0.000
Within Groups	45.083	15	3.006		
Total	16212.435	22			

*Sig. 0.000 < 0.05 indicates that all means of the data from at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N8 Results of Post Hoc test, Dunnett's T3 of growth inhibition (%) by using broth microdilution assay by comparing CMPE, its control and within group concentrations 0.78-50 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.78	-42.53000*	0.86000	0.037
	1.56	-51.82333*	0.71406	0.001
	3.13	-64.59333*	0.51615	0.000
	6.25	-71.76667*	0.92551	0.001
	12.5	-73.67000*	0.39552	0.000
	25	-73.55333*	0.88364	0.001
	50	-91.49000*	2.16730	0.003
0.78	control	42.53000*	0.86000	0.037
	1.56	-9.29333	1.11780	0.056
	3.13	-22.06333*	1.00300	0.019
	6.25	-29.23667*	1.26340	0.002
	12.5	-31.14000*	0.94659	0.019
	25	-31.02333*	1.23305	0.002
	50	-48.96000*	2.33169	0.004

1.56	control	51.82333*	0.71406	0.001
	0.78	9.29333	1.11780	0.056
	3.13	-12.77000*	0.88107	0.002
	6.25	-19.94333*	1.16895	0.001
	12.5	-21.84667*	0.81628	0.001
	25	-21.73000*	1.13608	0.001
	50	-39.66667*	2.28190	0.009
3.13	control	64.59333*	0.51615	0.000
	0.78	22.06333*	1.00300	0.019
	1.56	12.77000*	0.88107	0.002
	6.25	-7.17333*	1.05971	0.048
	12.5	-9.07667*	0.65026	0.002
	25	-8.96000*	1.02334	0.021
	50	-26.89667*	2.22792	0.027
6.25	control	71.76667*	0.92551	0.001
	0.78	29.23667*	1.26340	0.002
	1.56	19.94333*	1.16895	0.001
	3.13	7.17333*	1.05971	0.048
	12.5	-1.90333	1.00648	0.747
	25	-1.78667	1.27960	0.934
	50	-19.72333*	2.35665	0.038
12.5	control	73.67000*	0.39552	0.000
	0.78	31.14000*	0.94659	0.019
	1.56	21.84667*	0.81628	0.001
	3.13	9.07667*	0.65026	0.002
	6.25	1.90333	1.00648	0.747
	25	0.11667	0.96811	1.000
	50	-17.82000	2.20310	0.070

25	control	73.55333*	0.88364	0.001
	0.78	31.02333*	1.23305	0.002
	1.56	21.73000*	1.13608	0.001
	3.13	8.96000*	1.02334	0.021
	6.25	1.78667	1.27960	0.934
	12.5	-0.11667	0.96811	1.000
	50	-17.93667	2.34052	0.050
50	control	91.49000*	2.16730	0.003
	0.78	48.96000*	2.33169	0.004
	1.56	39.66667*	2.28190	0.009
	3.13	26.89667*	2.22792	0.027
	6.25	19.72333*	2.35665	0.038
	12.5	17.82000	2.20310	0.070
	25	17.93667	2.34052	0.050

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Different letters are significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table N9 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (10% DMSO) and within group concentrations 0.37-50 mg/ml.

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	4.298	8	45	0.001

*The data is a normal distribution. From the Based on Mean value, Sig. 0.001 < 0.05 which indicates that variances of the mean are unequal or equal variance is not assumed.

Table N10 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (10% DMSO) and within group concentrations 0.37-50 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47785.939	8	5973.242	1972.538	0.000
Within Groups	136.269	45	3.028		
Total	47922.208	53			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N11 Results of Post Hoc test, Dunnett's T3 of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (10% DMSO) and within group concentrations 0.37-50 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.37	-77.56167*	1.74325	0.000
	0.78	-80.11000*	1.00081	0.000
	1.56	-83.91000*	0.29542	0.000
	3.13	-90.53167*	0.59265	0.000
	6.25	-96.96500*	0.16229	0.000
	12.5	-98.96667*	0.13561	0.000
	25	-99.84333*	0.13111	0.000
	50	-99.94167*	0.03646	0.000

0.37	control	77.56167*	1.74325	0.000
	0.78	-2.54833	2.01011	0.990
	1.56	-6.34833	1.76811	0.190
	3.13	-12.97000*	1.84124	0.007
	6.25	-19.40333*	1.75079	0.002
	12.5	-21.40500*	1.74852	0.001
	25	-22.28167*	1.74817	0.001
	50	-22.38000*	1.74363	0.001
0.78	control	80.11000*	1.00081	0.000
	0.37	2.54833	2.01011	0.990
	1.56	-3.80000	1.04350	0.166
	3.13	-10.42167*	1.16312	0.000
	6.25	-16.85500*	1.01389	0.000
	12.5	-18.85667*	1.00996	0.000
	25	-19.73333*	1.00937	0.000
	50	-19.83167*	1.00148	0.000
1.56	control	83.91000*	0.29542	0.000
	0.37	6.34833	1.76811	0.190
	0.78	3.80000	1.04350	0.166
	3.13	-6.62167*	0.66220	0.000
	6.25	-13.05500*	0.33706	0.000
	12.5	-15.05667*	0.32506	0.000
	25	-15.93333*	0.32321	0.000
	50	-16.03167*	0.29766	0.000
3.13	control	90.53167*	0.59265	0.000
	0.37	12.97000*	1.84124	0.007
	0.78	10.42167*	1.16312	0.000
	1.56	6.62167*	0.66220	0.000
	6.25	-6.43333*	0.61447	0.001
	12.5	-8.43500*	0.60796	0.000
	25	-9.31167*	0.60698	0.000
	50	-9.41000*	0.59377	0.000

6.25	control	96.96500*	0.16229	0.000
	0.37	19.40333*	1.75079	0.002
	0.78	16.85500*	1.01389	0.000
	1.56	13.05500*	0.33706	0.000
	3.13	6.43333*	0.61447	0.001
	12.5	-2.00167*	0.21149	0.000
	25	-2.87833*	0.20864	0.000
	50	-2.97667*	0.16634	0.000
12.5	control	98.96667*	0.13561	0.000
	0.37	21.40500*	1.74852	0.001
	0.78	18.85667*	1.00996	0.000
	1.56	15.05667*	0.32506	0.000
	3.13	8.43500*	0.60796	0.000
	6.25	2.00167*	0.21149	0.000
	25	-.87667*	0.18863	0.023
	50	-.97500*	0.14043	0.010
25	control	99.84333*	0.13111	0.000
	0.37	22.28167*	1.74817	0.001
	0.78	19.73333*	1.00937	0.000
	1.56	15.93333*	0.32321	0.000
	3.13	9.31167*	0.60698	0.000
	6.25	2.87833*	0.20864	0.000
	12.5	.87667*	0.18863	0.023
	50	-0.09833	0.13609	1.000

50	control	99.94167*	0.03646	0.000
	0.37	22.38000*	1.74363	0.001
	0.78	19.83167*	1.00148	0.000
	1.56	16.03167*	0.29766	0.000
	3.13	9.41000*	0.59377	0.000
	6.25	2.97667*	0.16634	0.000
	12.5	.97500*	0.14043	0.010
	25	0.09833	0.13609	1.000

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Different letters are significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table N12 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (1% DMSO) and within group concentrations 0.016-2 mg/ml.

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	4.364	8	45	0.001

*The data is a normal distribution. From the Based on Mean value, Sig. 0.001 < 0.05 indicates that variances of the mean are unequal or equal variance is not assumed.

Table N13 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (1% DMSO) and within group concentrations 0.016-2 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	53259.578	8	6657.447	1263.256	0.000
Within Groups	237.153	45	5.270		
Total	53496.731	53			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N14 Results of Post Hoc test, Dunnett's T3 of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (1% DMSO) and within group concentrations 0.016-2 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.016	-14.35500*	1.41044	0.003
	0.031	-34.78333*	1.55897	0.000
	0.063	-57.35333*	0.98504	0.000
	0.125	-72.31833*	1.37317	0.000
	0.25	-79.93333*	0.49700	0.000
	0.5	-82.47500*	0.38260	0.000
	1	-86.27667*	0.38289	0.000
	2	-89.40833*	0.29914	0.000
0.016	control	14.35500*	1.41044	0.003
	0.031	-20.42833*	2.10231	0.000
	0.063	-42.99833*	1.72036	0.000
	0.125	-57.96333*	1.96849	0.000
	0.25	-65.57833*	1.49544	0.000
	0.5	-68.12000*	1.46141	0.000
	1	-71.92167*	1.46149	0.000
	2	-75.05333*	1.44181	0.000
0.031	control	34.78333*	1.55897	0.000
	0.016	20.42833*	2.10231	0.000
	0.063	-22.57000*	1.84410	0.000
	0.125	-37.53500*	2.07749	0.000
	0.25	-45.15000*	1.63627	0.000
	0.5	-47.69167*	1.60523	0.000
	1	-51.49333*	1.60530	0.000
	2	-54.62500*	1.58741	0.000

0.063	control	57.35333*	0.98504	0.000
	0.016	42.99833*	1.72036	0.000
	0.031	22.57000*	1.84410	0.000
	0.125	-14.96500*	1.68994	0.000
	0.25	-22.58000*	1.10332	0.000
	0.5	-25.12167*	1.05674	0.000
	1	-28.92333*	1.05684	0.000
	2	-32.05500*	1.02946	0.000
0.125	control	72.31833*	1.37317	0.000
	0.016	57.96333*	1.96849	0.000
	0.031	37.53500*	2.07749	0.000
	0.063	14.96500*	1.68994	0.000
	0.25	-7.61500*	1.46034	0.032
	0.5	-10.15667*	1.42548	0.008
	1	-13.95833*	1.42556	0.002
	2	-17.09000*	1.40538	0.001
0.25	control	79.93333*	0.49700	0.000
	0.016	65.57833*	1.49544	0.000
	0.031	45.15000*	1.63627	0.000
	0.063	22.58000*	1.10332	0.000
	0.125	7.61500*	1.46034	0.032
	0.5	-2.54167	0.62721	0.060
	1	-6.34333*	0.62738	0.000
	2	-9.47500*	0.58008	0.000
0.5	control	82.47500*	0.38260	0.000
	0.016	68.12000*	1.46141	0.000
	0.031	47.69167*	1.60523	0.000
	0.063	25.12167*	1.05674	0.000
	0.125	10.15667*	1.42548	0.008
	0.25	2.54167	0.62721	0.060
	1	-3.80167*	0.54128	0.001
	2	-6.93333*	0.48566	0.000

1	control	86.27667*	0.38289	0.000
	0.016	71.92167*	1.46149	0.000
	0.031	51.49333*	1.60530	0.000
	0.063	28.92333*	1.05684	0.000
	0.125	13.95833*	1.42556	0.002
	0.25	6.34333*	0.62738	0.000
	0.5	3.80167*	0.54128	0.001
	2	-3.13167*	0.48589	0.003
2	control	89.40833*	0.29914	0.000
	0.016	75.05333*	1.44181	0.000
	0.031	54.62500*	1.58741	0.000
	0.063	32.05500*	1.02946	0.000
	0.125	17.09000*	1.40538	0.001
	0.25	9.47500*	0.58008	0.000
	0.5	6.93333*	0.48566	0.000
	1	3.13167*	0.48589	0.003

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Different letters are significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table N15 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG (Std. MG), synthetic MG (Syn. MG), and CMPE 1 (Ext. MG) at concentrations 0.031-0.125 mg/ml and control (1% DMSO).

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	23.894	9	62	0.000

*The data is a normal distribution. From the Based on Mean value, Sig. 0.000 < 0.05 indicates that variances of the mean are unequal or equal variance is not assumed.

Table N16 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG (Std. MG), synthetic MG (Syn. MG), and CMPE 1 (Ext. MG) at concentrations 0.031-0.125 mg/ml and control (1% DMSO).

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29905.404	9	3322.823	562.388	0.000
Within Groups	366.322	62	5.908		
Total	30271.726	71			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N17 Results of Post Hoc test, Dunnett's T3 of growth inhibition (%) by using broth microdilution assay by comparing standard MG (Std. MG), synthetic MG (Syn. MG), and CMPE 1 (Ext. MG) at concentrations 0.031-0.125 mg/ml and control (1% DMSO).

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	Std MG 0.031	-39.12833*	0.50630	0.000
	Std MG 0.063	-49.83000*	0.49343	0.000
	Std MG 0.125	-65.19500*	1.36646	0.000
	Syn MG 0.031	-31.46833*	2.20635	0.001
	Syn MG 0.063	-37.44000*	1.10733	0.000
	Syn MG 0.125	-44.45500*	1.37262	0.000
	Ext MG 0.031	-33.27333*	0.84970	0.000
	Ext MG 0.063	-38.56500*	0.71997	0.000
	Ext MG 0.125	-46.56667*	0.79062	0.000
Std MG 0.031	control	39.12833*	0.50630	0.000
	Std MG 0.063	-10.70167*	0.70698	0.000
	Std MG 0.125	-26.06667*	1.45724	0.000
	Syn MG 0.031	7.66000	2.26370	0.247
	Syn MG 0.063	1.68833	1.21759	0.982
	Syn MG 0.125	-5.32667	1.46302	0.174
	Ext MG 0.031	5.85500*	0.98911	0.009
	Ext MG 0.063	0.56333	0.88017	1.000
	Ext MG 0.125	-7.43833*	0.93884	0.001

Std MG 0.063	control	49.83000*	0.49343	0.000
	Std MG 0.031	10.70167*	0.70698	0.000
	Std MG 0.125	-15.36500*	1.45282	0.001
	Syn MG 0.031	18.36167*	2.26085	0.006
	Syn MG 0.063	12.39000*	1.21230	0.001
	Syn MG 0.125	5.37500	1.45862	0.167
	Ext MG 0.031	16.55667*	0.98258	0.000
	Ext MG 0.063	11.26500*	0.87283	0.000
	Ext MG 0.125	3.26333	0.93196	0.163
Std MG 0.125	control	65.19500*	1.36646	0.000
	Std MG 0.031	26.06667*	1.45724	0.000
	Std MG 0.063	15.36500*	1.45282	0.001
	Syn MG 0.031	33.72667*	2.59523	0.000
	Syn MG 0.063	27.75500*	1.75881	0.000
	Syn MG 0.125	20.74000*	1.93683	0.000
	Ext MG 0.031	31.92167*	1.60910	0.000
	Ext MG 0.063	26.63000*	1.54453	0.000
	Ext MG 0.125	18.62833*	1.57870	0.000
Syn MG 0.031	control	31.46833*	2.20635	0.001
	Std MG 0.031	-7.66000	2.26370	0.247
	Std MG 0.063	-18.36167*	2.26085	0.006
	Std MG 0.125	-33.72667*	2.59523	0.000
	Syn MG 0.063	-5.97167	2.46864	0.574
	Syn MG 0.125	-12.98667*	2.59847	0.025
	Ext MG 0.031	-1.80500	2.36431	1.000
	Ext MG 0.063	-7.09667	2.32085	0.323
	Ext MG 0.125	-15.09833*	2.34373	0.013

Syn MG 0.063	control	37.44000*	1.10733	0.000
	Std MG 0.031	-1.68833	1.21759	0.982
	Std MG 0.063	-12.39000*	1.21230	0.001
	Std MG 0.125	-27.75500*	1.75881	0.000
	Syn MG 0.031	5.97167	2.46864	0.574
	Syn MG 0.125	-7.01500	1.76360	0.076
	Ext MG 0.031	4.16667	1.39577	0.296
	Ext MG 0.063	-1.12500	1.32081	1.000
	Ext MG 0.125	-9.12667*	1.36061	0.003
Syn MG 0.125	control	44.45500*	1.37262	0.000
	Std MG 0.031	5.32667	1.46302	0.174
	Std MG 0.063	-5.37500	1.45862	0.167
	Std MG 0.125	-20.74000*	1.93683	0.000
	Syn MG 0.031	12.98667*	2.59847	0.025
	Syn MG 0.063	7.01500	1.76360	0.076
	Ext MG 0.031	11.18167*	1.61434	0.003
	Ext MG 0.063	5.89000	1.54998	0.124
	Ext MG 0.125	-2.11167	1.58404	0.990
Ext MG 0.031	control	33.27333*	0.84970	0.000
	Std MG 0.031	-5.85500*	0.98911	0.009
	Std MG 0.063	-16.55667*	0.98258	0.000
	Std MG 0.125	-31.92167*	1.60910	0.000
	Syn MG 0.031	1.80500	2.36431	1.000
	Syn MG 0.063	-4.16667	1.39577	0.296
	Syn MG 0.125	-11.18167*	1.61434	0.003
	Ext MG 0.063	-5.29167*	1.11371	0.025
	Ext MG 0.125	-13.29333*	1.16064	0.000

	control	38.56500*	0.71997	0.000
	Std MG 0.031	-0.56333	0.88017	1.000
	Std MG 0.063	-11.26500*	0.87283	0.000
	Std MG 0.125	-26.63000*	1.54453	0.000
Ext MG 0.063	Syn MG 0.031	7.09667	2.32085	0.323
	Syn MG 0.063	1.12500	1.32081	1.000
	Syn MG 0.125	-5.89000	1.54998	0.124
	Ext MG 0.031	5.29167*	1.11371	0.025
	Ext MG 0.125	-8.00167*	1.06932	0.001
	control	46.56667*	0.79062	0.000
	Std MG 0.031	7.43833*	0.93884	0.001
	Std MG 0.063	-3.26333	0.93196	0.163
	Std MG 0.125	-18.62833*	1.57870	0.000
Ext MG 0.125	Syn MG 0.031	15.09833*	2.34373	0.013
	Syn MG 0.063	9.12667*	1.36061	0.003
	Syn MG 0.125	2.11167	1.58404	0.990
	Ext MG 0.031	13.29333*	1.16064	0.000
	Ext MG 0.063	8.00167*	1.06932	0.001

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Different letters are significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table N18 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (2% DMSO) and within group concentrations at 0.5-10 mg/ml.

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	10.225	11	96	0.000

*The data is a normal distribution. From the Based on Mean value, Sig. 0.000 < 0.05 indicates that variances of the mean are unequal or equal variance is not assumed.

Table N19 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (2% DMSO) and within group concentrations at 0.5-10 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73059.838	11	6641.803	2207.258	0.000
Within Groups	288.871	96	3.009		
Total	73348.710	107			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N20 Results of Post Hoc test, Games-Howell of growth inhibition (%) by using broth microdilution assay by comparing standard MG, its control (2% DMSO) and within group concentrations at 0.5-10 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.5	-73.88000*	1.24715	0.000
	1	-81.01889*	0.34771	0.000
	2	-83.61222*	1.26793	0.000
	3	-87.58556*	0.59443	0.000
	4	-90.27111*	0.46148	0.000
	5	-92.42889*	0.27919	0.000
	6	-94.09889*	0.19780	0.000
	7	-96.07222*	0.14771	0.000
	8	-97.38333*	0.07663	0.000
	9	-98.06444*	0.09592	0.000
	10	-98.85000*	0.08894	0.000

0.5	control	73.88000*	1.24715	0.000
	1	-7.13889*	1.29471	0.009
	2	-9.73222*	1.77849	0.002
	3	-13.70556*	1.38157	0.000
	4	-16.39111*	1.32979	0.000
	5	-18.54889*	1.27802	0.000
	6	-20.21889*	1.26274	0.000
	7	-22.19222*	1.25587	0.000
	8	-23.50333*	1.24950	0.000
	9	-24.18444*	1.25083	0.000
	10	-24.97000*	1.25032	0.000
1	control	81.01889*	0.34771	0.000
	0.5	7.13889*	1.29471	0.009
	2	-2.59333	1.31474	0.702
	3	-6.56667*	0.68866	0.000
	4	-9.25222*	0.57781	0.000
	5	-11.41000*	0.44592	0.000
	6	-13.08000*	0.40003	0.000
	7	-15.05333*	0.37778	0.000
	8	-16.36444*	0.35605	0.000
	9	-17.04556*	0.36070	0.000
	10	-17.83111*	0.35890	0.000

2	control	83.61222*	1.26793	0.000
	0.5	9.73222*	1.77849	0.002
	1	2.59333	1.31474	0.702
	3	-3.97333	1.40035	0.277
	4	-6.65889*	1.34930	0.016
	5	-8.81667*	1.29830	0.003
	6	-10.48667*	1.28326	0.001
	7	-12.46000*	1.27650	0.000
	8	-13.77111*	1.27024	0.000
	9	-14.45222*	1.27155	0.000
	10	-15.23778*	1.27104	0.000
3	control	87.58556*	0.59443	0.000
	0.5	13.70556*	1.38157	0.000
	1	6.56667*	0.68866	0.000
	2	3.97333	1.40035	0.277
	4	-2.68556	0.75253	0.078
	5	-4.84333*	0.65673	0.000
	6	-6.51333*	0.62647	0.000
	7	-8.48667*	0.61251	0.000
	8	-9.79778*	0.59935	0.000
	9	-10.47889*	0.60212	0.000
	10	-11.26444*	0.60105	0.000
4	control	90.27111*	0.46148	0.000
	0.5	16.39111*	1.32979	0.000
	1	9.25222*	0.57781	0.000
	2	6.65889*	1.34930	0.016
	3	2.68556	0.75253	0.078
	5	-2.15778*	0.53936	0.043
	6	-3.82778*	0.50208	0.000
	7	-5.80111*	0.48454	0.000
	8	-7.11222*	0.46780	0.000
	9	-7.79333*	0.47134	0.000
	10	-8.57889*	0.46997	0.000

5	control	92.42889*	0.27919	0.000
	0.5	18.54889*	1.27802	0.000
	1	11.41000*	0.44592	0.000
	2	8.81667*	1.29830	0.003
	3	4.84333*	0.65673	0.000
	4	2.15778*	0.53936	0.043
	6	-1.67000*	0.34215	0.008
	7	-3.64333*	0.31586	0.000
	8	-4.95444*	0.28951	0.000
	9	-5.63556*	0.29520	0.000
	10	-6.42111*	0.29301	0.000
6	control	94.09889*	0.19780	0.000
	0.5	20.21889*	1.26274	0.000
	1	13.08000*	0.40003	0.000
	2	10.48667*	1.28326	0.001
	3	6.51333*	0.62647	0.000
	4	3.82778*	0.50208	0.000
	5	1.67000*	0.34215	0.008
	7	-1.97333*	0.24686	0.000
	8	-3.28444*	0.21212	0.000
	9	-3.96556*	0.21983	0.000
	10	-4.75111*	0.21687	0.000
7	control	96.07222*	0.14771	0.000
	0.5	22.19222*	1.25587	0.000
	1	15.05333*	0.37778	0.000
	2	12.46000*	1.27650	0.000
	3	8.48667*	0.61251	0.000
	4	5.80111*	0.48454	0.000
	5	3.64333*	0.31586	0.000
	6	1.97333*	0.24686	0.000
	8	-1.31111*	0.16641	0.000
	9	-1.99222*	0.17612	0.000
	10	-2.77778*	0.17242	0.000

8	control	97.38333*	0.07663	0.000
	0.5	23.50333*	1.24950	0.000
	1	16.36444*	0.35605	0.000
	2	13.77111*	1.27024	0.000
	3	9.79778*	0.59935	0.000
	4	7.11222*	0.46780	0.000
	5	4.95444*	0.28951	0.000
	6	3.28444*	0.21212	0.000
	7	1.31111*	0.16641	0.000
	9	-.68111*	0.12277	0.002
	10	-1.46667*	0.11740	0.000
9	control	98.06444*	0.09592	0.000
	0.5	24.18444*	1.25083	0.000
	1	17.04556*	0.36070	0.000
	2	14.45222*	1.27155	0.000
	3	10.47889*	0.60212	0.000
	4	7.79333*	0.47134	0.000
	5	5.63556*	0.29520	0.000
	6	3.96556*	0.21983	0.000
	7	1.99222*	0.17612	0.000
	8	0.68111*	0.12277	0.002
	10	-0.78556*	0.13081	0.001

10	control	98.85000*	0.08894	0.000
	0.5	24.97000*	1.25032	0.000
	1	17.83111*	0.35890	0.000
	2	15.23778*	1.27104	0.000
	3	11.26444*	0.60105	0.000
	4	8.57889*	0.46997	0.000
	5	6.42111*	0.29301	0.000
	6	4.75111*	0.21687	0.000
	7	2.77778*	0.17242	0.000
	8	1.46667*	0.11740	0.000
	9	0.78556*	0.13081	0.001

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. From a group column, different letters are significantly different. Games-Howell was used due to a big population size and treatments have number of samples more than 5.

Table N21 Results of Homogeneity of Variances test (Based on Mean) of percentage of growth inhibition (%) by using broth microdilution assay by comparing between standard MG in 1% and 2% DMSO at 0.5-2 mg/ml.

Percentage of inhibition (%)	Levene Statistic	df1	df2	Sig.
Based on Mean	9.625	6	13	0.000

*The data is a normal distribution. From the Based on Mean value, Sig. 0.000 < 0.05 indicates that variances of the mean are unequal or equal variance is not assumed.

Table N22 Results of ANOVA test of percentage of growth inhibition (%) by using broth microdilution assay by comparing between standard MG in 1% and 2% DMSO at 0.5-2 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	25646.063	6	4274.344	953.621	0.000
Within Groups	58.269	13	4.482		
Total	25704.332	19			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table N23 Results of Post Hoc test, Dunnett's T3 of growth inhibition (%) by using broth microdilution assay by comparing between standard MG in 1% and 2% DMSO at 0.5-2 mg/ml.

Compound and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	std1_0.5	-82.47500*	0.12500	0.003
	std1_1	-86.28000*	0.59000	0.011
	std1_2	-89.41000*	0.50000	0.009
	std2_0.5	-73.88000*	1.91636	0.003
	std2_1	-81.01667*	0.40354	0.000
	std2_2	-83.61000*	2.38152	0.004
std1_0.5	control	82.47500*	0.12500	0.003
	std1_1	-3.80500	0.60310	0.249
	std1_2	-6.93500	0.51539	0.105
	std2_0.5	8.59500	1.92043	0.215
	std2_1	1.45833	0.42246	0.301
	std2_2	-1.13500	2.38480	1.000
std1_1	control	86.28000*	0.59000	0.011
	std1_0.5	3.80500	0.60310	0.249
	std1_2	-3.13000	0.77337	0.262
	std2_0.5	12.40000	2.00513	0.094
	std2_1	5.26333	0.71480	0.092
	std2_2	2.67000	2.45351	0.963
std1_2	control	89.41000*	0.50000	0.009
	std1_0.5	6.93500	0.51539	0.105
	std1_1	3.13000	0.77337	0.262
	std2_0.5	15.53000	1.98051	0.061
	std2_1	8.39333*	0.64253	0.020
	std2_2	5.80000	2.43344	0.549

std2_0.5	control	73.88000*	1.91636	0.003
	std1_0.5	-8.59500	1.92043	0.215
	std1_1	-12.40000	2.00513	0.094
	std1_2	-15.53000	1.98051	0.061
	std2_1	-7.13667	1.95839	0.288
	std2_2	-9.73000	3.05681	0.265
std2_1	control	81.01667*	0.40354	0.000
	std1_0.5	-1.45833	0.42246	0.301
	std1_1	-5.26333	0.71480	0.092
	std1_2	-8.39333*	0.64253	0.020
	std2_0.5	7.13667	1.95839	0.288
	std2_2	-2.59333	2.41547	0.964
std2_2	control	83.61000*	2.38152	0.004
	std1_0.5	1.13500	2.38480	1.000
	std1_1	-2.67000	2.45351	0.963
	std1_2	-5.80000	2.43344	0.549
	std2_0.5	9.73000	3.05681	0.265
	std2_1	2.59333	2.41547	0.964

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Different letters are significantly different. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Appendix O: Statistical analysis of lipase inhibition assay results.

Table O1 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by CME at concentrations 0.2-0.5 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	4.461	4	10	0.025

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.025 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O2 Results of ANOVA test of percentage of lipase inhibition (%) by CME at concentrations 0.2-0.5 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9388.361	4	2347.090	213.499	0.000
Within Groups	109.934	10	10.993		
Total	9498.295	14			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O3 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by CME at concentrations 0.2-0.5 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.2	-70.98333*	1.50591	0.002
	0.3	-58.80000*	3.31717	0.012
	0.4	-44.50000*	0.82614	0.001
	0.5	-26.00667*	2.09009	0.024
0.2	control	70.98333*	1.50591	0.002
	0.3	12.18333	3.64299	0.214
	0.4	26.48333*	1.71764	0.003
	0.5	44.97667*	2.57609	0.001
0.3	control	58.80000*	3.31717	0.012
	0.2	-12.18333	3.64299	0.214
	0.4	14.30000	3.41850	0.167
	0.5	32.79333*	3.92073	0.012
0.4	control	44.50000*	0.82614	0.001
	0.2	-26.48333*	1.71764	0.003
	0.3	-14.30000	3.41850	0.167
	0.5	18.49333*	2.24744	0.028
0.5	control	26.00667*	2.09009	0.024
	0.2	-44.97667*	2.57609	0.001
	0.3	-32.79333*	3.92073	0.012
	0.4	-18.49333*	2.24744	0.028

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O4 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by CME, CMPE, CDPE, and CHPE at concentrations 0.025-0.1 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	2.092	12	23	0.062

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.062 > 0.05 indicate that variances of the mean are equal or equal variance is assumed.

Table O5 Results of ANOVA test of percentage of lipase inhibition (%) by CME, CMPE, CDPE, and CHPE at concentrations 0.025-0.1 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12165.554	12	1013.796	38.844	0.000
Within Groups	600.277	23	26.099		
Total	12765.831	35			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O6 Results of Post Hoc test, Tukey HSD of lipase inhibition (%) by CME, CMPE, CDPE, and CHPE at concentrations 0.025-0.1 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	CME 0.025	-39.80500*	4.66360	0.000
	CME 0.05	-34.94500*	4.66360	0.000
	CME 0.1	-36.44500*	4.66360	0.000
	CMPE 0.025	-35.75667*	4.17125	0.000
	CMPE 0.05	-60.18000*	4.17125	0.000
	CMPE 0.1	-80.10667*	4.17125	0.000
	CDPE 0.025	-38.30333*	4.17125	0.000
	CDPE 0.05	-44.25333*	4.17125	0.000
	CDPE 0.1	-29.64000*	4.17125	0.000
	CHPE 0.025	-28.46333*	4.17125	0.000
	CHPE 0.05	-40.49000*	4.17125	0.000
	CHPE 0.1	-26.80333*	4.17125	0.000
CME 0.025	control	39.80500*	4.66360	0.000
	CME 0.05	4.86000	5.10872	0.999
	CME 0.1	3.36000	5.10872	1.000
	CMPE 0.025	4.04833	4.66360	0.999
	CMPE 0.05	-20.37500*	4.66360	0.011
	CMPE 0.1	-40.30167*	4.66360	0.000
	CDPE 0.025	1.50167	4.66360	1.000
	CDPE 0.05	-4.44833	4.66360	0.998
	CDPE 0.1	10.16500	4.66360	0.613
	CHPE 0.025	11.34167	4.66360	0.458
	CHPE 0.05	-0.68500	4.66360	1.000
	CHPE 0.1	13.00167	4.66360	0.272

CME 0.05	control	34.94500*	4.66360	0.000
	CME 0.025	-4.86000	5.10872	0.999
	CME 0.1	-1.50000	5.10872	1.000
	CMPE 0.025	-0.81167	4.66360	1.000
	CMPE 0.05	-25.23500*	4.66360	0.001
	CMPE 0.1	-45.16167*	4.66360	0.000
	CDPE 0.025	-3.35833	4.66360	1.000
	CDPE 0.05	-9.30833	4.66360	0.726
	CDPE 0.1	5.30500	4.66360	0.993
	CHPE 0.025	6.48167	4.66360	0.965
	CHPE 0.05	-5.54500	4.66360	0.990
	CHPE 0.1	8.14167	4.66360	0.856
CME 0.1	control	36.44500*	4.66360	0.000
	CME 0.025	-3.36000	5.10872	1.000
	CME 0.05	1.50000	5.10872	1.000
	CMPE 0.025	0.68833	4.66360	1.000
	CMPE 0.05	-23.73500*	4.66360	0.002
	CMPE 0.1	-43.66167*	4.66360	0.000
	CDPE 0.025	-1.85833	4.66360	1.000
	CDPE 0.05	-7.80833	4.66360	0.886
	CDPE 0.1	6.80500	4.66360	0.952
	CHPE 0.025	7.98167	4.66360	0.871
	CHPE 0.05	-4.04500	4.66360	0.999
	CHPE 0.1	9.64167	4.66360	0.683

CMPE 0.025	control	35.75667*	4.17125	0.000
	CME 0.025	-4.04833	4.66360	0.999
	CME 0.05	0.81167	4.66360	1.000
	CME 0.1	-0.68833	4.66360	1.000
	CMPE 0.05	-24.42333*	4.17125	0.000
	CMPE 0.1	-44.35000*	4.17125	0.000
	CDPE 0.025	-2.54667	4.17125	1.000
	CDPE 0.05	-8.49667	4.17125	0.701
	CDPE 0.1	6.11667	4.17125	0.950
	CHPE 0.025	7.29333	4.17125	0.855
	CHPE 0.05	-4.73333	4.17125	0.993
	CHPE 0.1	8.95333	4.17125	0.634
CMPE 0.05	control	60.18000*	4.17125	0.000
	CME 0.025	20.37500*	4.66360	0.011
	CME 0.05	25.23500*	4.66360	0.001
	CME 0.1	23.73500*	4.66360	0.002
	CMPE 0.025	24.42333*	4.17125	0.000
	CMPE 0.1	-19.92667*	4.17125	0.004
	CDPE 0.025	21.87667*	4.17125	0.001
	CDPE 0.05	15.92667*	4.17125	0.037
	CDPE 0.1	30.54000*	4.17125	0.000
	CHPE 0.025	31.71667*	4.17125	0.000
	CHPE 0.05	19.69000*	4.17125	0.005
	CHPE 0.1	33.37667*	4.17125	0.000

CMPE 0.1	control	80.10667*	4.17125	0.000
	CME 0.025	40.30167*	4.66360	0.000
	CME 0.05	45.16167*	4.66360	0.000
	CME 0.1	43.66167*	4.66360	0.000
	CMPE 0.025	44.35000*	4.17125	0.000
	CMPE 0.05	19.92667*	4.17125	0.004
	CDPE 0.025	41.80333*	4.17125	0.000
	CDPE 0.05	35.85333*	4.17125	0.000
	CDPE 0.1	50.46667*	4.17125	0.000
	CHPE 0.025	51.64333*	4.17125	0.000
	CHPE 0.05	39.61667*	4.17125	0.000
	CHPE 0.1	53.30333*	4.17125	0.000
CDPE 0.025	control	38.30333*	4.17125	0.000
	CME 0.025	-1.50167	4.66360	1.000
	CME 0.05	3.35833	4.66360	1.000
	CME 0.1	1.85833	4.66360	1.000
	CMPE 0.025	2.54667	4.17125	1.000
	CMPE 0.05	-21.87667*	4.17125	0.001
	CMPE 0.1	-41.80333*	4.17125	0.000
	CDPE 0.05	-5.95000	4.17125	0.959
	CDPE 0.1	8.66333	4.17125	0.677
	CHPE 0.025	9.84000	4.17125	0.502
	CHPE 0.05	-2.18667	4.17125	1.000
	CHPE 0.1	11.50000	4.17125	0.286

CDPE 0.05	control	44.25333*	4.17125	0.000
	CME 0.025	4.44833	4.66360	0.998
	CME 0.05	9.30833	4.66360	0.726
	CME 0.1	7.80833	4.66360	0.886
	CMPE 0.025	8.49667	4.17125	0.701
	CMPE 0.05	-15.92667*	4.17125	0.037
	CMPE 0.1	-35.85333*	4.17125	0.000
	CDPE 0.025	5.95000	4.17125	0.959
	CDPE 0.1	14.61333	4.17125	0.072
	CHPE 0.025	15.79000*	4.17125	0.040
	CHPE 0.05	3.76333	4.17125	0.999
	CHPE 0.1	17.45000*	4.17125	0.016
CDPE 0.1	control	29.64000*	4.17125	0.000
	CME 0.025	-10.16500	4.66360	0.613
	CME 0.05	-5.30500	4.66360	0.993
	CME 0.1	-6.80500	4.66360	0.952
	CMPE 0.025	-6.11667	4.17125	0.950
	CMPE 0.05	-30.54000*	4.17125	0.000
	CMPE 0.1	-50.46667*	4.17125	0.000
	CDPE 0.025	-8.66333	4.17125	0.677
	CDPE 0.05	-14.61333	4.17125	0.072
	CHPE 0.025	1.17667	4.17125	1.000
	CHPE 0.05	-10.85000	4.17125	0.362
	CHPE 0.1	2.83667	4.17125	1.000

CHPE 0.025	control	28.46333*	4.17125	0.000
	CME 0.025	-11.34167	4.66360	0.458
	CME 0.05	-6.48167	4.66360	0.965
	CME 0.1	-7.98167	4.66360	0.871
	CMPE 0.025	-7.29333	4.17125	0.855
	CMPE 0.05	-31.71667*	4.17125	0.000
	CMPE 0.1	-51.64333*	4.17125	0.000
	CDPE 0.025	-9.84000	4.17125	0.502
	CDPE 0.05	-15.79000*	4.17125	0.040
	CDPE 0.1	-1.17667	4.17125	1.000
	CHPE 0.05	-12.02667	4.17125	0.232
	CHPE 0.1	1.66000	4.17125	1.000
CHPE 0.05	control	40.49000*	4.17125	0.000
	CME 0.025	0.68500	4.66360	1.000
	CME 0.05	5.54500	4.66360	0.990
	CME 0.1	4.04500	4.66360	0.999
	CMPE 0.025	4.73333	4.17125	0.993
	CMPE 0.05	-19.69000*	4.17125	0.005
	CMPE 0.1	-39.61667*	4.17125	0.000
	CDPE 0.025	2.18667	4.17125	1.000
	CDPE 0.05	-3.76333	4.17125	0.999
	CDPE 0.1	10.85000	4.17125	0.362
	CHPE 0.025	12.02667	4.17125	0.232
	CHPE 0.1	13.68667	4.17125	0.112

CHPE 0.1	control	26.80333*	4.17125	0.000
	CME 0.025	-13.00167	4.66360	0.272
	CME 0.05	-8.14167	4.66360	0.856
	CME 0.1	-9.64167	4.66360	0.683
	CMPE 0.025	-8.95333	4.17125	0.634
	CMPE 0.05	-33.37667*	4.17125	0.000
	CMPE 0.1	-53.30333*	4.17125	0.000
	CDPE 0.025	-11.50000	4.17125	0.286
	CDPE 0.05	-17.45000*	4.17125	0.016
	CDPE 0.1	-2.83667	4.17125	1.000
	CHPE 0.025	-1.66000	4.17125	1.000
	CHPE 0.05	-13.68667	4.17125	0.112

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level.

Table O7 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by standard MG at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	6.679	3	8	0.014

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.014 < 0.05 indicate that variances of the mean are unequal or equal variances are not assumed.

Table O8 Results of ANOVA test of percentage of lipase inhibition (%) by standard MG at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3192.681	3	1064.227	35.381	0.000
Within Groups	240.634	8	30.079		
Total	3433.315	11			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O9 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by standard MG at concentrations 0.02-0.5 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	Std MG 0.02	-36.05333*	1.69018	0.007
	Std MG 0.1	-42.76333*	4.65942	0.035
	Std MG 0.5	-29.18667	3.94192	0.053
Std MG 0.02	control	36.05333*	1.69018	0.007
	Std MG 0.1	-6.71000	4.95650	0.710
	Std MG 0.5	6.86667	4.28899	0.597
Std MG 0.1	control	42.76333*	4.65942	0.035
	Std MG 0.02	6.71000	4.95650	0.710
	Std MG 0.5	13.57667	6.10319	0.328
Std MG 0.5	control	29.18667	3.94192	0.053
	Std MG 0.02	-6.86667	4.28899	0.597
	Std MG 0.1	-13.57667	6.10319	0.328

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O10 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by CMPE 2 at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	3.61693E + 30	3	4	0.000

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.000 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O11 Results of ANOVA test of percentage of lipase inhibition (%) by CMPE 2 at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	749.098	3	249.699	11.717	0.019
Within Groups	85.244	4	21.311		
Total	834.342	7			

*Sig. 0.019 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O12 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by CMPE 2 at concentrations 0.02-0.5 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	CMPE 2 0.02	-20.33000	3.95000	0.251
	CMPE 2 0.1	-16.09000	3.74000	0.297
	CMPE 2 0.5	-26.02000	3.61000	0.181
CMPE 2 0.02	control	20.33000	3.95000	0.251
	CMPE 2 0.1	4.24000	5.43968	0.937
	CMPE 2 0.5	-5.69000	5.35113	0.837
CMPE 2 0.1	control	16.09000	3.74000	0.297
	CMPE 2 0.02	-4.24000	5.43968	0.937
	CMPE 2 0.5	-9.93000	5.19805	0.509
CMPE 2 0.5	control	26.02000	3.61000	0.181
	CMPE 2 0.02	5.69000	5.35113	0.837
	CMPE 2 0.1	9.93000	5.19805	0.509

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O13 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) from CMPE 3 at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	5.753	3	8	0.021

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.021 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O14 Results of ANOVA test of percentage of lipase inhibition (%) by CMPE 3 at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1252.950	3	417.650	31.971	0.000
Within Groups	104.508	8	13.063		
Total	1357.458	11			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O15 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by CMPE 3 at concentrations 0.02-0.5 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	CMPE 3 0.02	-0.41000	0.41000	0.862
	CMPE 3 0.1	-19.88333	4.14311	0.120
	CMPE 3 0.5	-21.34333*	0.29077	0.001
CMPE 3 0.02	control	0.41000	0.41000	0.862
	CMPE 3 0.1	-19.47333	4.16335	0.123
	CMPE 3 0.5	-20.93333*	0.50264	0.000
CMPE 3 0.1	control	19.88333	4.14311	0.120
	CMPE 3 0.02	19.47333	4.16335	0.123
	CMPE 3 0.5	-1.46000	4.15330	0.998

CMPE 3 0.5	control	21.34333*	0.29077	0.001
	CMPE 3 0.02	20.93333*	0.50264	0.000
	CMPE 3 0.1	1.46000	4.15330	0.998

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O16 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by comparing standard MG, CMPE 2, and CMPE 3 at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	3.494	8	15	0.018

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.018 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O17 Results of ANOVA test of percentage of lipase inhibition (%) by CMPE 3 at concentrations 0.02-0.5 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3474.253	8	434.282	15.136	0.000
Within Groups	430.386	15	28.692		
Total	3904.639	23			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O18 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by CMPE 3 at concentrations 0.2-0.5 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
Std MG 0.02	Std MG 0.1	-6.71000	4.95650	0.943
	Std MG 0.5	6.86667	4.28899	0.879
	CMPE 2 0.02	15.72333	4.29642	0.421
	CMPE 2 0.1	19.96333	4.10418	0.293
	CMPE 2 0.5	10.03333	3.98608	0.614
	CMPE 3 0.02	35.64333*	1.73920	0.009
	CMPE 3 0.1	16.17000	4.47460	0.303
	CMPE 3 0.5	14.71000	1.71501	0.068
Std MG 0.1	Std MG 0.02	6.71000	4.95650	0.943
	Std MG 0.5	13.57667	6.10319	0.645
	CMPE 2 0.02	22.43333	6.10841	0.276
	CMPE 2 0.1	26.67333	5.97476	0.175
	CMPE 2 0.5	16.74333	5.89426	0.451
	CMPE 3 0.02	42.35333	4.67742	0.067
	CMPE 3 0.1	22.88000	6.23502	0.223
	CMPE 3 0.5	21.42000	4.66848	0.239

Std MG 0.5	Std MG 0.02	-6.86667	4.28899	0.879
	Std MG 0.1	-13.57667	6.10319	0.645
	CMPE 2 0.02	8.85667	5.58043	0.882
	CMPE 2 0.1	13.09667	5.43381	0.592
	CMPE 2 0.5	3.16667	5.34517	1.000
	CMPE 3 0.02	28.77667	3.96318	0.102
	CMPE 3 0.1	9.30333	5.71875	0.888
	CMPE 3 0.5	7.84333	3.95263	0.745
CMPE 2 0.02	Std MG 0.02	-15.72333	4.29642	0.421
	Std MG 0.1	-22.43333	6.10841	0.276
	Std MG 0.5	-8.85667	5.58043	0.882
	CMPE 2 0.1	4.24000	5.43968	0.999
	CMPE 2 0.5	-5.69000	5.35113	0.983
	CMPE 3 0.02	19.92000	3.97122	0.360
	CMPE 3 0.1	0.44667	5.72432	1.000
	CMPE 3 0.5	-1.01333	3.96069	1.000
CMPE 2 0.1	Std MG 0.02	-19.96333	4.10418	0.293
	Std MG 0.1	-26.67333	5.97476	0.175
	Std MG 0.5	-13.09667	5.43381	0.592
	CMPE 2 0.02	-4.24000	5.43968	0.999
	CMPE 2 0.5	-9.93000	5.19805	0.769
	CMPE 3 0.02	15.68000	3.76241	0.426
	CMPE 3 0.1	-3.79333	5.58148	1.000
	CMPE 3 0.5	-5.25333	3.75129	0.898

CMPE 2 0.5	std MG 0.02	-10.03333	3.98608	0.614
	Std MG 0.1	-16.74333	5.89426	0.451
	Std MG 0.5	-3.16667	5.34517	1.000
	CMPE 2 0.02	5.69000	5.35113	0.983
	CMPE 2 0.1	9.93000	5.19805	0.769
	CMPE 3 0.02	25.61000	3.63321	0.258
	CMPE 3 0.1	6.13667	5.49522	0.985
	CMPE 3 0.5	4.67667	3.62169	0.922
CMPE 3 0.02	Std MG 0.02	-35.64333*	1.73920	0.009
	Std MG 0.1	-42.35333	4.67742	0.067
	Std MG 0.5	-28.77667	3.96318	0.102
	CMPE 2 0.02	-19.92000	3.97122	0.360
	CMPE 2 0.1	-15.68000	3.76241	0.426
	CMPE 2 0.5	-25.61000	3.63321	0.258
	CMPE 3 0.1	-19.47333	4.16335	0.229
	CMPE 3 0.5	-20.93333*	0.50264	0.000
CMPE 3 0.1	Std MG 0.02	-16.17000	4.47460	0.303
	Std MG 0.1	-22.88000	6.23502	0.223
	Std MG 0.5	-9.30333	5.71875	0.888
	CMPE 2 0.02	-0.44667	5.72432	1.000
	CMPE 2 0.1	3.79333	5.58148	1.000
	CMPE 2 0.5	-6.13667	5.49522	0.985
	CMPE 3 0.02	19.47333	4.16335	0.229
	CMPE 3 0.5	-1.46000	4.15330	1.000

CMPE 3 0.5	std MG 0.02	-14.71000	1.71501	0.068
	Std MG 0.1	-21.42000	4.66848	0.239
	Std MG 0.5	-7.84333	3.95263	0.745
	CMPE 2 0.02	1.01333	3.96069	1.000
	CMPE 2 0.1	5.25333	3.75129	0.898
	CMPE 2 0.5	-4.67667	3.62169	0.922
	CMPE 3 0.02	20.93333*	0.50264	0.000
	CMPE 3 0.1	1.46000	4.15330	1.000

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O19 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) from CMPE 1 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	5.028	3	8	0.030

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.030 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O20 Results of ANOVA test of percentage of lipase inhibition (%) by CMPE 1 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1811.429	3	603.810	92.027	0.000
Within Groups	52.490	8	6.561		
Total	1863.919	11			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O21 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by CMPE 1 at concentrations 0.0008-0.02 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	CMPE 1 0.0008	0.00000	0.00000	
	CMPE 1 0.004	-18.82333*	1.70798	0.025
	CMPE 1 0.02	-28.41333*	2.41476	0.022
CMPE 1 0.0008	control	0.00000	0.00000	
	CMPE 1 0.004	-18.82333*	1.70798	0.025
	CMPE 1 0.02	-28.41333*	2.41476	0.022
CMPE 1 0.004	control	18.82333*	1.70798	0.025
	CMPE 1 0.0008	18.82333*	1.70798	0.025
	CMPE 1 0.02	-9.59000	2.95775	0.140
CMPE 1 0.02	control	28.41333*	2.41476	0.022
	CMPE 1 0.0008	28.41333*	2.41476	0.022
	CMPE 1 0.004	9.59000	2.95775	0.140

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O22 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) from CMPE 2 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	5.326	3	8	0.026

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.026 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O23 Results of ANOVA test of percentage of lipase inhibition (%) by CMPE 2 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	257.511	3	85.837	47.716	0.000
Within Groups	14.391	8	1.799		
Total	271.903	11			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O24 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by CMPE 2 at concentrations 0.0008-0.02 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	CMPE 2 0.0008	0.00000	0.00000	
	CMPE 2 0.004	-1.12333	1.12333	0.862
	CMPE 2 0.02	-11.02000*	1.06616	0.028
CMPE 2 0.0008	control	0.00000	0.00000	
	CMPE 2 0.004	-1.12333	1.12333	0.862
	CMPE 2 0.02	-11.02000*	1.06616	0.028
CMPE 2 0.004	control	1.12333	1.12333	0.862
	CMPE 2 0.0008	1.12333	1.12333	0.862
	CMPE 2 0.02	-9.89667*	1.54873	0.013

CMPE 2 0.02	control	11.02000*	1.06616	0.028
	CMPE 2 0.0008	11.02000*	1.06616	0.028
	CMPE 2 0.004	9.89667*	1.54873	0.013

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O25 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by CMPE 3 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	10.884	3	8	0.003

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.003 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O26 Results of ANOVA test of percentage of lipase inhibition (%) by CMPE 3 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.931	3	1.310	0.680	0.588
Within Groups	15.411	8	1.926		
Total	19.342	11			

*Sig. 0.588 > 0.05 indicates that all means of the data are not significantly different at 95% ($p \leq 0.05$).

Table O27 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by comparing CMPE 1, CMPE 2, and CMPE 3 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	3.789	8	18	0.009

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.009 < 0.05 indicate that variances of the mean are unequal or equal variance is not assumed.

Table O28 Results of ANOVA test of percentage of lipase inhibition (%) by comparing CMPE 1, CMPE 2, and CMPE 3 at concentrations 0.0008-0.02 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2594.907	8	324.363	70.949	0.000
Within Groups	82.292	18	4.572		
Total	2677.199	26			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O29 Results of Post Hoc test, Dunnett's T3 of lipase inhibition (%) by comparing CMPE 1, CMPE 2, and CMPE 3 at concentrations 0.0008-0.02 mg/ml.

Extract and concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
CMPE 1 0.0008	CMPE 1 0.004	-18.82333*	1.70798	0.047
	CMPE 1 0.02	-28.41333*	2.41476	0.042
	CMPE 2 0.0008	0.00000	0.00000	
	CMPE 2 0.004	-1.12333	1.12333	0.989
	CMPE 2 0.02	-11.02000	1.06616	0.054
	CMPE 3 0.0008	-0.96000	0.96000	0.989
	CMPE 3 0.004	0.00000	0.00000	
	CMPE 3 0.02	-1.28333	1.28333	0.989

CMPE 1 0.004	CMPE 1 0.0008	18.82333*	1.70798	0.047
	CMPE 1 0.02	-9.59000	2.95775	0.321
	CMPE 2 0.0008	18.82333*	1.70798	0.047
	CMPE 2 0.004	17.70000*	2.04428	0.020
	CMPE 2 0.02	7.80333	2.01343	0.220
	CMPE 3 0.0008	17.86333*	1.95929	0.022
	CMPE 3 0.004	18.82333*	1.70798	0.047
	CMPE 3 0.02	17.54000*	2.13639	0.019
CMPE 1 0.02	CMPE 1 0.0008	28.41333*	2.41476	0.042
	CMPE 1 0.004	9.59000	2.95775	0.321
	CMPE 2 0.0008	28.41333*	2.41476	0.042
	CMPE 2 0.004	27.29000*	2.66326	0.022
	CMPE 2 0.02	17.39333	2.63965	0.074
	CMPE 3 0.0008	27.45333*	2.59859	0.025
	CMPE 3 0.004	28.41333*	2.41476	0.042
	CMPE 3 0.02	27.13000*	2.73460	0.019
CMPE 2 0.0008	CMPE 1 0.0008	0.00000	0.00000	
	CMPE 1 0.004	-18.82333*	1.70798	0.047
	CMPE 1 0.02	-28.41333*	2.41476	0.042
	CMPE 2 0.004	-1.12333	1.12333	0.989
	CMPE 2 0.02	-11.02000	1.06616	0.054
	CMPE 3 0.0008	-0.96000	0.96000	0.989
	CMPE 3 0.004	0.00000	0.00000	
	CMPE 3 0.02	-1.28333	1.28333	0.989

CMPE 2 0.004	CMPE 1 0.0008	1.12333	1.12333	0.989
	CMPE 1 0.004	-17.70000*	2.04428	0.020
	CMPE 1 0.02	-27.29000*	2.66326	0.022
	CMPE 2 0.0008	1.12333	1.12333	0.989
	CMPE 2 0.02	-9.89667*	1.54873	0.038
	CMPE 3 0.0008	0.16333	1.47766	1.000
	CMPE 3 0.004	1.12333	1.12333	0.989
	CMPE 3 0.02	-0.16000	1.70553	1.000
CMPE 2 0.02	CMPE 1 0.0008	11.02000	1.06616	0.054
	CMPE 1 0.004	-7.80333	2.01343	0.220
	CMPE 1 0.02	-17.39333	2.63965	0.074
	CMPE 2 0.0008	11.02000	1.06616	0.054
	CMPE 2 0.004	9.89667*	1.54873	0.038
	CMPE 3 0.0008	10.06000*	1.43468	0.028
	CMPE 3 0.004	11.02000	1.06616	0.054
	CMPE 3 0.02	9.73667	1.66843	0.055
CMPE 3 0.0008	CMPE 1 0.0008	0.96000	0.96000	0.989
	CMPE 1 0.004	-17.86333*	1.95929	0.022
	CMPE 1 0.02	-27.45333*	2.59859	0.025
	CMPE 2 0.0008	0.96000	0.96000	0.989
	CMPE 2 0.004	-0.16333	1.47766	1.000
	CMPE 2 0.02	-10.06000*	1.43468	0.028
	CMPE 3 0.004	0.96000	0.96000	0.989
	CMPE 3 0.02	-0.32333	1.60267	1.000

CMPE 3 0.004	CMPE 1 0.0008	0.00000	0.00000	
	CMPE 1 0.004	-18.82333*	1.70798	0.047
	CMPE 1 0.02	-28.41333*	2.41476	0.042
	CMPE 2 0.0008	0.00000	0.00000	
	CMPE 2 0.004	-1.12333	1.12333	0.989
	CMPE 2 0.02	-11.02000	1.06616	0.054
	CMPE 3 0.0008	-0.96000	0.96000	0.989
	CMPE 3 0.02	-1.28333	1.28333	0.989
CMPE 3 0.02	CMPE 1 0.0008	1.28333	1.28333	0.989
	CMPE 1 0.004	-17.54000*	2.13639	0.019
	CMPE 1 0.02	-27.13000*	2.73460	0.019
	CMPE 2 0.0008	1.28333	1.28333	0.989
	CMPE 2 0.004	0.16000	1.70553	1.000
	CMPE 2 0.02	-9.73667	1.66843	0.055
	CMPE 3 0.0008	0.32333	1.60267	1.000
	CMPE 3 0.004	1.28333	1.28333	0.989

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level. Dunnett's T3 was used due to a small population size with less different sample size and some treatments have number of samples lower than 5.

Table O30 Results of Homogeneity of Variances test (Based on Mean) of percentage of lipase inhibition (%) by standard MG at concentrations 0.063-1 mg/ml.

Percentage of inhibition (%)	Levene's Statistic	df1	df2	Sig.
Based on Mean	2.352	5	12	0.104

*The data is a normal distribution. From the Based on Mean value, all Sig. 0.104 > 0.05 indicate that variances of the mean are equal or equal variance is assumed.

Table O31 Results of ANOVA test of percentage of lipase inhibition (%) by standard MG at concentrations 0.063-1 mg/ml.

Percentage of inhibition (%)	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2592.245	5	518.449	63.118	0.000
Within Groups	98.568	12	8.214		
Total	2690.813	17			

*Sig. 0.000 < 0.05 indicates that all means of the data for at least two groups are significantly different at 95% ($p \leq 0.05$).

Table O32 Results of Post Hoc test, Tukey HSD of lipase inhibition (%) by standard MG at concentrations 0.063-1 mg/ml.

Concentration (mg/ml)		Mean Difference (I-J)	Std. Error	Sig.
I	J			
control	0.063	-30.24667*	2.34008	0.000
	0.125	-31.46333*	2.34008	0.000
	0.25	-34.92333*	2.34008	0.000
	0.5	-25.90000*	2.34008	0.000
	1	-33.78333*	2.34008	0.000
0.063	control	30.24667*	2.34008	0.000
	0.125	-1.21667	2.34008	0.994
	0.25	-4.67667	2.34008	0.396
	0.5	4.34667	2.34008	0.469
	1	-3.53667	2.34008	0.664

0.125	control	31.46333*	2.34008	0.000
	0.063	1.21667	2.34008	0.994
	0.25	-3.46000	2.34008	0.683
	0.5	5.56333	2.34008	0.238
	1	-2.32000	2.34008	0.912
0.25	control	34.92333*	2.34008	0.000
	0.063	4.67667	2.34008	0.396
	0.125	3.46000	2.34008	0.683
	0.5	9.02333*	2.34008	0.022
	1	1.14000	2.34008	0.996
0.5	control	25.90000*	2.34008	0.000
	0.063	-4.34667	2.34008	0.469
	0.125	-5.56333	2.34008	0.238
	0.25	-9.02333*	2.34008	0.022
	1	-7.88333*	2.34008	0.049
1	control	33.78333*	2.34008	0.000
	0.063	3.53667	2.34008	0.664
	0.125	2.32000	2.34008	0.912
	0.25	-1.14000	2.34008	0.996
	0.5	7.88333*	2.34008	0.049

*Subscript of symbol " * " represents the mean difference is significant at the 0.05 level.

VITA

NAME	Kawisara Konsila
DATE OF BIRTH	23 January 1998
PLACE OF BIRTH	Chiang Rai
INSTITUTIONS ATTENDED	Chulalongkorn university
HOME ADDRESS	44 Mueang Chum, Wiang Chai District, Chiang Rai Province 57210, Thailand.
AWARD RECEIVED	The 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund)