ANTIFUNGAL ACTIVITY OF SELECTED BIOACTIVE COMPOUNDS AGAINST YEAST-HYPHAL TRANSITION OF ORAL ASSOCIATED *CANDIDA* SPECIES

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ABSTRACT

Most of fungal infections in oral cavity are caused by *Candida* spp. Ability to alternate between yeast and hyphae helps dimorphic *Candida* adhere to epithelial cells and support the biofilm formation and causes oral candidiasis. Oral rinse is used to control the growth of microorganism in the oral cavity. However, most oral rinse contains active ingredients with limited antifungal activity, particularly against hyphae development. Thus, this has encouraged us to search for bioactive compounds from natural sources that are effective against preventing the morphology transition of the dimorphic *Candida*. Antifungal activity of selected bioactive compounds of bakuchiol, hydroxychavicol, pseudolaric acid B, luteolin and sakuranetin against several species of oral associated *Candida* were screened before the evaluation on their potency in inhibition of hyphal formation. Identified potent inhibitor was then further investigated on its effectiveness through a brief treatment test on germination, adhesion, cellular morphology and gene expression. Bakuchiol, hydroxychavciol and pseudolaric acid B showed antifungal activity while luteolin and sakuranetin were determined to be inactive against the *Candida* at concentration below than 1 mg/ml. Hydroxychavicol was determined as a potent inhibitor against hyphal growth of the identified dimorphic *Candida* species namely, *C. albicans*. Hydroxychavicol delayed germination process of *C. albicans* by affecting on the expression of *RAS1, NRG1* and *HWP1* genes up to 1 hour after the treatment. In addition, the treatment caused slight changes to the cellular morphological structure. However, hydroxychavicol showed poor anti-adherence activity against germinated cells. In summary, hydroxychavicol possessed antifungal activity and was able to prevent the hyphal formation in *C. albicans.*

ABSTRAK

Kebanyakkan jangkitan kulat di dalam rongga mulut adalah disebabkan oleh spesis *Candida.* Kebolehan untuk bertukar daripada yis kepada hifa membantu *Candida* dimorfik melekat pada sel epitelium bagi menyokong kepada pembentukan biofilm serta menyebabkan jangkitan kandidiasis mulut. Ubat kumur digunakan untuk mengawal pertumbuhan mikroorganisma dalam rongga mulut. Namun, kebanyakkan ubat kumur mengandungi komponen-komponen aktif dengan kegiatan antikulat terhad, khususnya dalam kegiatan merencat pembentukkan hifa. Justeru, hal ini telah mendorong kami untuk mengkaji komponen-komponen bioaktif daripada unsur semulajadi yang dapat menghalang perubahan morfologi pada spesies *Candida* dimorfik. Ujian saringan kegiatan anti-kulat pada komponen-komponen bioaktif yang dipilih iaitu bakuciol, hydroksikavikol, asid pseudolarik B, luteolin dan sakuranetin terhadap beberapa spesies *Candida* terlebih dahulu dibuat sebelum menilai tahap kekuatan mereka dalam perencatan pembentukan hifa. Perencat kuat yang telah dikenalpasti kemudiannya diuji lebih lanjut keberkesanannya melalui satu ujian rawatan singkat terhadap percambahan, lekatan, morfologi sel dan ekspresi gen. Bakuciol, hydroksikavikol, dan asid pseudolarik B menunjukkan kegiatan anti-kulat manakala luteolin dan sakuranetin dikesan tidak giat menentang *Candida* di bawah kepekatan 1 mg/ml. Hydroksikavikol telah dikenalpasti sebagai satu komponen perencat kuat yang menentang pertumbuhan hifa spesies *Candida* dimorfik iaitu, *C. albicans*. Hydroksikavikol melambatkan proses percambahan *C. albicans* sehingga 1 jam selepas rawatan dengan mempengaruhi eskpresi gen *RAS1*, *NRG1* dan *HWP1*. Selain itu juga, rawatan tersebut menyebabkan sedikit perubahan pada struktur morfologi sel. Walau bagaimanapun, hydroksikavikol menunjukkan kegiatan anti-lekatan yang lemah terhadap

sel yang telah bercambah. Kesimpulannya, hydroksikavikol mempunyai kegiatan antikulat dan berupaya menghalang pembentukan hifa pada *C. albicans*.

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TABLE OF CONTENTS

ABSTRACT iii

ABSTRAK iv

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS vii

TABLES AND FIGURES xii

LIST OF TABLES xii

LIST OF FIGURES xiii

LIST OF SYMBOLS AND ABBREVIATIONS xvi

APPENDIX xix

CHAPTER 1: INTRODUCTION AND RESEARCH OBJECTIVES 1

1.1 Introduction 1

1.2 Research Objectives 3

CHAPTER 2: LITERATURE REVIEW 4

2.1 Oral Cavity and the Indigenous Microbes 4

2.2 Indigenous Oral Fungi 4

2.3 *Candida* and its Characteristics 5

2.4 Pathogenic *Candida* 5

2.5 *Candida* Species and Its Emergence 6

2.5.1 *Candida albicans* 6

2.5.2 *Candida dubliniensis* 6

2.5.3 *Candida glabrata* 7

2.5.4 *Candida krusei* 7

2.5.5 *Candida lusitaniae* 7

2.5.6 *Candida parapsilosis* 8

2.5.7 *Candida tropicalis* 8

2.6 Virulence factors of *Candida* 9
2.6.1 Dimorphic transition (Morphogenesis) 10
2.6.1.1 Filamentous Form (Hyphae and Pseudohyphae) 10
2.6.1.2 Yeast-to-hyphae transition 11
2.6.1.3 Dimorphic *Candida* 11
2.6.1.4 Host Recognition and Adhesion 12
2.6.1.5 Hyphae-Related Genes 13
2.6.1.6 Ras-like protein 1 (*RAS1*) 13
2.6.1.7 Negative Regulator of Glucose-repressed 1 (*NRG1*) 14
2.6.1.8 Adhesin-like Hyphal Cell Wall Protein 1 (*HWP1*) 15
2.7 Oral Rinse, Active Ingredients and Its Limitation 16
2.8 Natural Products as Source of Antifungal: Bioactive compound 16
2.9 Potential Bioactive Compounds 17
2.9.1 Bakuchiol 17
2.9.1.1 General Information 17
2.9.1.2 Medicinal Value 17
2.9.2 Hydroxychavicol 18
2.9.2.1 General Information 18
2.9.2.2 Medicinal Value 18
2.9.3 Luteolin 19
2.9.3.1 General Information 19
2.9.3.2 Medicinal Value 20
2.9.4 Pseudolaric Acid B (PAB) 20
2.9.4.1 General Information 20
2.9.4.2 Medicinal Value 21
2.9.5 Sakuranetin 21

2.9.5.1 General Information 21
2.9.5.2 Medicinal Value 22
CHAPTER 3: MATERIALS AND METHODOLOGY 23
3.1 MATERIALS 23
3.1.1 Bioactive Compounds 23
3.1.2 *Candida* Species 23
3.1.3 Culture Media 23
3.1.4 Chemical Reagents 24
3.1.5 Commercial Kits 24
3.1.6 Consumables 24
3.1.7 Equipments 25
3.2 METHODOLOGY 27
3.2.1 Selection and preparation of bioactive compounds 28
3.2.2 Preparation of Culture Media 29
3.2.3 Stock Culture Preparation of *Candida* Species 29
3.2.4 Preparation of standard cell suspension 30
3.2.5 Screening for antifungal activity of bioactive compounds against *Candida* species 30
3.2.5.1 Microdilution broth assay 30
3.2.5.2 Determination of minimum inhibition concentration (MIC) 31
3.2.6 Inhibitory Activity of bioactive compounds against Germination 33
3.2.6.1 Screening of germinated Candida 33
3.2.6.1.1 Preparation of growth induction media 33
3.2.6.1.2 Determination of germinated cells and its percentage 33
3.2.6.1.3 Determination of Inhibition Concentration (IC_{50}) of Germination 34
3.2.6.1.4 Colorimetric Assay 34

3.2.7 Brief Exposure of Compounds 35
3.2.7.1 Effect of Compound on Germination 35
3.2.7.1.1 Sample preparation 35
3.2.7.2 Effect of Compound on Ultrastructure of the Germinated *Candida* Cells. 37
3.2.7.2.1 Sample preparation 37
3.2.7.2.2 Determination of Ultrastructural Changes under SEM..... 37
3.2.7.3 Effect of Compound on the Adherence of Germinated *Candida* cells..... 38
3.2.7.3.1 Sample preparation 38
3.2.7.3.2 Determination of Anti-Adherence Activity of Compound 39
3.2.7.4 Effect of Compound on the expression of hyphae related genes 39
3.2.7.4.1 Sample preparation 39
3.2.7.4.2 Total RNA extraction and quantification..... 40
3.2.7.4.3 cDNA synthesis 40
3.2.7.4.4 Development of primers 41
3.2.7.4.5 Real Time PCR 42
3.2.8 Data Analysis 42
CHAPTER 4: RESULTS 43
4.1 Determination of Antifungal Activity of Selected Bioactive Compounds 43
4.1.1 Minimal Inhibitory Concentration (MIC) and Susceptibility of *Candida* species 43
4.2 Determination of Inhibitory Activity against Germinated *Candida* 45
4.2.1 Germinated *Candida* 45
4.2.2 Inhibitory Concentration (IC_{50}) on Germination 47
4.2.3 Inhibition potency of the Antifungal Bioactive Compounds..... 49
4.3 Brief Treatment of Potent Inhibitor (Hydroxychavicol)..... 51
4.3.1 Germination of Hydroxychavicol-treated *C. albicans*..... 51

4.3.2 Ultrastructure of Hydroxychavicol-treated *C. albicans* 54

4.3.3 Adherence of Germinated *C. albicans* on Hydroxychavicol-treated Salivary Pellicle 56

4.3.4 Gene Expression of Hydroxychavicol-treated *C. albicans* during Germination 58

CHAPTER 5: DISCUSSION..... 63

5.1 Antifungal Activity of bakuchiol, hydroxychavciol, luteolin, PAB and Sakuranetin 63

5.2 Hydroxychavicol is A Potent Agent against Germination of *C. albicans* 64

5.3 Brief Treatment of Hydroxychavicol Affected Germination Activity 66

5.4 Cellular Morphological Changes of germinated *Candida* after Treated with Hydroxychavicol 67

5.5 Hydroxychavciol is a Poor Anti-Adherence Agent against Germinated *C. albicans* 68

5.6 Hydroxychavicol Altered the Expression of *RAS1*, *NRG1* and *HWP1* 69

CHAPTER 6: SUMMARY AND CONCLUSIONS..... 72

REFERENCES..... 73

TABLES AND FIGURES

LIST OF TABLES

Table 1. A list of the selected bioactive compounds used with their natural sources and reported antifungal properties. 28

Table 2. Details of forward and reverse primer of *ACT1, RAS1*, *NRG1* and *HWP1* used for gene expression analysis 41

Table 3. The MIC of the selected bioactive compounds against seven *Candida* strains. 44

Table 4. Germination test of seven *Candida* strains in induction media of YPDS [YPD + 10% serum (v/v)]..... 46

Table 5. IC_{50} with 95% CI of the antifungal bioactive compounds (compounds with antifungal activity) and CHX against germ tube formation of *C. albicans* 48

Table 6. IC_{50}/MIC ratio and preferential growth inhibition of antifungal bioactive compounds and CHX against *C. albicans*..... 50

LIST OF FIGURES

Figure 1. An illustration of virulence factors of *C. albicans* involves in pathogenesis of candidiasis on a mucosal surface (Calderone & Fonzi, 2001). 10

Figure 2. Intergrative network of cAMP-PKA pathway in the regulation of cell cycle in yeast (Vinod et al., 2008)..... 14

Figure 3. Intergrative network of hyphae related genes (*RAS1, NRG1, HWP1*) and cAMP-PKA signaling pathway in the regulation of hyphal formation (Biswas et al., 2007)..... 15

Figure 4. Chemical structure of bakuchiol..... 17

Figure 5. Chemical structure of hydroxychavicol..... 18

Figure 6. Chemical structure of luteolin 19

Figure 7. Chemical structure of PAB..... 21

Figure 8. Chemical structure of sakuranetin 22

Figure 9. Outline of research methodology applied in this study. 27

Figure 10. An illustration of microdilution broth assay protocol that has been performed in the experiment for determination of MIC. Symbols meaning; ↓ = dispenses, ↑ = withdraw, ∩ = serially dilute, + = positive control, - = negative control and S1 – S10 = Serial dilution 1st to 10th. 32

Figure 11. Methodology for short period treatment with compound on the ability to form germ tube of *C. albicans* ATCC 14053..... 36

Figure 12. Germination percentage of hydroxychavicol-treated *C. albicans*. Each data point represents the mean ± SD (n = 6). The statistical significance of difference was determined by independent t-test. Asterisks (*) label indicates p-value of < 0.05. 52

Figure 13. Microscopic observation of germination of hydroxychavicol-treated *C. albicans*. The image was captured at magnification 200X using Moticam Digital Microscope Camera with capture resolution at 1280 x 800. 53

Figure 14. Ultrastructure images of germinated cells of the hydroxychavicol-treated *C. albicans*. All images visualized at 15 kV. Image A showed a number of germinated cell at magnification of 1,500X. Image B showed the structure of mother cell (M) and germ tube (GT) of a germinated cell at magnification of 3,000X. Images C and D showed the structure of neck (N), germ tube body (GB) and tail (T) of a germinated cell at magnification of 10,000X. In the image, it showed the rough surface of the cell as indicate by the black arrow. 55

Figure 15. Percentage of adherence of germinated *C. albicans* on hydroxychavicol-treated salivary pellicle. Each data point represents the mean ± SD (n = 6). The statistical significance of difference was determined by independent t-test..... 57

Figure 16. Gel electrophoresis images. (A) total RNA extraction with lane A1: λ *E. coli* + *Hind III* ladder, lane A2: total RNA of *C. albican*s. (B) PCR products of *C. albicans* with B1: 100bp ladder, B2: ACT1, B3: *RAS*, B4: *NRG1*, B5: *HWP1* and B6; negative control (without cDNA template). 60

Figure 17. Relative expression of (A) *RAS1*, (B) *NRG1 and* (C) *HWP1* during germination of *C. albicans*. Each data point represents the mean ± SD (n = 3). The statistical significance of difference was determined by ANOVA with Bonferroni post-hoc test. Asterisks (*) and (**) label indicate p-value of < 0.05 and < 0.01, respectively. 61

Figure 18. Relative expression of (A) *RAS1*, (B) *NRG1* and (C) *HWP1* after treatment with hydroxychavicol. Each data point represents the mean ± SD (n = 3). The

statistical significance of difference was determined by ANOVA with Bonferroni post-hoc test. Asterisks (*) and (***) label indicate p-value of < 0.05 and < 0.001, respectively. 62

Figure 19. An illustration of the suggested mechanism how hydroxychavicol delayed yeast to hyphae transition process after brief treatment on *C. albicans.* Label indicate of downregulation (red arrow), upregulation (green arrow), suppression (red cross) and promoting (green right). 71

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
>	More than
<	Less than
≤	Less than and equal to
+	Positive
-	Negative
/	Per
1x	One time
µg	Microgram
µg/mL	Microgram per mililiter
µL	Microliter
µm	Micrometer
°C	Degree of celsius
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CHX	Chlorhexidine gluconate
CI	Confidence interval
C. albicans	*Candida albicans*
C. dubliniensis	*Candida dubliniensis*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. lusitaniae	*Candida lusitaniae*
C. parapsilosis	*Candida parapsilosis*
C. tropicalis	*Candida tropicalis*
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
F	Forward
et. al	And others
E. floccosum	*Epidermophyton floccosum*
FBS	Fetal bovine serum
g	Gram

g/mol	Gram per mole
GlcNAc	N-Acetylglucosamine
GTPase	Guanosine triphosphate enzyme
h	Hour
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
H. pylori	*Helicobacter pylori*
IC_{50}	Half maximal inhibitory concentration
kV	Kilovolts (Voltage)
L	Liter
m	Meter
mg	Milligram
mL	Milliliter
MAP	Mitogen-activated protein
MIC	Minimal inhibitory concentration
min	Minute
mol	Mole
M. gypseum	*Microsporum gypseum*
N	Normality
nm	Nanometer
NCAC	Non- *Candida albicans Candida*
OD	Optical density
PA	Phytoalexin
PAB	Pseudolaric acid B
pH	Power of hydrogen
PKA	Protein Kinase A
P. fruticosa	*Polymnia fruticosa*
P. kaempferi	*Pseudolarix kaempferi*
R	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
RQ	Relative quantification
RT	Reverse transcription
s	Second
SEM	Scanning electron micrograph
SPE	Short period exposure
SPSS	Statistical package for the social sciences
spp.	Several species
S. aureus	*Staphylococcus aureus*

T. chebula	*Terminalia chebula*
T. rubrum	*Trichophyton rubrum*
T. mentagrophytes	*Trichophyton rubrum*
Tm	Melting point
USA	United State of America
UV	Ultraviolet
v	Volume
V	Voltage
v/v	Volume per volume
WS	Water serum
YPD	Yeast peptone dextrose
YPDS	Yeast peptone dextrose serum

APPENDIX

Appendix A. Protocol for Total RNA Extraction using Ribo Pure Yeast RNA Purification Kit 90

Appendix B. DNA Sequence of Hyphae Related Genes 95

Appendix C. Details of Designed Primers 99

Appendix D. Comparative Technical Analysis of qPCR 101

CHAPTER 1: INTRODUCTION AND RESEARCH OBJECTIVES

1.1 Introduction

Candida yeast is commensal fungi that inhabit human oral cavity, gastrointestinal and urogenital tract (Huffnagle & Noverr, 2013). In the oral cavity, any alteration to the ecological balance enable the switching of *Candida* opportunistic characteristic to become pathogenic and leads to infection such as candidiasis (Chandra, Retuerto, Mukherjee, & Ghannoum, 2016). Candidiasis caused high cases of nosocomial infections in immunocomprimsed patients (Suleyman & Alangaden, 2016). Although *C. albicans* remains the most pathogenic species among the oral Candida species, there has been an increased in infections caused by non-*Candida albicans Candida* (NCAC) species (Papon, Courdavault, Clastre, & Bennett, 2013).

An overgrowth of *Candida* could lead to biofilm formation and candidiasis in the oral cavity. The most common *Candida* species isolated from oral cavity is *C. albicans*, while other species are infrequently but consistently isolated (Patil, Rao, Majumdar, & Anil, 2015). *C. albicans* is a dimorphic fungus which is able to alternate between yeast and hyphae in certain environmental conditions (Lu, Su, & Liu, 2014). Dimorphism is crucial in pathogenicity since hyphae plays a major role in biofilm formation and including in invasive infection (Susewind, Lang, & Hahnel, 2015; W. Yang, Yan, Wu, Zhao, & Tang, 2014). Other than *C. albicans, C. dubliniensis, C. tropicalis* and *C. lusitenae* are also able to produce hyphae (Yu, Chang, & Chen, 2015)

Most of the available antifungal have several limitations due to the adverse side effects, drug-drug interactions and variable pharmacokinetics (Denning & Hope, 2010). In the meantime, drug resistance is becoming an increasing problem that brings to the unsuccessful chemotherapy of *Candida* infections (Pfaller et al., 2010; Presterl, Daxböck, Graninger, & Willinger, 2007). Some of oral healthcare products such as oral rinse are incompetent when most of the active ingredients formulated has limited antifungal activity, particularly against hyphae development as well as biofilm formation (Aroonrerk & Dhanesuan, 2007). This condition hasled scientists to search for new compounds (I. Ali, Satti, Dutt, Prasad, & Khan, 2016; Ferreira Mdo et al., 2014). Therefore, this research has focused on the rich chemical diversity of natural products for the discovery of bioactive compounds that work as potent inhibitor on *Candida* morphogenesis. Thus, this study could be the fundamental research in searching for a better template for the development of novel antifungal in reducing yeast-hyphal transition activity.

1.2 Research Objectives

The aim of this study is to identify bioactive compounds that possess antifungal activity and act as potent inhibitors of germination and adhesion of *Candida* spp.

Specific objectives of this study are:

1. To determine antifungal activity (minimal inhibitory concentration) of the selected bioactive compounds; bakuchiol, hydroxychavicol, luteolin, pseudolaric acid B and sakuranetin against seven common oral *Candida* species
2. To determine inhibitory concentration (IC_{50}) and inhibitory potency of the bioactive compounds that possessed antifungal activity against germination of dimorphic *Candida*
3. To assess the effect of brief exposure of the potent inhibitor on the germination of *Candida* cells and its morphological changes
4. To investigate the adhesion of germinated *Candida* cells on the treated salivary pellicle
5. To assess the effect of potent inhibitor on the expression of hyphae related genes

CHAPTER 2: LITERATURE REVIEW

2.1 Oral Cavity and the Indigenous Microbes

The mouth is the first lining of the human digestive system. It comprises of an oral cavity that includes lips, cheeks, teeth, gums, tongue and palate. Unlike other organ, mouth is directly in contact to the external environment of the body, giving opportunity for microbial species, including eubacteria, archaea, fungi, mycoplasmas and protozoa to inhabit (L. P. Samaranayake, 2006). It has been described that there are approximately more than 700 species of the indigenous oral microbes. Both the indigenous oral microbes and the innate host tissues live in commensalism in mutual relationship (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005).

2.2 Indigenous Oral Fungi

Apart from bacteria, fungi are also categorized as influential oral microbes. It is estimated that more than 80 different species of indigenous oral fungi, including *Candida*, *Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium* and *Cryptococcu*s being frequently discovered in oral healthy individual (Ghannoum et al., 2010). Among those fungi, clinically, *Candida* is found to be the most significance species since it causes infection, particularly in immunocompromised patients (Ship, Vissink, & Challacombe, 2007). *Candida* infection in the oral cavity is commonly associated with oral candidiasis, an overgrowth of yeast on the mucous membrane (Akpan & Morgan, 2002).

2.3 *Candida* and its Characteristics

Candida is the genus of yeast, under family of *Saccharomycetaceae* which represent the unique asexual reproductive budding yeast. There is a total of 163 species that has been listed under the genus of *Candida* which most of them display almost identical characteristics (Sardi, Scorzoni, Bernardi, Fusco-Almeida, & Mendes Giannini, 2013). In general, *Candida* grows in blastospore form which is illustrated as globular, ellipsoidal, and triangular in shape. Even though the morphological appearance is nearly similar, there is variation in shapes and sizes of blastospore for each of the *Candida* species. Besides, some of *Candida* species are able to grow in the filamentous form (hyphae or pseudohyphae) which appeared as elongated multicellular cells (Yu et al., 2015). Another form of *Candida* growth is chlamydospore, a spherical and thick-walled unicellular cell, produced during pseudohyphae phase.

2.4 Pathogenic *Candida*

Candida is one of the indigenous oral microbes that live as normal microflora in the oral cavity, gaining nutrient most from fermentation of carbohydrates that is present in saliva (Ghasempour, Sefidgar, Eyzadian, & Gharakhani, 2011). However, *Candida* may turn into opportunistic pathogen under favourable condition that give chance for the organism to invade the mucosal surface and causes oral infection (Singh, Verma, Murari, & Agrawal, 2014). This change usually derived from several factors, including local (degradation of saliva and oral mucosal), systemic (immunodeficiency and malnutrition) or iatrogenic (antimicrobial resistance) factors that final result in impaired host defence and immunity (Nittayananta, Chanowanna, Sripatanakul, & Winn, 2001). *C. albicans* is a well known

pathogenic *Candida* species to cause oral candidiasis. However, recently there are several species of non-*Candida albicans Candida* (NCAC) which have emerged as potential pathogenic *Candida* species.

2.5 *Candida* Species and Its Emergence

2.5.1 *Candida albicans*

Candida albicans is the most common yeast isolated from the oral cavity of both healthy and infected individuals (Rautemaa, Rusanen, Richardson, & Meurman, 2006). Due to high prevalence in clinical cases, *C. albicans* has been extensively studied for its pathogenicity (Akpan & Morgan, 2002). Major contributor for the pathogenicity is discovered to arise from its virulence factors, especially the ability to switch its growth of budding yeast to a filamentous form. This morphological switch known as dimorphic transition accommodates for cell adhesion to the host (Calderone & Fonzi, 2001).

2.5.2 *Candida dubliniensis*

Candida dubliniensis is phenotypically closer to but genetically different from *C. albicans* (Ells, Kock, & Pohl, 2011). Even though *C. dubliniensis* is rarely isolated in the oral cavity, it is one of the emerging NCAC species that cause oral infection in HIV (Dar et al., 2015). *C. dubliniensis* has the capability to produce germ tube but it is less efficient compared to *C. albicans* (Moran, MacCallum, Spiering, Coleman, & Sullivan, 2007).

2.5.3 *Candida glabrata*

Candida glabrata is one of the most prevalent species of NCAC (Gabaldon & Carrete, 2016). Despite it is considered as a non-pathogenic *Candida* in healthy individuals, its implication discovered in immunocompromised patients has increased concerns among physicians (Dar et al., 2015). *C. glabrata* has distinct characteristic compared to other species which is non-dimorphic, incapable to form pseudohyphae but consistently grow in yeast (Glockner & Cornely, 2015)

2.5.4 *Candida krusei*

Candida krusei species is considered to be one of the emerging NCAC species since it is found to cause infection in humans (Tan et al., 2016). Even though it was discovered to cause nosocomial bloodstream infections or candidemia (Hachem, Hanna, Kontoyiannis, Jiang, & Raad, 2008), the clinical occurrence is still rare and less frequent compared to other *Candida* species (Pfaller et al., 2008). Distinct from other species, *C. krusei* has the unique morphology of blastospore with elongated cell and its appearance resemble "long grain rice". *C. krusei* can exist either in yeast or pseudohyphae form, but both are frequently present simultaneously in growing media (Y. H. Samaranayake & Samaranayake, 1994).

2.5.5 *Candida lusitaniae*

Candida lusitaniae is also categorised as NCAC and has been infrequently reported as an opportunistic pathogen. Even though it is rarely isolated from clinical occurrence (Viudes

et al., 2002), but most previous cases of serious infection created by *C. lusitaniae* have led to fatal. It has also shown to be gradually emerging as a pathogenic *Candida* species to cause invasive candidiasis.

2.5.6 *Candida parapsilosis*

Among NCAC species, *C. parapsilosis* is the most frequently isolated *Candida* from clinical samples and it is found to be associated with oral candidiasis mostly in cancer patients (Devrim et al., 2015). It has an affinity to adhere to artificial surfaces such as intravascular catheter and prosthetic devices, making it easy to spread its infection (Kabach, Zaiem, Valluri, & Alrifai, 2016). *C. parapsilosis* do grow in both yeast and pseudohypal forms.

2.5.7 *Candida tropicalis*

Candida tropicalis is the most pathogenic NCAC that cause candidiasis in adults (Nucci & Colombo, 2007). Interestingly, it has the capability to invade mucosal surfaces more successful than *C. albicans* and is able to change its morphology and growth in yeast, pseudohyphae and hyphae form. In addition, it also has the ability to switch its phenotype that contributes to the extracellular proteolytic activity for its colonization (Porman, Alby, Hirakawa, & Bennett, 2011).

2.6 Virulence factors of *Candida*

Virulence factor is described as the activity of the pathogen that contributes to the replication and dissemination within a host in part by weakening or evading host defences (Cross, 2008). The virulence of *Candida* is normally associated with host recognition and adhesion, secretion of degradative enzymes, morphogenesis and phenotypic switching ability (Calderone & Fonzi, 2001).

Host recognition is a key factor to *Candida* adhesion. Adhesion to the host allows further grow of the organism and lead to its colonization (Hiller et al., 2011). In addition, extracellular hydrolytic enzymes such as aspartyl proteases and phospholipases are well-known virulence attributes. Since protein and phospholipid represent the major constituent of cell membranes, these enzymes seem to play an important role in the decomposition of the host cell components to obtain nutrients as well as to facilitate invasion (Ghannoum, 2000). Reversible transition between yeast and filamentous form (morphogenesis) provide advantages for *Candida* to expand the nutrient excavation and then support the development of thicker biofilm structure (Brand, 2012; Jackson, Coulthwaite, Loewy, Scallan, & Verran). Additionally, a filamentous form encourages *Candida* to penetrate into mucosa cell and blood vessels, causing yeast cells to seed into the bloodstream and eventually spread the infection (Gow, van de Veerdonk, Brown, & Netea, 2012). In addition, a characteristic change in *Candida* which is known as phenotypic switching has been proposed to contribute to its virulence. The changes include adherence properties, antigen expression and biomolecule affinities which promote tissue-specific interaction with the host (Calderone & Fonzi, 2001).

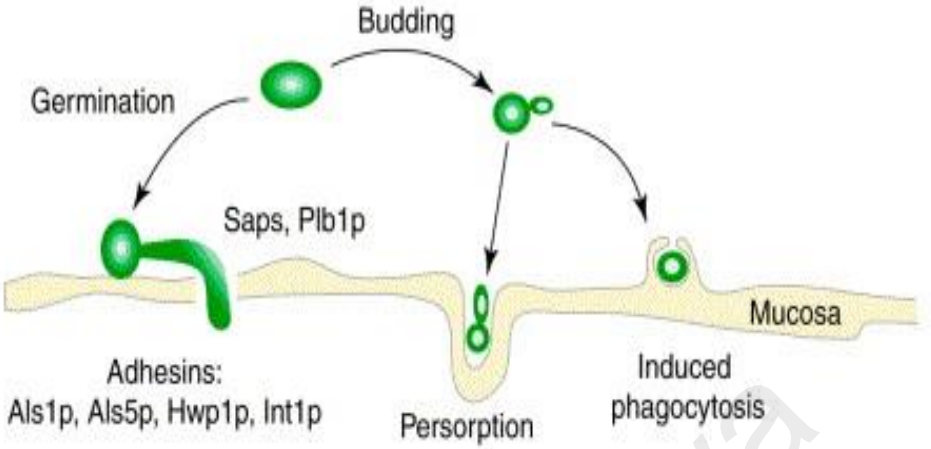


Figure 1. An illustration of virulence factors of *C. albicans* involves in pathogenesis of candidiasis on a mucosal surface (Calderone & Fonzi, 2001).

The yeast cell of *C. albicans* is able to undergo either budding or germination (Figure 1). At the mucosal surface, persorption of yeast cells (centre) results in the uptake of budding cells into the submucosa, whereas on the far right, phagocytosis of the yeast was induced by the mucosal cells. However, germination of yeast cells allows penetration of the mucosal layer. These events are promoted by adhesins and enzymes. The germ tube is pictured as expressing different antigens compared with the yeast cell. The contribution of phenotypic switching to pathogenesis could be in promoting tissue-specific affinities for the organism as well as antigenic changes.

2.6.1 Dimorphic transition (Morphogenesis)

2.6.1.1 Filamentous Form (Hyphae and Pseudohyphae)

Most of the previous literature generally used the terms hyphae and pseudohyphae interchangeably. In point of fact, they are clearly different. Hyphae are also known as 'true' hyphae, and are basically filamentous cells that look like threads. It is long and thin, separated by a cross-wall (septa) with no obvious constrictions between cells. Germ tube

forms in the initial stage of hyphae development before septation occur (P. E. Sudbery, 2001). Pseudohyphae are generally elongated cells with ellipsoid shape. It is formed when incomplete budding cell remains attached after division phase and constriction is clearly observed between the cells. Both hyphae and pseudohyphae are invasive form since they are capable to penetrate into agar substratum growth media. This property may associate with the activity of the *Candida* in tissue penetration. Even pseudohyphae exhibits similarities more to yeast, its tendency in virulent is debilitated compared to hyphae (P. Sudbery, Gow, & Berman, 2004).

2.6.1.2 Yeast-to-hyphae transition

The ability of *Candida* to switch from yeast to hyphae growth is essential for its virulence. Several lines of evidence suggest that hyphae morphology plays a key role in adhesion to epithelial cells to cause oral candidiasis (D. W. Williams et al., 2013) and it also contribute to formation of biofilms (Douglas, 2003). Yeast-to-hyphae transition can be induced by multiple factors such as serum, N-acetylglucosamine (GlcNAc), neutral pH, high temperature, starvation, CO_2, and adherence (Biswas, Van Dijck, & Datta, 2007). Yeast cultured in media supplemented with serum at 37°C described to be the optimum condition for hyphae growth since it mimics the physiological environment of human host.

2.6.1.3 Dimorphic *Candida*

Most of *Candida* species are dimorphic, capable to change between yeast and pseudohyphae form, except *C. glabrata* that grows only in yeast. However, *C. albicans*, *C. dubliniensis* and *C. tropicalis* are discovered to form hyphae as well (Thompson, Carlisle,

& Kadosh, 2011). Distinct from *C. albicans* and *C. dubliniensis*, *C. tropicalis* is reported to display two different characteristics, which are germ tube positive and negative strains (Hilmioglu, Ilkit, & Badak, 2007; Martin & White, 1981). Culture media such as nutrient broth that is supplemented with serum is known to be inducer for germ tube production of the three *Candida* species (Hilmioglu et al., 2007). However, *C. dubliniensis* is described to form germ tube better in water serum (WS) rather than in culture media serum (O'Connor, Caplice, Coleman, Sullivan, & Moran, 2010).

2.6.1.4 Host Recognition and Adhesion

Host recognition helps the *Candida* adheres to mucosal tissues (D. W. Williams et al., 2013). It triggers the organism to interact with mucosal cells to hold to each other, in order to avoid from clearance by the salivary flow and finally leading to the successful colonization in the oral cavity. In general, the interaction of *Candida* to the host is described to have different types of mechanism. The interaction includes specific mechanism that involve ligand-receptor interactions (Filler, 2006) and non-specific mechanism that require electrostatic charge force. In oral cavity, specific binding forms between the *Candida* cell surface and acquired pellicle, a thin film composed of salivary glycoproteins that cover the oral surface (Custodio, Silva, Paes Leme, Cury, & Del Bel Cury, 2015). In comparison of the morphological appearance, hyphae of *C. albicans* have higher affinity to adhere to various surfaces rather than yeast (D. W. Williams et al., 2013). This factor may indicate the role of hyphae in strengthening the structure of *Candida* biofilm on mucosal surfaces.

2.6.1.5 Hyphae-Related Genes

Candida species have extensively developed its virulence traits for survival. *C. albicans* particularly evolved with morphological changes in morphogenesis providing further advantages for tissue invasion. At molecular level, yeast-to-hyphae transition involve the expression of hyphae-specific genes (Figure 3) which is controlled by regulatory genes such as Ras-like Protein 1 (*RAS1*) and Negative Regulator of Glucose-repressed 1(*NRG1*) (Biswas et al., 2007). Additionally, the presence of hyphae also facilitates the adhesion activity which is mediated by Adhesin-like Hyphal Cell Wall Protein 1 (*HWP1*) (Orsi et al., 2014).

2.6.1.6 Ras-like protein 1 (*RAS1*)

RAS1 is a signal transduction GTPase protein that functions upstream of both MAP kinase and cAMP-PKA signaling pathway (Biswas et al., 2007). The pathway regulates yeast-to-hyphae transition (Leberer et al., 2001) and several biological processes (Figure 2) including cell cycle progression and metabolism (Doedt et al., 2004; Vinod, Sengupta, Bhat, & Venkatesh, 2008). Serum is found to act as inducer of yeast-to-hyphae transition in *C. albicans* through the Ras-mediated signal transduction pathway (Feng, Summers, Guo, & Fink, 1999). *RAS1* is also identified in *C. dubliniensis* which shares 83% identity at the amino acid sequence level with its *C. albicans* orthologue. Opposite to *C. albicans*, stimulation of the cAMP-PKA pathway in *C. dubliniensis* does not promote yeast-to-hyphae transition (Moran et al., 2007).

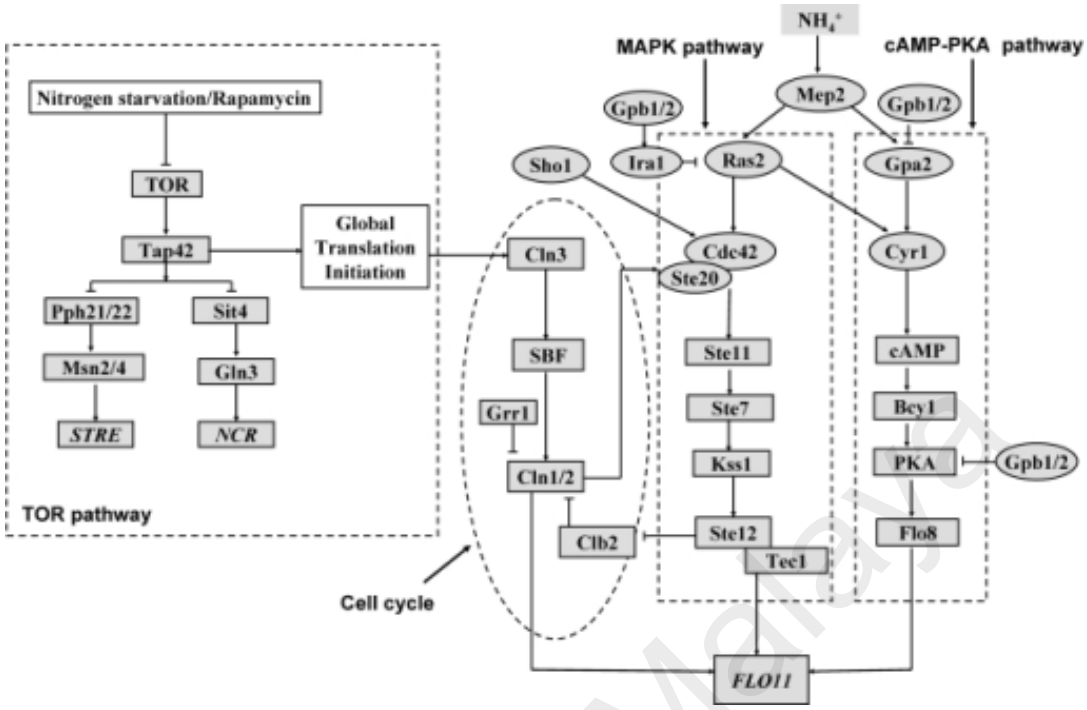


Figure 2. Intergrative network of cAMP-PKA pathway in the regulation of cell cycle in yeast (Vinod et al., 2008)

2.6.1.7 Negative Regulator of Glucose-repressed 1 (*NRG1*)

NRG1 is a zinc finger domain DNA-binding protein that represses the yeast-to-hyphae transition of *C. albicans* (Braun, Kadosh, & Johnson, 2001; Murad et al., 2001). During hyphae formation, transcription level of *NRG1* decrease and it indicates that transition is induced by downregulation of *NRG1* in *C. albicans* (Braun et al., 2001). Activation of the cAMP-PKA pathway is discovered to initiate the downregulation of *NRG1* (Lu, Su, Wang, & Liu, 2011). *C. dubliniensis NRG1* shares 86% the identity of amino acid sequence with its *C. albicans* orthologue. Deletion of *NRG1* in *C. dubliniensis* causes increment rate of filamentation, suggesting that expression of *NRG1* represses the morphological transition activity (Moran et al., 2007).

2.6.1.8 Adhesin-like Hyphal Cell Wall Protein 1 (*HWP1*)

HWP1 is an adhesin, cell surface protein that is bound to the *Candida* cell wall (Staab, Bahn, Tai, Cook, & Sundstrom, 2004). It is abundantly found in *C. albicans* (Sundstrom, 2002). This protein functions in generating the adhesion of *C. albicans* to host tissue surfaces. The existence of adhesin is essential for the *Candida* pathogenesis particularly in candidiasis infection (Staab, Bradway, Fidel, & Sundstrom, 1999; Sundstrom, Balish, & Allen, 2002). *HWP1* is also crucial to strengthen the biofilm structure (Nobile, Nett, Andes, & Mitchell, 2006). Since *HWP1* is hyphae-specific, its expression is highly regulated in hyphae rather than yeast form (Nantel et al., 2002).

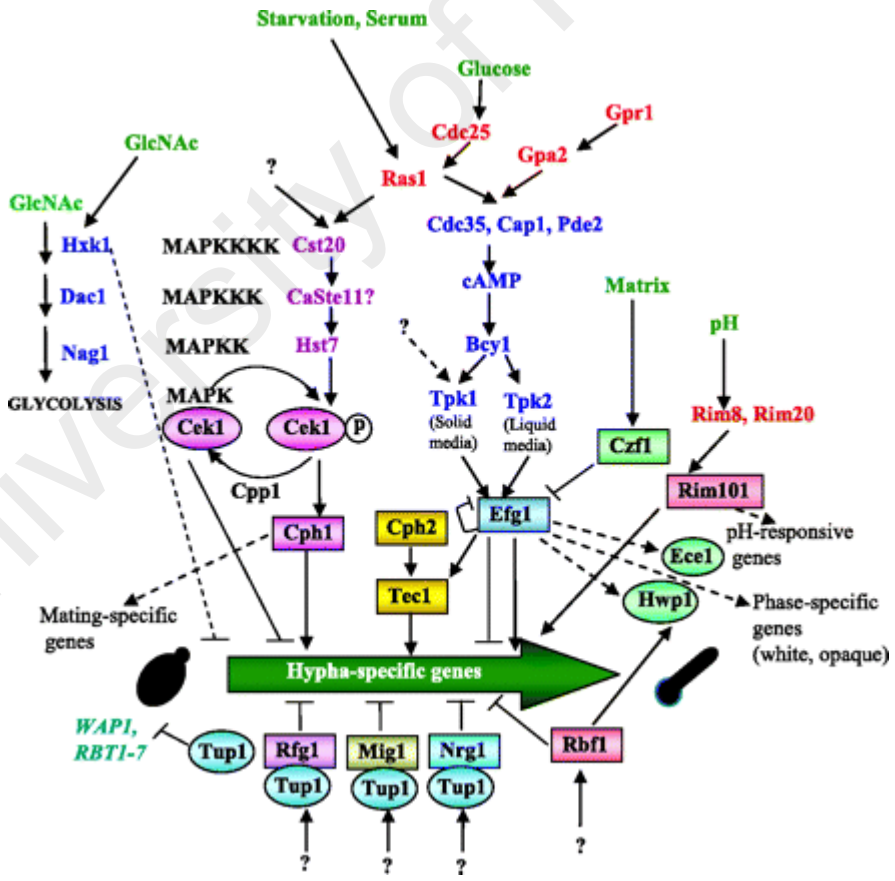


Figure 3. Intergrative network of hyphae related genes (*RAS1, NRG1, HWP1*) and cAMP-PKA signaling pathway in the regulation of hyphal formation (Biswas et al., 2007)

2.7 Oral Rinse, Active Ingredients and Its Limitation

In the past few years, the use of oral rinse as a hygiene practice has increased dramatically. Oral rinse is a solution for oral surface cleansing and controlling dental caries. Oral rinse requires only a short exposure time of between 1-2 minute for each gargle. Formulated oral rinse contains various active ingredients that work as antimicrobial agents. Certain active ingredients in oral rinse such as sodium monofluorophosphate and thymol, has been discovered to have weak activity against hyphae formation of *C. albicans* (Aroonrerk & Dhanesuan, 2007). This may contributes to resistance of *Candida* biofilm formation in the oral cavity and limits the usage of the oral rinses in the treatment of oral candidiasis as well.

2.8 Natural Products as Source of Antifungal: Bioactive compound

Natural products are important sources for the development of new antimicrobial agents. Therefore, many studies have now focused on searching novel bioactive compounds from the natural products as an alternative to the current antimicrobial agents (Arif et al., 2009; Termentzi, Fokialakis, & Skaltsounis, 2011). Plants are the most common source of natural products for this purpose. For many years, various bioactive compounds with antimicrobial activity were discovered from different plants. However, actual potency of certain bioactive compounds as antifungal agents remains scarce and insufficient. Thus, further study is recommended to investigate their antifungal activities against the common oral *Candida* species and its virulence capability involving hyphae growth. Oral rinse that is formulated with potent antifungal developed from bioactive compounds is hoped to be reliable in the prevention of oral candidiasis.

2.9 Potential Bioactive Compounds

2.9.1 Bakuchiol

2.9.1.1 General Information

Bakuchiol is a meroterpene compound which has a partial terpenoid on its structure (Figure 4) and it is classified under terpenophenol compound group. Chemical nomenclature of bakuchiol is 4-[(1E,3S)-3-ethenyl-3,7-dimethylocta-1,6-dienyl]phenol. The other name of the compound is (+) - bakuchiol. It has 256.38 g/mol of molar mass with $C_{18}H_{24}O$ of molecular formula. Bakuchiol is commonly found in *Psoralea corylifolia* and *Oenothera pubescens*.

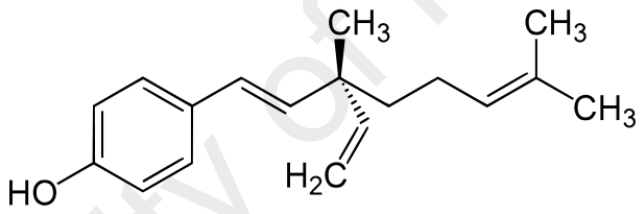


Figure 4. Chemical structure of bakuchiol

2.9.1.2 Medicinal Value

Psoralea corylifolia is a tree herb that has since long been used in Ayurveda and traditional Chinese medicine for treatment of various skin disease. Its medicinal value has been proven from antimicrobial activities of its polar extracts against gram positive and negative bacteria as well as yeast (Erazo, González, Zaldivar, & Negrete, 1997). Chemical profiling identified that bakuchiol is one of the major bioactive compounds of its crude extract other than psoralen, psoralidin, corylin and corylifolin (Jiangning, Xinchu, Hou, Qinghua, & Kaishun, 2005). It has many biological activity including anti-inflammation (Choi et al.,

2010; Ferrandiz et al., 1996), anti-tumor (Chen, Feng, & Li, 2010) and hepatoprotective effects (Cho et al., 2001). Bakuchiol has antibacterial activity against oral and skin bacteria (Hsu, Miller, & Berger, 2009; Katsura, Tsukiyama, Suzuki, & Kobayashi, 2001). This compound has been widely used commercially as an active ingredient in cosmetic product.

2.9.2 Hydroxychavicol

2.9.2.1 General Information

Hydroxychavicol is a phenylpropene compound which has benzene ring with an allyl group on its structure (Figure 5). Chemical nomenclature of hydroxychavicol is 4-prop-2-enylbenzene-1,2-diol. The other name of the compound is 1,2-dihydroxyallylbenzene, 1,2-dihydroxy-4-allylbenzene and 2-hydroxychavicol. It has 150.17 g/mol of molar mass with $C_9H_{10}O_2$ of molecular formula. Hydroxychavicol is commonly found in *Piper betle.*

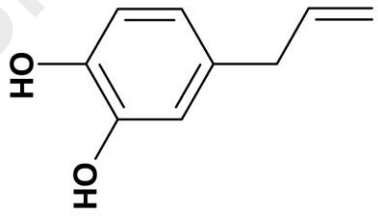


Figure 5. Chemical structure of hydroxychavicol

2.9.2.2 Medicinal Value

Piper betle has been extensively used in traditional remedies in most Asian countries. The herbs plant is believed to have many medicinal uses. It is discovered that hydroxychavicol is the major component of *Piper betle.* Hydroxychavicol has been reported to possess

several biological activities in human body such as anti-nitrosation, anti-mutagenic and anti-carcinogenic (Chang et al., 2002). It also exhibited other useful properties which include anti-inflammatory, anti-platelet and anti-thrombotic (Chang et al., 2007). In addition, hydroxychavicol possess antibacterial activities against oral pathogens especially on biofilm-forming bacteria (Sharma et al., 2009), and displayed antifungal effect on fungal species such as *Candida* and *Aspergillus* (Intzar Ali et al., 2010).

2.9.3 Luteolin

2.9.3.1 General Information

Luteolin is a flavone compound which is one of the flavonoid groups and its structure as shown in Figure 6. Chemical nomenclature of luteolin is 2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone. The other name of the compound is luteolol, digitoflavone, flacitran and luteoline. It has 286.24 g/mol of molar mass with $C_{15}H_{10}O_6$ of molecular formula. Luteolin is commonly found in *Terminalia chebula*, some fruits and vegetables.

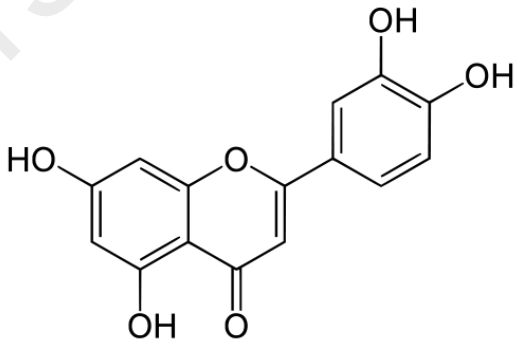


Figure 6. Chemical structure of luteolin

2.9.3.2 Medicinal Value

As a highly prevalent flavonoid in fruits and vegetables, luteolin exhibits favorable biological activity such as anti-tumor (Horinaka et al., 2005; Ueda, Yamazaki, & Yamazaki, 2003) and anti-inflammatory (Ueda, Yamazaki, & Yamazaki, 2002, 2004). In addition, it is a protective agent, able to prevent damage of endothelium that induced by superoxide anion (Ma et al., 2008). Furthermore, luteolin displayed antibacterial activity against *S. aureus* by inhibition of DNA topoisomerase (Wang & Xie, 2010). It also has antifungal effect on dermatophyte fungi such as *Epidermophyton floccosum, Trichophyton rubrum and Trichophyton mentagrophytes* (Sartori et al., 2003).

2.9.4 Pseudolaric Acid B (PAB)

2.9.4.1 General Information

Pseudolaric acid B is a diterpene compound which composed of four isoprene units on its structure (Figure 7). Chemical nomenclature of pseudolaric acid B is 4a-(acetyloxy)-3-[(1E, 3E)-4-carboxy-1,3-pentadien-1-yl]-3R,4S,4aS,5,6,9-hexahydro-3-methyl-1-oxo-7methylester;1H-4,9aR ethanocyclohepta[c]pyran-7-carboxylic acid. The other name of the compound is (-)-pseudolaric acid B and pseudolarix acid B. It has 432.46 g/mol of molar mass with $C_{23}H_{28}O_8$ of molecular formula. Pseudolaric acid B is commonly found in *Pseudolarix kaempferi.*

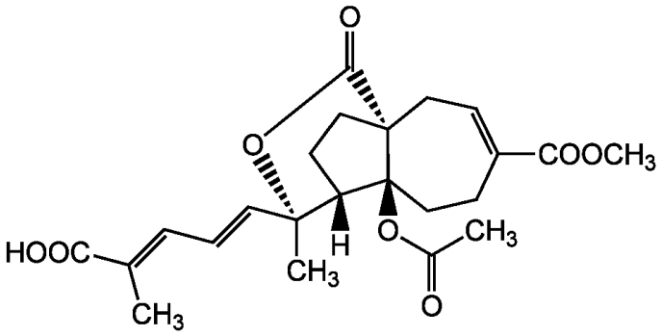


Figure 7. Chemical structure of PAB

2.9.4.2 Medicinal Value

In Chinese traditional medicine, the root of *Pseudolarix kaempferi* has been used as an alternative for treatment of skin fungal infections. Further investigation has found that PAB is the main antifungal constituent of *Pseudolarix kaempferi* (S. P. Yang, Wu, & Yue, 2002). PAB has antifungal activity against several fungal species including *Trichophyton mentagrophytes, Torulopsis petrophilum, Microsporum gypseum* and *Candida* spp (Li, Clark, & Hufford, 1995). The report also showed that PAB decreased the number of recovered colony-forming units significantly at different concentrations in mice of disseminated candidiasis (Li et al., 1995).

2.9.5 Sakuranetin

2.9.5.1 General Information

Sakuranetin is a flavanone compound which consists of glycosides unit on its structure (Figure 8). Chemical nomenclature of sakuranetin is (2S)-5-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-2,3-dihydrochromen-4-one. The other name of the compound is (S)-(−)-4′-5-Dihydroxy-7-methoxyflavanone, 7-O-methylnaringenin and naringenin 7-O-methyl ether.

It has 286.27 g/mol of molar mass with $C_{16}H_{14}O_5$ of molecular formula. Sakuranetin is commonly found in *Polymnia fruticosa* and rice.

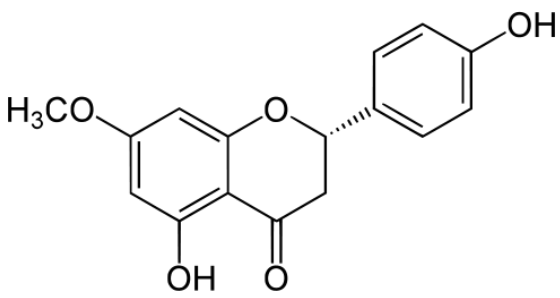


Figure 8. Chemical structure of sakuranetin

2.9.5.2 Medicinal Value

Sakuranetin is phytoalexin (PA), a compound that is induced by plant to overcome environmental stresses including microbial infection. PA is also defined as low molecular weight antimicrobial compounds synthesized by host plants (Jeandet, Clement, Courot, & Cordelier, 2013). Even though sakuranetin is known as antimicrobial, it also exhibits various biological activities including anti-inflammatory (Hernandez, Recio, Manez, Giner, & Rios, 2007) and anti-mutagenic (Miyazawa, Kinoshita, & Okuno, 2003). Its role in maintenance of glucose homeostasis results in alleviation of diabetes disease (Saito, Abe, & Sekiya, 2008). As an antimicrobial agent, sakuranetin have antibacterial activity against *H. pylori* (Zhang et al., 2008) and antifungal activity against blast fungus-infected rice leaves (Hasegawa et al., 2014). In addition, it is effective against parasitic protozoa such as leishmania and trypanosoma (Grecco Sdos et al., 2012).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 MATERIALS

3.1.1 Bioactive Compounds

Bakuchiol, Hydroxychavicol, Luteolin, Pseudolaric Acid B and Sakuranetin were purchased from ChromaDex, California, USA. A 0.12% w/v chlorhexidine gluconate (CHX) in oral rinse product was purchased from Oradex.

3.1.2 *Candida* Species

The strains of *Candida albicans* ATCC 14053, *Candida dubliniensis* ATCCMYA 2975, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 14243, *Candida lusitaniae* ATCC 64125, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 13803 were purchased from American Types Cultures Collection (ATCC), Virginia, USA.

3.1.3 Culture Media

Ultrapure water (Millipore), Fetal Bovine Serum (Gibco), Yeast Peptone Dextrose Agar (BD Difco™) and Yeast Peptone Dextrose Broth (BD Difco™) was used for preparation of culture media.

3.1.4 Chemical Reagents

Chemical reagents such was Acid Hydrochloric, Agarose, Dimethyl Sulphoxide, Ethidium Bromide, Gluteraldehyde, Osmium tetroxide, Glycerol, Phosphate Buffer Saline and Sorensen phosphate buffer were purchased from Sigma Aldrich, Acetone, Crystal violet, Ethanol, Isopropanol were purchased from Merck Millipore, RNase Free Water from Ambion, Sodium Dodecyl Sulphate from Bio Rad, Tris/Borate/EDTA Buffer from Thermo Fisher Scientific.

3.1.5 Commercial Kits

The commercial kits used in this study are DNase I treatment kit from Ambion, RiboPure™ Yeast RNA Purification Kit from Ambion, SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen and SYBR® Select Master Mix kit from Applied Biosystem

3.1.6 Consumables

The consumables used in this study are aluminium foil, gas cartridge, disposable pipette tips, falcon tube 15 ml, filter paper, latex examination glove, microtitre 96-well plate, parafilm and syringe

3.1.7 Equipments

The list of equipments were available in Balai Ungku Aziz Research Laboratory (BUARL), Faculty of Dentistry, and used in the study.

- 7500 Fast Real-Time PCR System (ABI, USA)
- Autoclave (HICLA VE HVE-50 Hirayama, Japan)
- Analytical Balance (Mettler AJ100J, USA)
- Analytical Balance (Denver XL-1810, USA)
- Centrifuge (Jouan A14, France)
- Centrifuge (Refrigerated) (Jouan GR20 22, France)
- Chiller (4°C) (Mutiara, Malaysia)
- Freezer (-20°C) (Zanussi, Germany)
- Freezer (-80°C) (Hetofrig Cl410, Denmark)
- Gel Image Analyzer (Media Cybernetics, USA)
- Gradient Thermocycler (Eppendorf, Germany)
- Hotplate / Stirrer (Thermolyne)
- Incubator (Memmert, Germany)
- Incubator (Binder, Germany)
- Ion Sputter Coater (JOEL JFC1100: JOEL, Tokyo, Japan)
- Laminar Flow Unit (ERLA CFM Series, Australia)
- NanoDrop 2000 UV–vis Spectrophotometer (Thermo Scientific)
- Scanning Electron Microscope (SEM) (Phillips, Japan)
- Spectrophotometer (Shimadzu UV160A, Japan)
- Vortex Mixer (Snijders Scientific, Holland)

- Water Bath (Grants SS-40-A2, Cambridge, England)
- Water Distiller (J Bibby Merit)

3.2 METHODOLOGY

Research methodology was applied according to Figure 9.

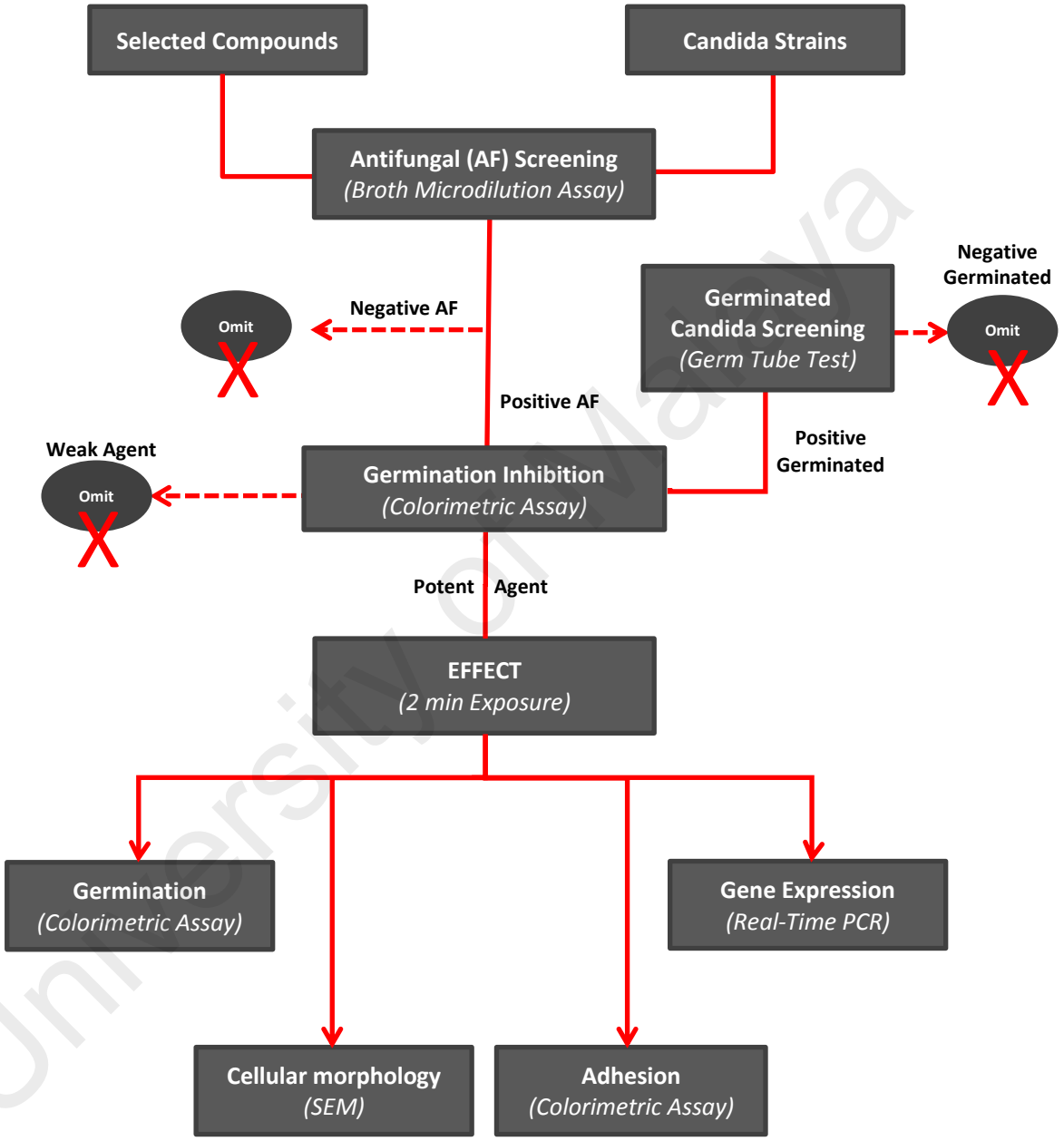


Figure 9. Outline of research methodology applied in this study.

3.2.1 Selection and preparation of bioactive compounds

Five identified bioactive compounds (Table 1) known to possess antifungal properties were selected to be used in this study. Bakuchiol, hydroxychavicol, luteolin, PAB and sakuranetin were purchased from ChromaDex Inc. All the selected bioactive compounds were delivered in lyophilised form in vials. Each bioactive compounds was prepared in 1% dimethyl sulphoxide (DMSO) to obtain concentration of 4 mg/ml and kept as stock solution at -20°C. A stock of 0.12% w/v chlorhexidine gluconate (CHX) in oral rinse product was used as positive control in the study.

Table 1. A list of the selected bioactive compounds used with their natural sources and reported antifungal properties.

Bioactive compound	Sources (Plant)	Antifungal activity against species
Bakuchiol	*P. corylifolia*	*T. mentagrophytes, T. rubrum, P. variotii* (Lau et al., 2010)
Hydroxychavicol	*P. betle*	*C. albicans, C. glabrata, C. krusei C. parapsilosis, C. tropicalis, C. neoformans, A. flavus, A. fumigatus, A. niger, A. parasiticus, E. floccosum, M. canis, M. gypsium, T. mentagrophytes, T. rubrum* (Intzar Ali et al., 2010)
Luteolin	*T. chebula, fruits*	*E. floccosum, T. rubrum and T. mentagrophytes* (Sartori et al., 2003)
PAB	*P. kaempferi*	*C. albicans, C. glabrata, C. krusei C. dubliniensis, C. tropicalis*, and C. *guilliermondii*, (Guo et al., 2011; Yan, Hua, Xu, & Samaranayake, 2012)
Sakuranetin	*P. fruticosa, rice*	*M. oryzae* (Hasegawa et al., 2014)

3.2.2 Preparation of Culture Media

Yeast Peptone Dextrose (YPD) agar and broth were used as the growth media for all *Candida* strains in the study. The powder form of the stock media was initially dissolved in distilled water before further sterilized at 121ºC for 15 min in an autoclave. The agar media was poured into 15 mm agar plates while broth media was kept in Schott bottle before stored at 4ºC for later use.

3.2.3 Stock Culture Preparation of *Candida* Species

All seven *Candida* species; *Candida albicans* ATCC 14053, *Candida dubliniensis* ATCCMYA-2975, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 14243, *Candida lusitaniae* ATCC 64125, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 13803 were purchased from American Type Culture Collection (ATCC) and the samples were delivered in lyophilised form in vials. The lyophilised cells were rehydrated in sterile distilled water, followed by inoculation onto Yeast Peptone Dextrose (YPD) agar media and then incubated overnight at 37°C for 24 h to recover the cell growth as recommended on the product sheet of ATCC. Following incubation, a single colony from the agar was sub-cultured onto fresh YPD agar slants before being kept at 4ºC for short term storage stock culture. For long term storage, stock culture was prepared in 20% v/v glycerol and kept at -70ºC. During the experiment, working cultures were prepared on YPD agar and maintained for two weeks at 4ºC. Routine sub-culturing was carried out before being used in any experiment to ensure the viability of cells. The growth colonies from the culture were used in cell suspension preparation.

3.2.4 Preparation of standard cell suspension

The cell suspension was adjusted to a 0.5 McFarland standard contain approximately 1×10^6 yeast cells/ml. Briefly, a loopful of single colony from fresh culture agar was inoculated into 5 ml of YPD broth and mixed by vortex. The turbidity of cell suspensions was then adjusted spectrophotometrically to achieve an optical density (OD_{550nm}) of 0.144 which is equivalent to the 0.5 McFarland standards.

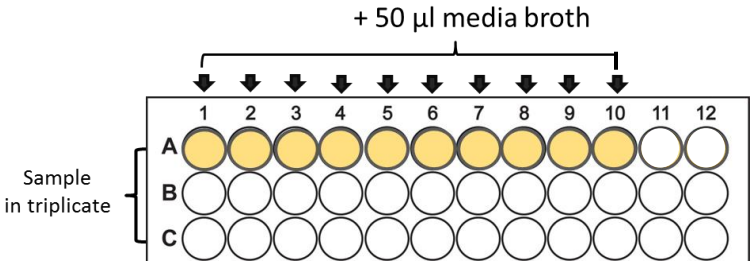
3.2.5 Screening for antifungal activity of bioactive compounds against *Candida* species

3.2.5.1 Microdilution broth assay

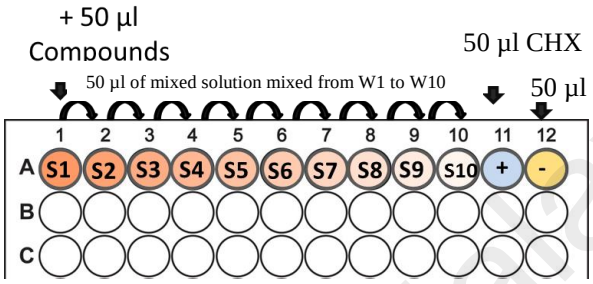
Twofold microdilution broth method (Jorgensen et al., 1999) in Figure 10 was used to determine the MIC. In this context, MIC is the lowest concentration of the compounds that inhibit *Candida* cell growth. Briefly, 50 µL of YPD broth was dispensed into 96 wells microdilution plate marked as Well 1 (W1) to Well 10 (W10). After that, 50 µL of stock solution (4 mg/ml) of the compounds was added into W1 and twofold serial dilution was carried out from W1 to W10. A 50 µL of 0.12% w/v CHX was dispensed into W11 and used as positive control, while 50 µL of YPD was dispensed into W12 used as the negative control. Then, 50 µL of *Candida* cell suspension (1×10^6 yeast cells/ml) was added to W1 through W12. Hence, the final concentrations of the compounds in W1 to W10 were 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 µg/ml in the respective wells and final concentration of CHX in W11 was 0.06% w/v (600 µg/ml). Triplicate samples were performed for each test concentration. The microdilution plates were incubated overnight at 37°C..

3.2.5.2 Determination of minimum inhibition concentration (MIC)

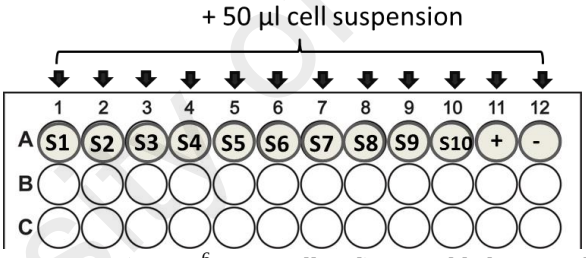
The growth of *Candida* cells in microdilution wells was measured using spectrophotometer and compared to the well of positive control, CHX. Concentration of the suspension, which showed an absorbance reading almost or equal to the positive control was determined as the MIC.



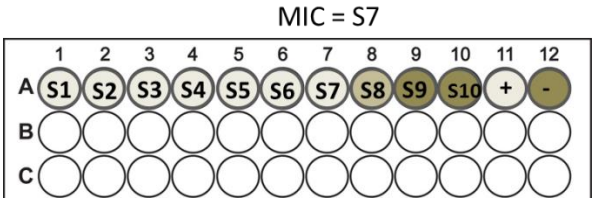
1. 50 µL of YPD broth was dispensed into well 1 (W1) to well 10 (W10).



2. 50 µL of the compounds (4 mg/ml) was dispensed into W1. Twofold serial dilution was performed from W1 to W10.
3. 0.12 % CHX was dispensed in W11 represented for positive control. W12 contain only YPD broth represented for the negative control.



4. 50 µL of *Candida* cell suspension (1×10^6 yeast cells/ml) was added to W1 through W12. Concentration of the compounds from S1 to S10 after dilution was 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 µg/ml and final concentration of CHX in W11 was 0.06% w/v (600 µg/ml). The plate was incubated overnight at 37°C.



5. After incubation, concentration of the compounds which showed no growth indicated by a clear broth similar to positive control was determined as the MIC.

Figure 10. An illustration of microdilution broth assay protocol that has been performed in the experiment for determination of MIC. Symbols meaning; ↓ = dispenses, ↑ = withdraw, ∩ = serially dilute, + = positive control, - = negative control and S1 – S10 = Serial dilution 1^{st} to 10^{th}.

3.2.6 Inhibitory Activity of bioactive compounds against Germination

3.2.6.1 Screening of germinated Candida

3.2.6.1.1 Preparation of growth induction media

An induction medium was prepared to stimulate the germination of *Candida* cells. The induction media is a mixture of yeast peptone dextrose (YPD) broth with 10% fetal bovine serum (FBS) and named as YPDS. During preparation, YPD growth media and FBS were sterilized separately. YPD was sterilized through heat sterilization using autoclave while FBS was sterilized through microfiltration using 0.22µm membrane filter. Following sterilization, YPD was left to cool to about 50-60°C before being added with 10% FBS to produce YPDS. YPDS was freshly prepared each time prior to the experiment.

3.2.6.1.2 Determination of germinated cells and its percentage

Yeast cells suspension of each strain at concentration 1 x 10^6 cells per ml were initially incubated in the respective YPDS induction media at 37 °C for 3 h. Following incubation, an adequate amount of the sample was loaded in the counting chamber of hemacytometer slide. Percentage of germinated cells were calculated from the total count of 100 cells. Pseudohyphae cells (incomplete budding cell with a constriction between the cells) were counted as non-germinated cells. Experiment was repeated three times to get the average of the cell count.

3.2.6.1.3 Determination of Inhibition Concentration (IC_{50}) of Germination

Germ tube formation assay (Brayman and Wilks et al., 1999) was used to determine IC_{50} value. In this context, IC_{50} is the concentration of compounds that is required for 50% inhibition of germinated *Candida* strains. Briefly, 50µL of 1 x 10^6 yeast cells per ml was added into a 96 well microtitre plate containing 50µL of the induction media that has been 2-fold serially diluted with the respective compounds (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 µg/ml final concentration) and CHX (600, 300, 150, 75, 37.5, 18.75, 9.38, 4.69, 2.34 and 1.17 µg/ml). The plate then was incubated at 37 °C for 3 h to induce germ tube formation.

3.2.6.1.4 Colorimetric Assay

After incubation, the medium was discarded. The cells were washed once by immersing the whole plate in 70% ethanol. Then, 200µL of 0.25% sodium dodecyl sulphate (SDS) was added to each well, discarded and followed by three times washing using sterile distilled water (immersing the whole plate). After that, 100µL of 0.02% crystal violet (prior to the experiment, a crystal violet stock solution which was prepared in phosphate-buffered saline and filtered through a 0.22 µm pore size filter to remove precipitated dye particles) was added into the wells to stain the cells. After 15 min, crystal violet solution was removed by inverting the plate. The plate was washed thrice, once with 0.25% SDS, and twice with sterile distilled water, and left to dry at room temperature. A 200µL of isopropanol containing 0.04 N HCl and 50µL of 0.25% SDS was added to the wells and mixed briefly on the orbital shaker for 15 min, and the absorbance of the mixture was measured at 590 nm using spectrophotometer. Fifty percent inhibitory concentrations (IC_{50}) and 95%

confidence interval (CI) of the IC_{50} were determined using GraphPad Prism version 3.00 for Windows (GraphPad Software, USA).

3.2.7 Brief Exposure of Compounds

Since the usage and recommendations for using oral rinse for each gargle are practically between 1-2 minutes, thus, we take into consideration of using similar exposure period of the bioactive compounds in this study. This experiment is basically assessing the post effect on germinated *Candida* cells (germination, cellular morphology and gene expression) after being exposed to the bioactive compounds for 2 minutes.

3.2.7.1 Effect of Compound on Germination

3.2.7.1.1 Sample preparation

The procedure was described in Figure 11. Briefly, 1ml of 1 x 10^6 cells/ml of *Candida* yeast suspension was incubated in the presence of hydroxychavicol at IC_{50}. An untreated yeast suspension, which acts as the control of the experiment, was prepared similarly without the addition of hydroxychavicol. After that, the cells were subsequently washed twice with PBS and cell pellet was collected by centrifugation at 3,000 x g at 37 °C for 10 min. Hydroxychavicol-treated cells were then suspended in 1 ml of induction media to induce germ tube formation (GTF). A 100µl of the mixtures were added into 96 well plates and incubated at 37 °C. The percentage of germ tube formation was checked every hour for the first three hours of induction using germ tube formation assay (Colorimetric Assay). Reduction percentage of germ tube formation was then calculated as follows:

% of GTF Reduction = ($Abs_{non\text{-}treated}$ - $Abs_{treated}$ / $Abs_{non\text{-}treated}$) x 100%.

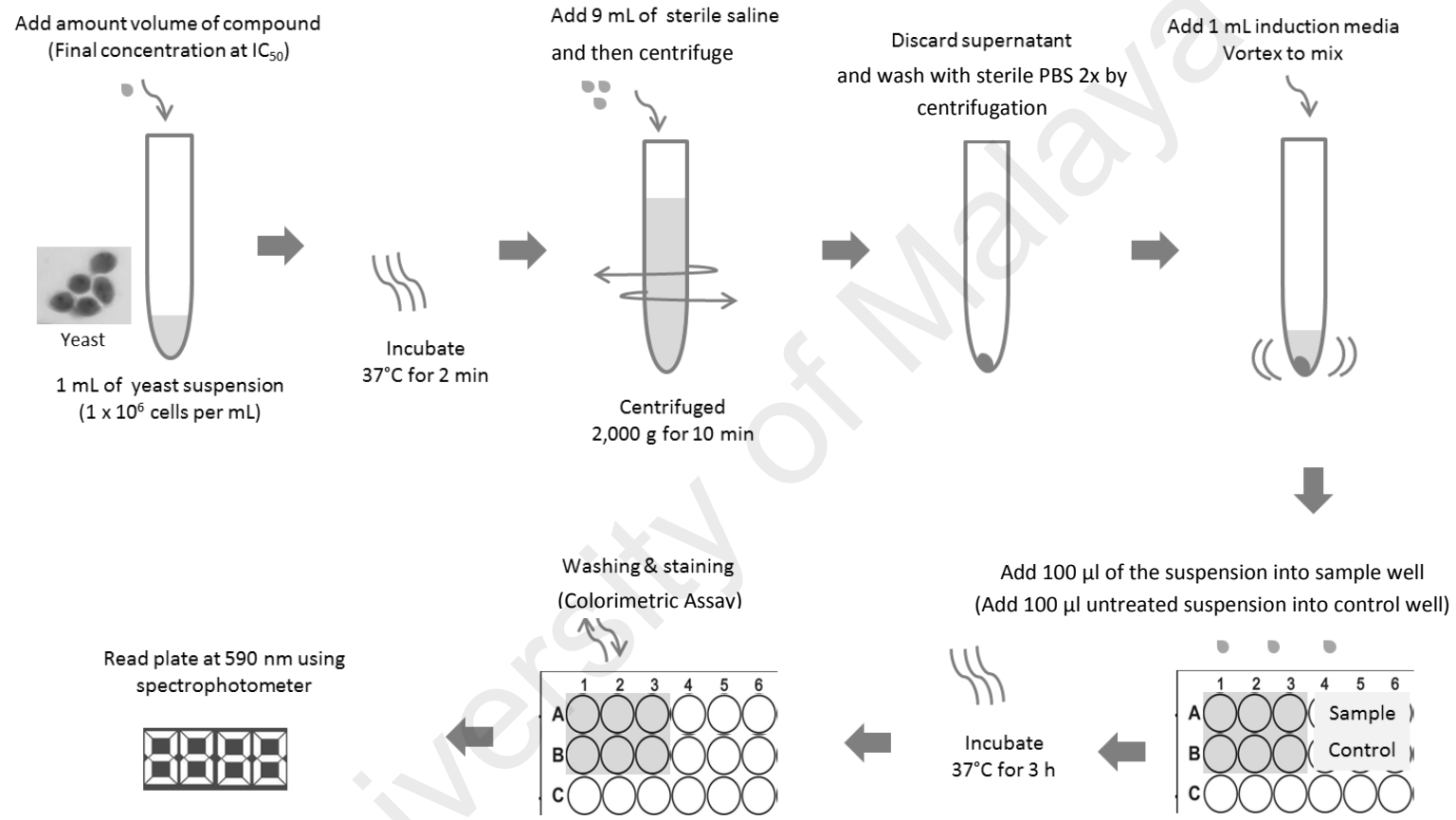


Figure 11. Methodology for short period treatment with compound on the ability to form germ tube of *C. albicans* ATCC 14053

3.2.7.2 Effect of Compound on Ultrastructure of the Germinated *Candida* Cells

3.2.7.2.1 Sample preparation

Briefly, yeast cells of germinated *Candida* strains at a concentration of 1 x 10^6 cells per ml were initially incubated with hydroxychavicol at the concentration of IC_{50} for 2 min. An untreated yeast suspension, which acts as the control of the experiment, was prepared similarly without the addition of compound. After incubation, the cells were subsequently washed with PBS and collected by centrifugation. Inhibitor-treated yeast cells were then suspended in 1 ml of induction media and then incubated at 37 °C for 3 h. After incubation, the cells were ready to be used in sample processing of SEM.

3.2.7.2.2 Determination of Ultrastructural Changes under SEM

The samples were initially immersed in a mixture of gluteraldehyde 8% and Sorenson Phosphate Buffer solution (1:1 ratio) for 1 h to fix the cells. After that, the solution was discarded and the samples were washed with a mixture of Sorenson Phosphate Buffer solution and distilled water (1:1 ratio). Samples were further immersed in a mixture of osmium tetraoxide 4% and distilled water (1:3 ratio) at 4˚C for 14 h in fridge to coat the cells. After pipetting out the solution, the samples were then immersed for 15 min in distilled water, from ethanol 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% until 100% in the fume hood for dehydration steps. After that, samples were immersed in mixture of 100% ethanol and 100% acetone (ethanol : acetone) from 3:1, 1:1 until 1:3 ratio for 20 min. Samples were immersed in acetone 100%, v/v for 20 min for four times and dried using critical drying point (CPD) technique. Once the samples were completely dried,

they were mounted on aluminium stubs, with copper tape and coated with gold under low-pressure with an ion sputter coater. Three samples from each treatment were visualized with a scanning electron microscope in high-vacuum mode at 15kV, and the images processed. Any considerable changes on the morphology between the untreated and treated cells were observed.

3.2.7.3 Effect of Compound on the Adherence of Germinated *Candida* cells

This experiment applied the method of adhesion assay using crystal violet staining following Negri et al. (2010) with slight modification by coating the well with saliva to develop pellicle which mimic the environment of oral cavity. Exposure method was carried out according to Nordin, Wan Harun, and Abdul Razak (2013) which salivary pellicle was exposed to the compound for two minute and been removed through washing step as applied in oral rinse practice as well.

3.2.7.3.1 Sample preparation

In the experiment, unstimulated saliva was collected by expectoration from a healthy donor between 8.00 am to 11.00 am and kept in ice-chilled test tube. A single donor was used throughout the study to minimize variations in saliva composition that may arise between different individuals. The donor was asked to gargle with distilled water for a few seconds to clean and reduce bacterial contamination before the collection begins. The saliva was centrifuged at 17,000 x g for 30 min and the pelleted debris was discarded. The saliva was then sterilized through microfiltration using 0.22µm membrane filter. Collection from different batches was then pooled and stored at -20ºC until further use.

3.2.7.3.2 Determination of Anti-Adherence Activity of Compound

A 100µL of the saliva was added in 96 well plate for 1 h to allow the formation of salivary pellicle in the wells. Saliva was then removed by gentle pipetting and the formed salivary pellicle was then incubated with 100µL of the hydroxychavicol at IC_{50} for 2 min. Untreated salivary pellicle which acts as the control of the experiment, was prepared similarly without the addition of hydroxychavicol. The salivary pellicles were subsequently washed twice with PBS. A 100µL of 1 x 10^6 cells per ml of induced yeast cells, which was prepared prior to the experiment was added into the wells and then incubated at 37 °C. The adherence of germinated cells to the salivary pellicle was checked every hour for the first three hours of exposure using crystal violet staining (Colorimetric Assay) as described in section 3.2.6.1.4. Reduction percentage of adherence was then calculated as follows:

% of Adherence Reduction = ($Abs_{non\text{-}treated}$ - $Abs_{treated}$ / $Abs_{non\text{-}treated}$) x 100%.

3.2.7.4 Effect of Compound on the expression of hyphae related genes

3.2.7.4.1 Sample preparation

Briefly, yeast cells suspension of *Candida* was standardized at a concentration of 1 x 10^6 cells/ml. The cells were then incubated in the presence of the hydroxychavicol at IC_{50} for 2 min at 37 °C in the incubator. After incubation, the cells were subsequently washed twice with PBS and collected by centrifugation at 3,000 x g at 37 °C for 10 min. Untreated yeast cells of *Candida* act as the control of the experiment, was prepared similarly without the addition of hydroxychavicol. The cells were then suspended in 1 ml of induction media and then incubated at 37 °C. The sample was processed for RNA extraction every hour of incubation, from first to third hour.

3.2.7.4.2 Total RNA extraction and quantification

Total RNA was extracted using RiboPure™ Yeast RNA Purification Kit according to the manufacturer's instructions (Appendix A). To avoid genomic DNA contamination, the final isolated RNA was treated with DNase I treatment kit. For verification purpose, the samples were subjected to electrophoresis on 0.7 % agarose gels in 1x TBE buffer for 60 minutes at 90 V. The gel then was stained with ethidium bromide dye and the RNA was observed using Gel Image Analyzer. Total RNA concentrations and purity ratios (260/280 and 260/230) were measured using NanoDrop 2000 UV–vis Spectrophotometer. Finally, the concentration of total RNA was standardized to 5 µg by sample dilution before use in cDNA synthesis step.

3.2.7.4.3 cDNA synthesis

cDNA synthesis was performed using SuperScript® VILO™ cDNA Synthesis Kit. Briefly, 1 µL of of total RNA (5 µg) was mixed with 4 µL of 5X VILO™ Reaction Mix, 2 µL of SuperScript® Enzyme Mix and 13 µL of RNAse free water in a sterile PCR tube to obtain a total volume of 20 µL. Reverse transcription (RT) reaction was carried out using thermocycler at 25°C for 10 min and followed by 60 min at 42°C. Finally, The RT reaction was terminated at 85°C for 5 min. The synthesized cDNA was kept in -20°C prior to use.

3.2.7.4.4 Development of primers

Candidate genes that are related to hyphae; *RAS1, NRG1* and *HWP1* were selected for gene expression analysis (Table 2). DNA sequences of the related genes were obtained from the *Candida* Genome Database (Appendix B). Primers were designed using National Center for Biotechnology Information (NCBI) Primer-Blast online software (Appendix C). Housekeeping gene, *actin1* (*ACT1*) was used as a control for normalization in real time PCR experiments. Universal primer of *ACT1* for yeast was obtained from previous study (Sandini et al., 2011). For optimization purposes, the primers were subjected to standard PCR reaction which were carried out at 95°C for 5 min, followed by 30 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Gel electrophoresis was performed on 1.5 % agarose gels in 1x TBE buffer for 60 minutes at 90 V and the gel was stained with ethidium bromide dye to visualize the PCR product using Gel Image Analyzer.

Table 2. Details of forward and reverse primer of *ACT1, RAS1, NRG1* and *HWP1* used for gene expression analysis

Sets	Gene	Direction	Primers (5' – 3')	Tm (°C)	Product size (bp)
1	*ACT1*	F	CGATAACGGTTCTGGTATG	52.5	97
		R	CCTTGATGTCTTGGTCTAC	50.6	
2	*RAS1*	F	TTGTTGGAGGTGGTGGTGTT	59.7	212
		R	AAACCCTTCACCGGTTCTCA	59.2	
3	*NRG1*	F	CCCCATCCTTCCCAAGTACC	59.8	228
		R	TGGGTCTTTGCTTTGGGTGT	59.8	
4	*HWP1*	F	TGAACCTTCCCCAGTTGCTC	59.3	237
		R	GCAGATGGTTGCATGAGTGG	58.5	

3.2.7.4.5 Real Time PCR

Real time PCR was performed in MicroAmp® Fast 8-Tube Strip, 0.1 ml and run using the 7500 Fast Real-Time PCR System. The SYBR green assay was used as a dye for the quantification of PCR product using SYBR® Select Master Mix kit. Briefly, 2 µL of 1:10 diluted cDNA, 10 µL of SYBR®Select Master Mix (2X), 2 µL of each primers and 4 µL of RNAse free water was added into the tubes for a total of 20 µL. Real-time PCR reaction was performed at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Following PCR, samples were subjected to incubations of an ascending temperature starting from 60°C to 95°C to obtain melt curves. Control reaction was performed with RNA that had not been reverse transcribed in order to ensure that no genomic DNA amplified during the PCR reactions. Triplicate reactions were performed for each sample. Comparative technical analysis was performed using Applied Biosystem (ABI) 7500 software v2.3 after data collection from this experiment (appendix D).

3.2.8 Data Analysis

Every test was run in triplicate and the data was recorded and analysed using IBM SPSS statistics version 20 software. The statistical significance of difference was determined by independent t-test and ANOVA with the value acceptance is $p < 0.05$.

CHAPTER 4: RESULTS

4.1 Determination of Antifungal Activity of Selected Bioactive Compounds

4.1.1 Minimal Inhibitory Concentration (MIC) and Susceptibility of *Candida* species

MIC of the selected bioactive compounds against the *Candida* strains was determined as shown in Table 3. Out of five, three selected bioactive compounds; bakuchiol, hydroxychavicol and PAB, have antifungal activity against all seven *Candida* strains. The MIC of bakuchiol, hydroxychavicol and PAB were determined within the range of 31.3-125, 250-500 and 3.9-250 µg/ml, respectively. Bakuchiol exhibited the most effective antifungal activity with the lowest range of MIC followed by PAB and hydroxychavicol. Meanwhile, MIC of both luteolin and sakuranetin were unsuccessfully determined at concentration ≤1mg/ml.

Susceptibility of the *Candida* species showed variations. *C. dubliniensis* was the most susceptible to each bioactive compound. Meanwhile, *C. glabrata* and *C. krusei* were the least susceptible compared to other strains.

Table 3. The MIC of the selected bioactive compounds against seven *Candida* strains.

Compounds	[a]Minimum Inhibitory Concentration (MIC) (µg/mL)						
	C. albicans ATCC 14053	*C. dubliniensis* ATCC MYA-2975	*C. glabrata* ATCC 90030	*C. krusei ATCC* 14243	*C. lusitenaie* ATCC 64125	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC13803
Bakuchiol	125	31.3	125	62.5	62.5	62.5	62.5
Hydroxychavicol	250	250	250	500	500	500	250
Luteolin	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND
Pseudolaric acid B	3.9	3.9	250	250	125	15.6	125
Sakuranetin	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND	[b]ND

[a]MIC value was determined through observation of clear optical wells of sample compared to positive control.

[b]MIC was unsuccessful determined at concentration ≤1mg/mL.

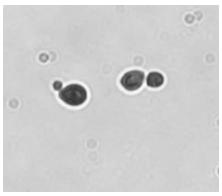
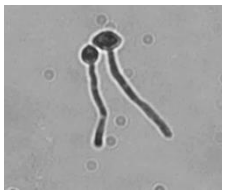
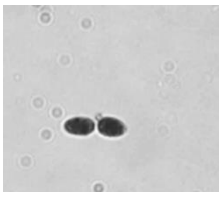
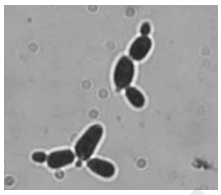
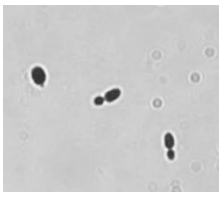
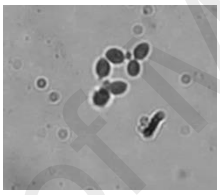
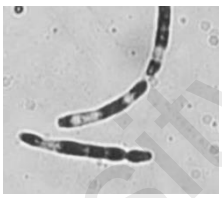
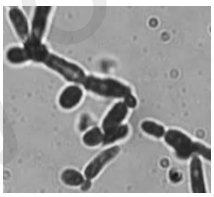
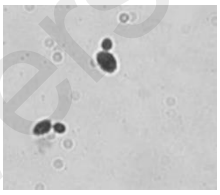
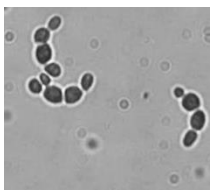
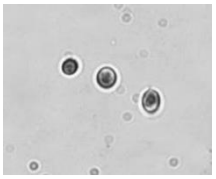
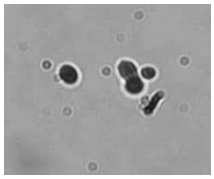
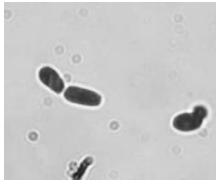
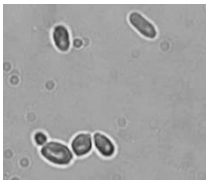
ND = Not determined.

4.2 Determination of Inhibitory Activity against Germinated *Candida*

4.2.1 Germinated *Candida*

Germinated *Candida* in YPDS was determined as shown in Table 4. Out of the seven *Candida* strains, only one strain; *C. albicans* was positive in germination. *C. albicans* produced 98% of germinated cells in the induction media within 3 h incubation period. Meanwhile, other strains did not display any morphological changes but showed an increase in cell number and grew in budding yeast form.

Table 4. Germination test of seven *Candida* strains in induction media of YPDS [YPD + 10% serum (v/v)].

Strain	Before Induction (0 h)	After Induction (3 h)	Germination	Percentage of Germinated Cell (%)
C. albicans ATCC 14053			Positive	98 ± 2
C. dubliniensis ATCCMYA-2975			Negative	0
C. glabrata ATCC 90030			Negative	0
C. krusei ATCC 14243			Negative	0
C. lusitenaie ATCC 64125			Negative	0
C. parapsilosis ATCC 22019			Negative	0
C. tropicalis ATCC 13803			Negative	0

4.2.2 Inhibitory Concentration (IC_{50}) on Germination

Inhibitory concentration (IC_{50}) of the antifungal bioactive compounds and CHX on germination was determined as shown in Table 5. IC_{50} of bakuchiol, hydroxychavicol, PAB and CHX were 642.6, 151.7, 502.9 and 15.8 µg/ml respectively. CHX demonstrated the lowest IC_{50} followed by hydroxychavicol, PAB and bakuchiol.

Table 5. IC_{50} with 95% CI of the antifungal bioactive compounds (compounds with antifungal activity) and CHX against germ tube formation of *C. albicans*

Compounds	Inhibition of germ tube formation (IC_{50} with 95% CI) (µg/ml)	
	[a]IC_{50}	[b]95% CI
Bakuchiol	642.6	507.2 - 814.2
Hydroxychavicol	151.7	100.5 - 228.9
Pseudolaric Acid B	502.9	354.7 - 713.0
[c] 0.12%w/v Chlorhexidine Gluconate (CHX)	15.8	11.58 - 21.46

[a]Concentration of the compounds that is required for 50% inhibition of germ tube formation.
[b]95% confidence interval for the IC_{50}s concentration range determined.
[c]Active compound in oral rinse used as comparative control

4.2.3 Inhibition potency of the Antifungal Bioactive Compounds

The inhibition potency of the bioactive compounds was determined and is shown in Table 6. Hawser (1999) has described that potency or strength of an agent in inhibiting either budding or hyphal growth can be determined by calculating the IC_{50}/MIC ratio. It has been suggested that an agent preferentially inhibit hyphal growth if the ratio is less than 1. If the ratio is more than 2, an agent is considered to have low ability to inhibit hyphal growth, but exert their activity by inhibiting the budding yeast growth. In contrast, if the ratio is between 1 to 2, an agent is considered to have approximately equal effects on both activities.

The IC_{50}/MIC ratio of bakuchiol, hydroxychavicol, PAB and CHX was determined at 5.1, 0.6, 128.6 and 1.7, respectively. Hydroxychavicol has the lowest ratio followed by CHX, bakuchiol and PAB. The ratio for hydroxychavicol was below than 1 while for bakuchiol and PAB were more than 2. The ratio of CHX was between 1 to 2. Therefore, hydroxychavicol was considered to preferentially inhibit hyphal growth while bakuchiol and PAB were considered to preferentially inhibit budding yeast growth. CHX is considered to have approximately equal effects on budding and hyphal growth.

Table 6. IC_{50}/MIC ratio and preferential growth inhibition of antifungal bioactive compounds and CHX against *C. albicans*

Compounds	Preferential Growth Inhibition based on IC_{50}/MIC ratio			
	IC_{50} (µg/ml)	MIC (µg/ml)	IC_{50}/MIC ratio	Preferential Growth Inhibition
Bakuchiol	642.6	125	5.1	Budding yeast
Hydroxychavicol	151.7	250	0.6	Hyphal growth
Pseudolaric Acid B	502.9	3.9	128.6	Budding yeast
[c] 0.12%w/v Chlorhexidine Gluconate (CHX)	15.8	9.4	1.7	Budding yeast & Hyphal growth

Determination is based on IC_{50}/MIC ratio. This ratio determines the relative potency of each agent to inhibit budding and hyphal growth: (i) ratio < 1 (agents which preferentially inhibit hyphal growth), (ii) ratio of 1–2 (those with approximately equal effects on both) and (iii) ratio > 2 (agents with a lower ability to inhibit the hyphal growth but exert their activity by inhibiting the budding yeast growth).

4.3 Brief Treatment of Potent Inhibitor (Hydroxychavicol)

4.3.1 Germination of Hydroxychavicol-treated *C. albicans*

Figure 12 showed the germination of hydroxychavicol-treated *C. albicans* within 3 h incubation period. Percentage of germinated cells were significantly reduced by 65%, within the first hour post-treatment. However, only 8% and 10% reduction were respectively determined within the second and third hour post-treatment. Microscopic observation in Figure 13 demonstrated the development of germinated cell within each hour. The picture showed some differences in populations of germinated cells between treated and non-treated samples especially within the first hour after treatment.

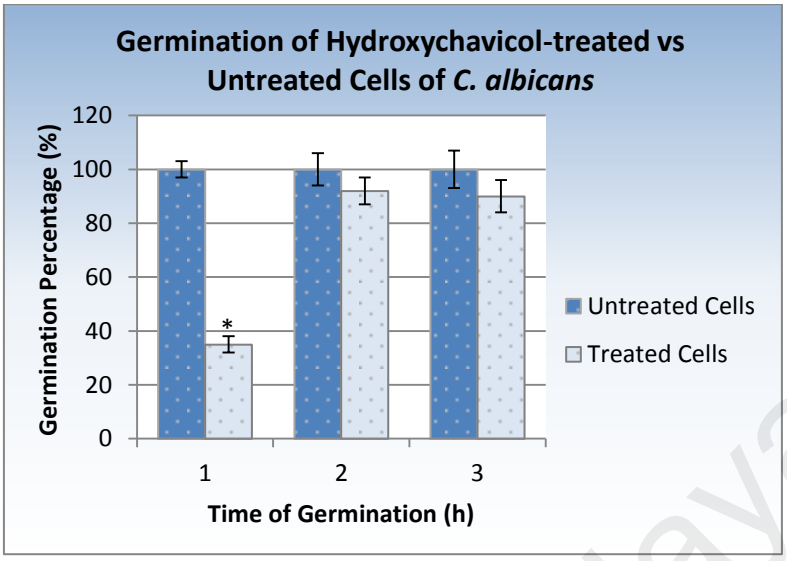


Figure 12. Germination percentage of hydroxychavicol-treated *C. albicans*. Each data point represents the mean ± SD (n = 6). The statistical significance of difference was determined by independent t-test. Asterisks (*) label indicates p-value of < 0.05.

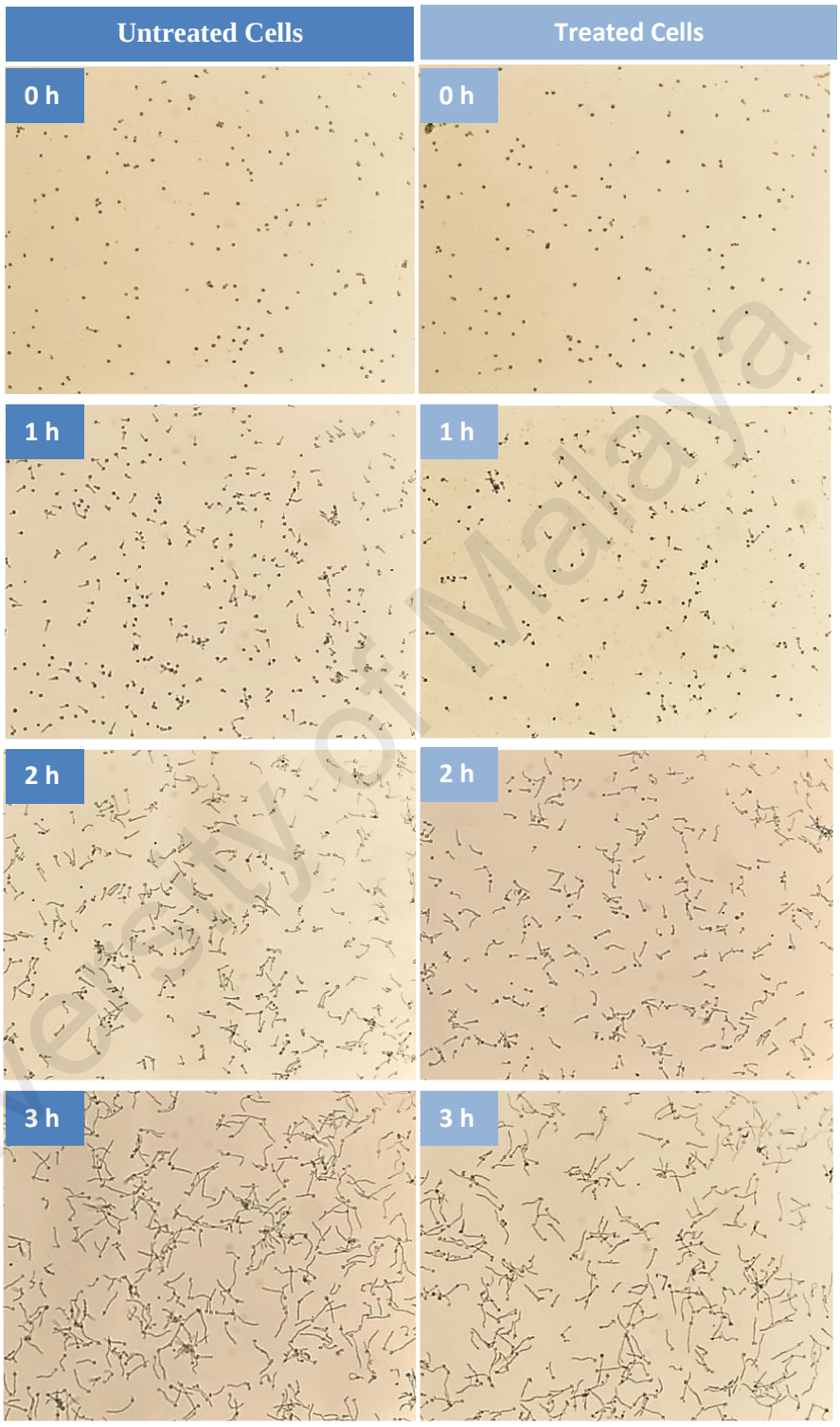


Figure 13. Microscopic observation of germination of hydroxychavicol-treated *C. albicans*. The image was captured at magnification 200X using Moticam Digital Microscope Camera with capture resolution at 1280 x 800.

4.3.2 Ultrastructure of Hydroxychavicol-treated *C. albicans*

The activity of hydroxychavicol was further studied for their effect on the morphological appearance of the germinated cells by using a scanning electron microscope (SEM) and the result was shown in Figure 14. For the untreated control sample, it can be concluded that the cells had developed the optimal size, rounded shape and had a smooth surface of the germinated tube. Following the treatment with hydroxychavicol, similar observation was seen as showed in the untreated sample but some of the cells have minimal changes such a constriction at the germ tube neck and had rough surface morphology on the whole surface of its body and tail.

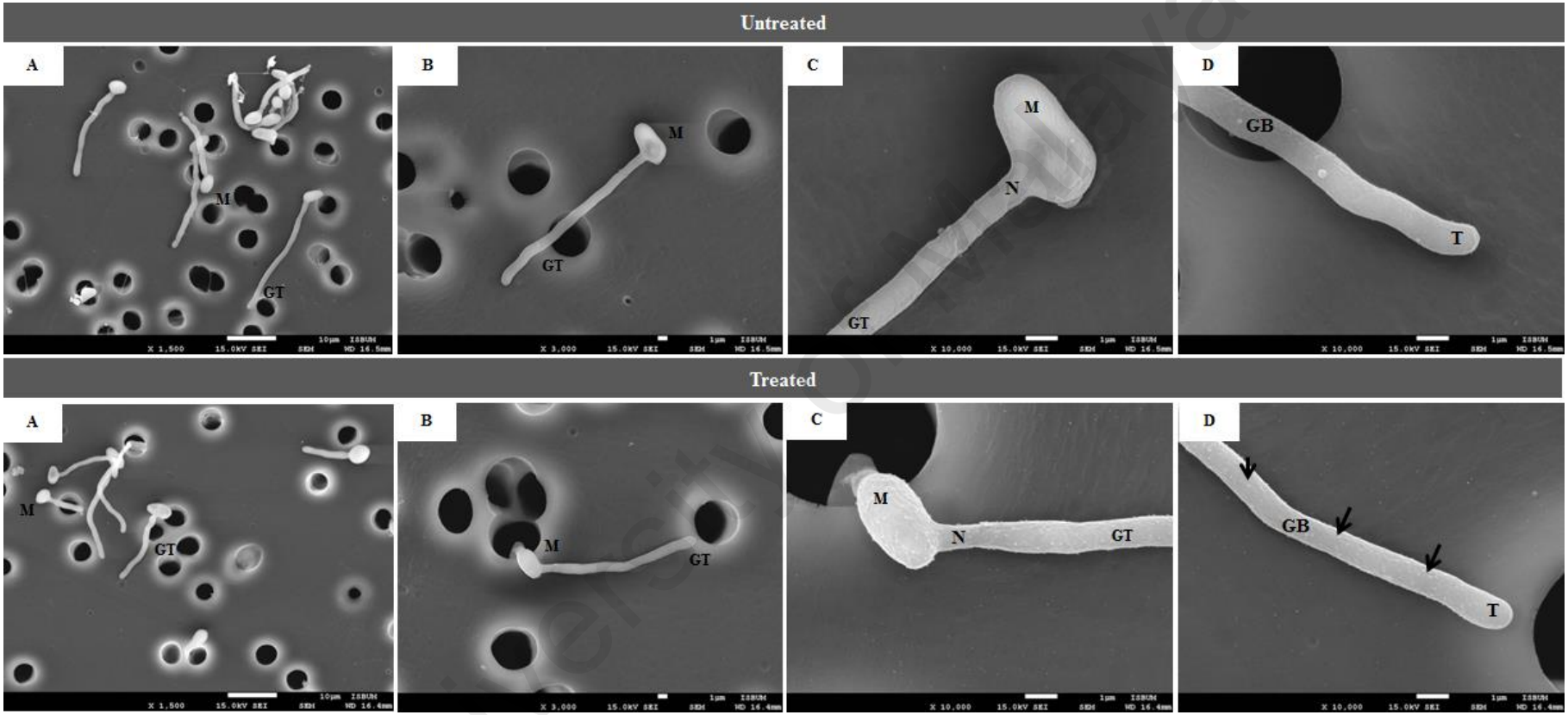


Figure 14. Ultrastructure images of germinated cells of the hydroxychavicol-treated *C. albicans*. All images were visualized at 15 kV. Image A showed a number of germinated cell at magnification of 1,500X. Image B showed the structure of mother cell (M) and germ tube (GT) of a germinated cell at magnification of 3,000X. Images C and D showed the structure of neck (N), germ tube body (GB) and tail (T) of a germinated cell at magnification of 10,000X. In the image, it showed the rough surface of the cell as indicate by the black arrow.

4.3.3 Adherence of Germinated *C. albicans* on Hydroxychavicol-treated Salivary Pellicle

Figure 15 showed the percentage of adherence of germinated *C. albicans* on the hydroxychavicol-treated salivary pellicle within 3 h incubation period. The reduction percentage for the first, second hour and third hour post-treatment was 2%, 3% and 1%, respectively. It showed that hydroxychavicol was not capable to effectively reduce the adherence affinity of the germinated cells to the treated salivary pellicle. It is, therefore, suggested that hydroxychavicol has poor anti-adherence activity of germinated cells.

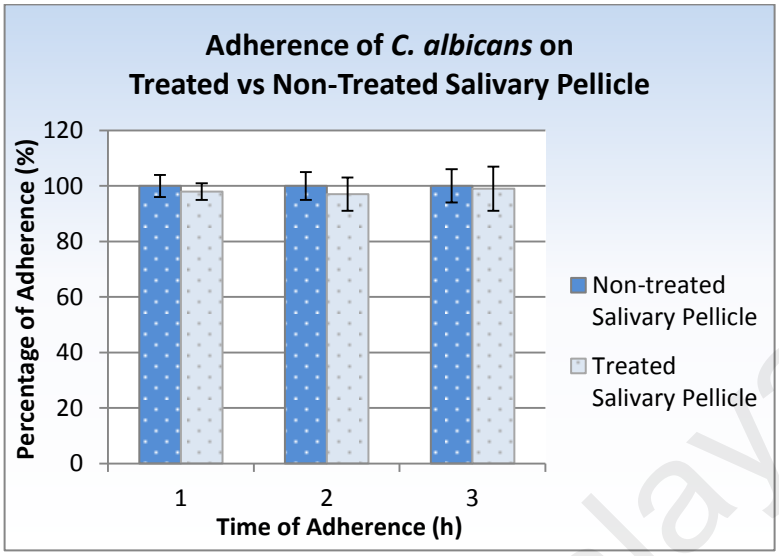


Figure 15. Percentage of adherence of germinated *C. albicans* on hydroxychavicol-treated salivary pellicle. Each data point represents the mean ± SD (n = 6). The statistical significance of difference was determined by independent t-test.

4.3.4 Gene Expression of Hydroxychavicol-treated *C. albicans* during Germination

Total RNA of *C. albicans* was successfully isolated as shown in Figure 16. The image comprised of 28s and 18s rRNA band that was indicative of intact RNA. Concentration of the RNA and purity of the RNA were also determined. cDNA was successfully synthesized from the standardized total RNA. PCR reaction was optimized using the tested reaction parameters and the designed primers (Table 2) to yield precise size of amplicons at 97, 212, 228 and 237 bp for *ACT1*, *RAS1*, *NRG1* and *HWP1* genes, respectively. The optimised reaction parameters was further used in real-time PCR (qPCR) experiment and the gene expression analysis was successfully determined as shown in Figure 17 and 18.

Figure 17 showed the relative expression of *RAS1*, *NRG1* and *HWP1* of *C. albicans* during germination (1-3 h) compared before germination (0 h). Expression of these genes was found to be significantly changed ($p<0.05$ and $p<0.01$) during germination period, which showed the necessity of the genes in the germination process of *C. albicans*. Expression of *RAS 1* was significantly upregulated ($p <0.05$) with 2.6 fold changes during the first hour and also upregulated 1.5 fold changes both during the subsequent of germination (2^{nd} and 3^{rd} hour). Expression of *NRG1* was significantly downregulated ($p<0.05$) with 0.2, 0.3 and 0.3 fold changes during each hour of germination (1^{st}, 2^{nd} and 3^{rd} hour, respectively). Meanwhile, *HWP1* was significant highly upregulated ($p<0.01$) with approximately 350, 180, 210 fold changes during each hour of germination (1^{st}, 2^{nd} and 3^{rd} hour, respectively).

Figure 18 showed the relative expression of *RAS1*, *NRG1* and *HWP1* of the hydroxychavicol-treated *C. albicans* compared the untreated control. During the treatment (0 h), expression of these genes were initially downregulated with 0.7, 0.9 and 0.7 fold changes for *RAS1*, *NRG1* and *HWP1*, respectively. However, the expression of *RAS1*

begins to upregulate with 1.4 fold changes both within the first and second hour, but then downregulated with 1.0 fold changes within the third hour post-treatment. Meanwhile, the expression of *NRG1* begins to upregulate within the first hour with 1.4 fold changes, but then downregulated with 1.0 and 0.9 fold changes within the second and third hour post-treatment, respectively. In contrast, expression of *HWP1* still downregulated with 1.0 fold changes within the first hour, but begin to upregulate with 1.5 and 1.2 fold changes within the second and third hour post-treatment, respectively.

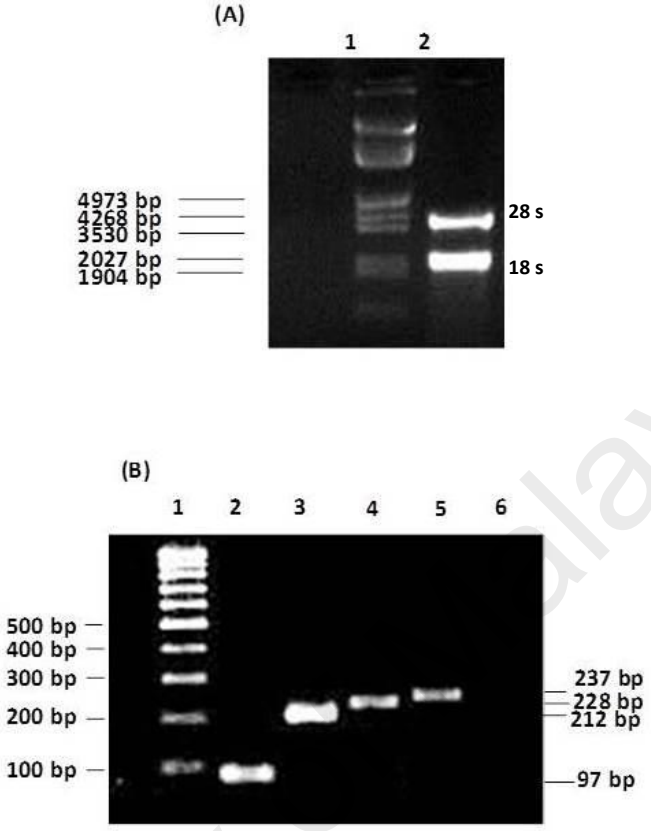


Figure 16. Gel electrophoresis images. (A) total RNA extraction with lane A1: λ *E. coli* + *Hind III* ladder, lane A2: total RNA of *C. albican*s. (B) PCR products of *C. albicans* with B1: 100bp ladder, B2: ACT1, B3: *RAS*, B4: *NRG1*, B5: *HWP1* and B6; negative control (without cDNA template).

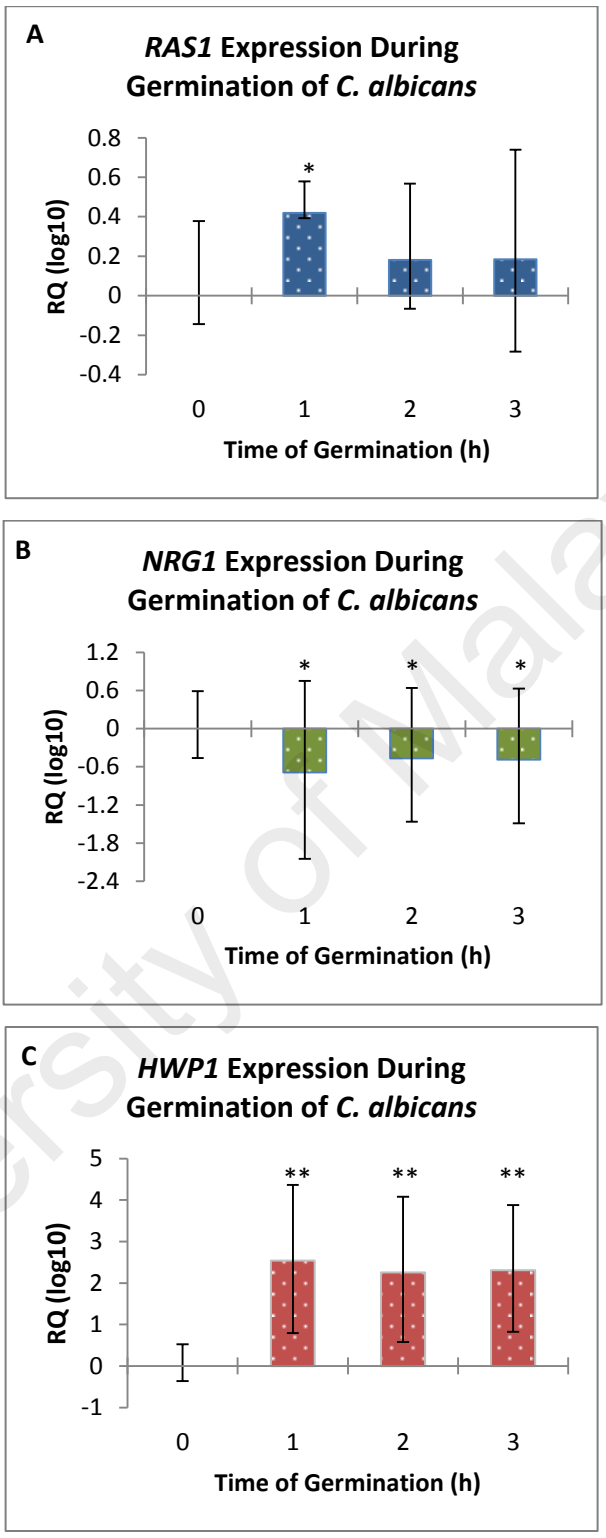


Figure 17. Relative expression of (A) *RAS1*, (B) *NRG1 and* (C) *HWP1* during germination of *C. albicans*. Each data point represents the mean ± SD (n = 3). The statistical significance of difference was determined by ANOVA with Bonferroni post-hoc test. Asterisks (*) and (**) label indicate p-value of < 0.05 and < 0.01, respectively.

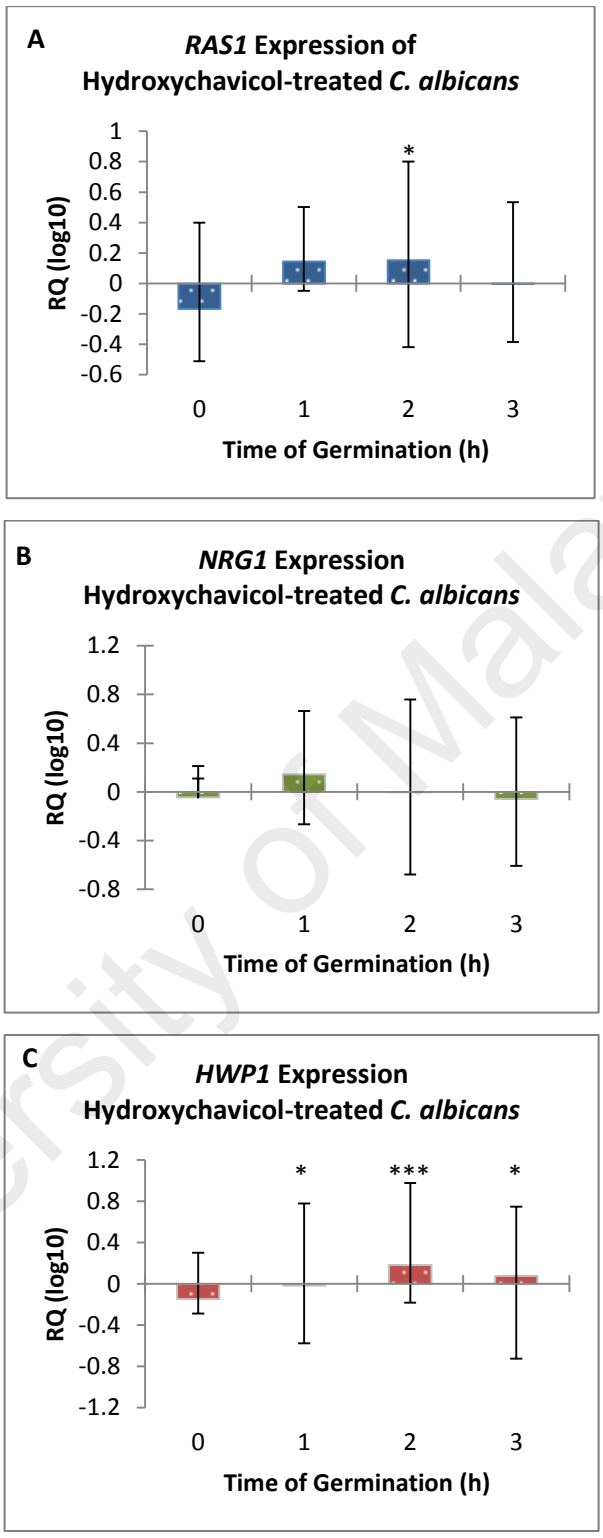


Figure 18. Relative expression of (A) *RAS1*, (B) *NRG1* and (C) *HWP1* after treatment with hydroxychavicol. Each data point represents the mean ± SD (n = 3). The statistical significance of difference was determined by ANOVA with Bonferroni post-hoc test. Asterisks (*) and (***) label indicate p-value of < 0.05 and < 0.001, respectively.

CHAPTER 5: DISCUSSION

5.1 Antifungal Activity of bakuchiol, hydroxychavciol, luteolin, PAB and Sakuranetin

Firstly anti-candida activity was screened from five bioactive compounds (bakuchiol, hydroxychavciol, luteolin, PAB and sakuranetin) reported to possess antifungal activities based on literatures (Table 1) against seven different strains of oral–associated *Candida*. Compounds that were not anti-candida agent was excluded in further experiment. MIC value was used for the ratio calculation of inhibition potency and also used as a guided dosage in other experiments. So the dose that we used below than MIC and not kill the yeast but control its growth and allow the inhibition assessment of the compounds. In this experiment, CHX was used as a positive control. CHX has a very broad antimicrobial spectrum and it been used in the dental practice over the years (Sajjan, Laxminarayan, & Kar, 2016). Dose of CHX at 600 µg/ml used was highly above the reported MIC value against *Candida* species (Fathilah, Himratul-Aznita, Fatheen, & Suriani, 2012; Salim, Moore, Silikas, Satterthwaite, & Rautemaa, 2013) and this validated the usage of CHX as reference for positive control in this experiment.

As a result, bakuchiol, hydroxychavciol and PAB were determined to exhibit anti-candidal activity while luteolin and sakuranetin were found to be inactive against the *Candida* species at concentration below 1 mg/ml. Luteolin and sakuranetin were previously described elsewhere to be active against dermatophytes and rice blast fungal (Hasegawa et al., 2014; Sartori et al., 2003), however, both were found to be inactive against *Candida* species tested in our study. Thus this findings suggests that both compounds of luteolin and sakuranetin are not an anti-candida agent.

Based on the chemical structure, the selected bioactive compounds are categorized into three different groups which is terpenoid (bakuchiol and PAB), flavonoid (luteolin and sakuranetin) and phenylpropanoid (hydroxychavicol). Most of the terpenoid (carvacrol, farnesol, geraniol, linalool, menthol, menthone, terpinen-4-ol, and α-terpineol) and phenylpropenoid (eugenol and tyrosol) are known to be active against isolates of *Candida* species (Carrasco et al., 2012; Marcos-Arias, Eraso, Madariaga, & Quindos, 2011) but certain flavonoids (catechin, quercetin and epigallocatechin) need to be coupled with other commercial drugs to be fully active against the species (da Silva et al., 2014).

5.2 Hydroxychavicol is A Potent Agent against Germination of *C. albicans*

Seven *Candida* strains were screened for germ tube formation at the tested condition that closely resemble the human body (present of serum and incubation at temperature of 37°C). *C. albicans* was the only strain that was positive in germination. *C. dubliniensis* and *C. tropicalis* were unable to germinate in the tested condition eventhough they are reported as potential germinated species (O'Connor et al., 2010). This probably arises from the different sensing mechanism of the strains on the environmental nutrient which determine their germination activity. Nutrient depletion was found to be one of factors for *C. dubliniensis* to germinate (Falsetta et al., 2014) and maybe also for *C. tropicalis*. Since YPDS is a nutrient-rich media, one or more of the nutrient component may block the expression of certain genes or proteins and then inhibit the transition process of the strains. The other strain exhibited normal cell growth by budding growth and they seem not to be affected by the environmental conditions.

Following identification of germinated *Candida*, the bioactive compounds that showed antifungal activity in earlier experiment were further subjected to germination inhibition experiment. The capacity of bioactive compounds as inhibitor was measured based on their concentration to inhibit 50% of germinated cells. Since the germ tube is the initial stage of hyphal growth, inhibition of germination could help in prevention of disease related to hyphae such as oral candidiasis (D. Williams & Lewis, 2011).

Each compound has tended to act as an inhibitor against germination. However, certain compounds such as bakuchiol and PAB have a higher concentration range of IC_{50} which is greater than the MIC determined. This variance may indicate the limited inhibitory activity of the compound against hyphae. Thus, in order to describe the differences better, (Hawser & Islam, 1999) have formulated a calculation based on IC_{50}/MIC ratio. This ratio can be used to determine the potency of each agent in the inhibition of either budding yeast or germination of hyphal growth.

Therefore, based on the IC_{50}/MIC ratio, we found that the bioactive compounds are categorized into three different potencies; hydroxychavicol was active against germination, bakuchiol and PAB were active against budding yeast and CHX was active against both growth modes. What makes the potency different between the compounds could be possibly derived from their specific action mechanism on the biological target.

In biological reaction, hydroxychavicol and CHX are capable to alter the cell membrane structure and disrupt the permeability barrier of the membrane (Intzar Ali et al., 2010; Komljenovic, Marquardt, Harroun, & Sternin, 2010). Consequently, the injured cells are incapable to perform normal biological activity and indirectly inhibit the germination as well as budding growth. PAB is known to inhibit the synthesis of mitotic spindles during

mitosis (Wong et al., 2005). Mitosis is crucial during cell growth in living yeast, but its process is variable in different growth mode. During budding growth, yeast cells will initially undergo mitosis stage before further buds off daughter from parent cells (Becker et al., 2017). Meanwhile, in germination, yeast cells will start to germinate before producing elongated tube through mitosis stage (P. E. Sudbery, 2001). Thus, these factors may explain the inefficient activity of PAB against germination.

5.3 Brief Treatment of Hydroxychavicol Affected Germination Activity

Nowadays using oral rinses as daily oral hygiene has been practiced by many people due to the increase in oral health awareness. One of the function of ral rinse is used to eliminate dental biofilm which is colonized by microorganisms in the oral cavity. *Candida* is one of the microorganisms that form dental biofilm community. In fact, matured biofilm may consists different type of cellular morphology of *C. albicans* and majority constituent is occupied by hyphal form (Hirota et al., 2017). In particular, oral rinse should be able to inhibit hyphal growth in order to reduce the presence and attachment of this opportunistic microbe in the oral cavity. Oral healthcare practice requires a limited time exposure of the oral rinse in mouth. Therefore, we applied a brief treatment method in this study in order to investigate the suitability of hydroxychavicol to be used in the oral healthcare products.

Hydroxychavicol exhibited suppression effect on the germination activity of *C. albicans.* Significant inhibition can be seen within the first hour, but not in the subsequent hours post-treatment. The presence of hydroxychavicol during the brief treatment delayed the process of elongated tube production, but then it started to return to its normal biological activity after the first hour post-treatment. Hydroxychavicol may cause injury to the cell

through disruption of cell membrane (Intzar Ali et al., 2010), causes rapid loss nutrients and will inhibit the growth activity of cells. However, the cells are able to recover within a certain period after hydroxychavicol was removed and it begins to continue germinating. This factor could be the reason why its growth activity is limited by time of exposure. Since two-minute exposure gave effect for one hour inhibition, we expected that longer time inhibition could be achieved when the exposure time increased.

5.4 Cellular Morphological Changes of germinated *Candida* after Treated with Hydroxychavicol

The activity of hydroxychavicol was further studied for their effect on the morphological appearance of the germinated cells by using a scanning electron microscope (SEM). For the untreated control sample, it can be concluded that the cells had developed with optimized size, rounded shape and had a smooth surface of the germinated tube. Following treatment with hydroxychavicol, some of the cells were unable to retain their normal morphological structure, probably affected by the suppression of metabolic activity, especially on the production of hyphal wall and cytoskeletal network protein. Hydroxychavicol may cause environmental stress towards cell growth, which would restrict the ions and nutrients uptake mechanisms that normally take place on the cell surfaces. Some hyphal cell wall constituents which are less firmly bound to the rigid glucan-chitin network may be lost during the treatments which then causes uneven cell surface. It has been suggested that the loss of a few wall components could lead to the loss of structural integrity (de Billerbeck, Roques, Bessiere, Fonvieille, & Dargent, 2001). From SEM analysis, it seems that the brief treatment with hydroxychavicol caused slight changes to the structure of the germinated

cells. This is in agreement with previous study which has reported that many antifungal drugs can affect the morphology of yeasts (Nakamura, Abe, Hirata, & Shimoda, 2004).

5.5 Hydroxychavciol is a Poor Anti-Adherence Agent against Germinated *C. albicans*

One of the main purpose of oral rinse is to eliminate the attachment of microbes on oral surfaces. This function can be achieved by reducing the binding affinity between cells and the oral surfaces itself. Oral surface comprises of salivary pellicle, a covering protein film that has a selective binding of glycoproteins. Cell membrane of *C. albicans* have non-polar cell surface proteins and the cells are able to bind to the pellicle through hydrophobic interaction (Bujdáková, Didiášová, Drahovská, & Černáková, 2013). Rationally, if the cells are in hyphae form, it has more adherence capacity to pellicle layers due to the large surface area of the elongated membrane.

A brief treatment of the experimental pellicle with hydroxychavicol at IC_{50} (152 µg/ml) was found to exhibit a slight reduction in the adhering capacity of the germinated *C. albicans*. A concentration of 152 µg/ml was applied due to its potential reduction against the germination in the earlier study. We found that hydroxychavicol have shown poor anti-adherence effects against the germinated *C. albicans*. It has been described that certain antifungal agents were able to alter the protein receptors on salivary pellicle, resulting in failure recognition of *Candida* cells (Nordin et al., 2013). Furthermore, physiochemical forces from cell surface hydrophobicity (CSH) of the cells may be implicated in assisting its adherence to treated pellicle. It has been suggested that CSH is directly involved in adherence since it ease the binding process of microbes to epithelial cells (Y. H. Samaranayake, Wu, Samaranayake, & So, 1995). In this case, the CSH was increased in

germinated cells since the elongated tube has large surface area compared to yeast form. This situation increases the physiochemical forces and strengthen the binding affinity of the cells, even to the treated salivary pellicle.

5.6 Hydroxychavicol Altered the Expression of *RAS1*, *NRG1* and *HWP1*

C. albicans change its morphology from yeast to hyphae through germination process. In untreated condition, normal yeast-hyphae transition involved *RAS1* gene that works as a signalling transducer for cAMP-PKA pathway (Biswas et al., 2007) being upregulated when the cells being cultured in YPDS (Feng et al., 1999). As a result, cAMP signaling pathway is activated. The upregulation of *RAS1* has indirectly downregulated the expressions of *NRG1*, a repressor gene, which functions to block the transition activity of *Candida* yeast (Lu et al., 2011). Thus, the lowered expression of repressor gene *NRG1* has encouraged yeast transition activity and resulted in the formation of germ tubes. Changes in the regulatory genes have finally resulted in a higher expression of hyphae specific genes including *HWP1*. *HWP1* is a gene that encodes for a cell wall adhesion protein which helps *C. albicans* to attach strongly to oral surfaces (Orsi et al., 2014). The presence of hyphae does not only increase the surface areas of *Candida*, but has exhibited an increased of the *HWP1* gene during its formation. The upregulation of *HWP1* gene resulted in producing more cell wall adhesion protein, which assist in adhesion ability of *Candida*.

Apart from causing damage to the cell membrane, our study has found that hydroxychavicol inhibited cell germination by regulating the expression of hyphae related genes (Figure 19), especially *RAS1*, and caused inhibition to the growth of cells. Since *RAS1* plays an important role in cell sustainability, this could be the reason why the cells

experienced immediate upregulation of *RAS1* gene for restoration of its normal physiological condition after the exposure to hydroxychavicol.

The downregulation of RASI gene expression lead to the inactivation of cAMP-PKA signaling pathway. However, with time, the expression of *NRG1* was found to be downregulated and cells begin to regain its ability to germinate. This finding has answered the reason why the number of germinated cells was substantially elevated in the second and third hour after exposure to hydroxychavicol. Within this period, biological processes including cell cycle progression, cell proliferation and metabolism were recovered by upregulation of RASI gene expression (Doedt et al., 2004; Vinod et al., 2008).

During the transition activity, we found that *HWP1* gene was upregulated. Thus, the cell could produce abundant of adhesin on its hyphal wall which then generate high adherence affinity and strengthen the binding even to the modified glycoprotein of salivary pellicle. As observed in our study, when *C.albicans* was exposed to hydroxychavicol, *HWP1* was immediately downregulated but regained to its normal level of expression at 1 hour and continued to be upregulated with time. In fact, the existence of adhesion is essential for the *C. albians* pathogenesis particularly in candidiasis infection (Sundstrom et al., 2002).

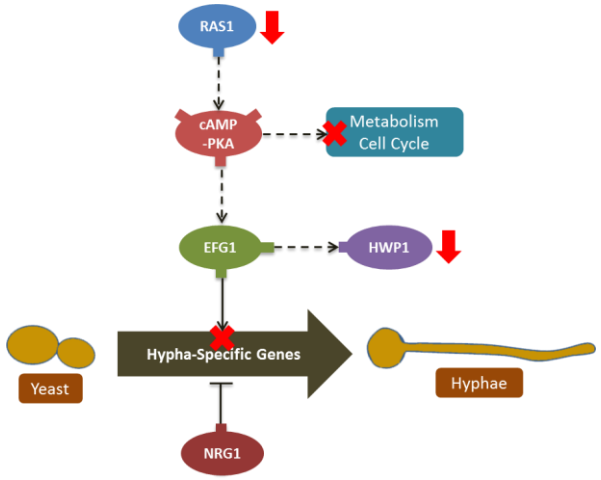
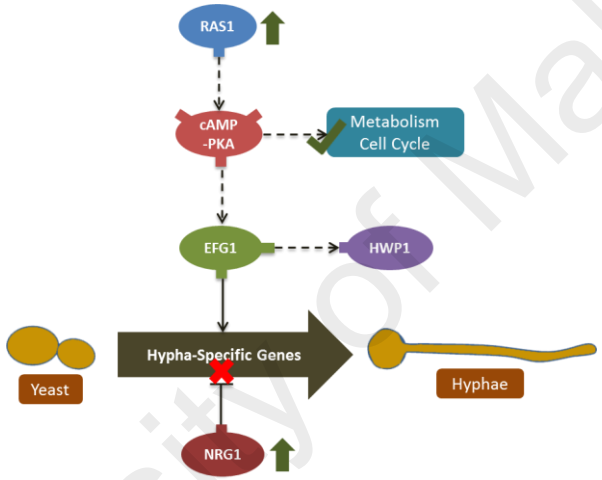
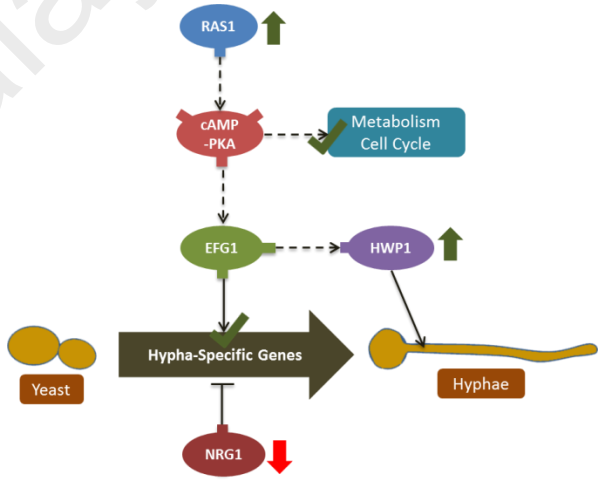
A. During Treatment (0 h)	B. Post Treatment (1st h)	C. Post Treatment (2nd and 3rd h)
		
Expression of *RAS1* was downregulated and caused the suppression on *HWP1* gene, metabolism, cell cycle and hyphal formation process.	After removal of hydroxychavicol, *RAS1* was highly expressed and resulted in promoting the cell cycle but not in hyphal formation since expression *NRG1* was upregulated (suppressed hyphal formation). This occurrance may be because of cells in a recovery process to regain normal physiological condition before it can change to hyphae.	After recovered, *RAS1* remain expressed, but *NRG1* was now downregulated resulted in promoting hyphal formation. At the same time, *HWP1* was highly expressed which support the development of hyphal wall protein of the germinated cell.

Figure 19. An illustration of the suggested mechanism how hydroxychavicol delayed yeast to hyphae transition process after brief treatment on *C. albicans.* Label indicate of downregulation (red arrow), upregulation (green arrow), suppression (red cross) and promoting (green right).

CHAPTER 6: SUMMARY AND CONCLUSIONS

Listed below are the conclusions of our findings :

- Bakuchiol, hydroxychavicol and PAB possessed antifungal activity against the seven oral associated *Candida* spp used in this study.
- Bakuchiol and PAB are more active against budding yeast growth while hydroxychavicol is more active and act as a potent inhibitor against hyphal growth of *C. albicans*
- Hydroxychavicol delayed germination process of *C. albicans*. Longer exposure will prolonged the suppression effect. However, it has poor anti-adherence activity against germinated cells.
- Hydroxychavicol could slightly modify and change the morphological structure of *C. albicans* germinated cells. The changes might arise from the affected physiological conditions of the cells.
- Hydroxychavicol delayed germination process of *C. albicans* by affecting the expression of *RAS1*, *NRG1* and *HWP1*.

In overall, hydroxychavicol is one of the identified bioactive compounds that possessed antifungal properties, potent in inhibition of germination process by affecting the morphological and gene regulation of *C. albicans*. Since hydroxychavicol has poor anti-adherence activity against germinated cells, it is recommended for further study of the synergistic potential with other bioactive compounds to improve its properties towards preventing colonization of *C. albicans* in the oral cavity.

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