PRELIMINARY EVALUATION ON ANTIFUNGAL PROPERTIES OF RED LEAF EXTRACT OF *Syzygium campanulatum*

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ABSTRACT

There's a need for more effective antifungals and one of the ways of obtaining them is to test natural flora for their potential antifungal capability. The family Syzygium of plants have long been associated to contain many bioactive compounds including antifungals. In this study, we aimed to obtain *Syzygium campanalatum* (SC) crude leaf extract through ethanol maceration, to separate SC crude leaf extract into the respective fractions and to test antifungal effect of both SC crude leaf extract and fractions against the growth of *Aspergillus brasiliensis* (AB) suspension. The extraction of SC crude extract was performed with ethanol maceration for 24 hours and obtained 45% yield of crude extract. An Amberlite column chromatography was performed on SC crude extract with three solvent systems, namely methanol (MeOH), ethylacetate (EA) and MeOH:EA). MeOH:EA and EA solvent systems separated fractions with the absorbance that have similar absorbance peaks with tested chalcone standard fractions which were fraction-15 and fraction-24 which indicated high possibility of both fractions containing chalcone compound. Based on chalcone standard curve, the chalcone concentrations in both fraction-15 and fraction-24 have 0.011 mg/mL of chalcone. Antifungal assays measured at 620 nm (A_{620}) involving AB suspensions treated for both SC crude extract and fractions through microdilution method. 20 µL to 100 µL of crude extract have antifungal inhibition against AB growth within 24 hours. Whereas all three SC fractions treatment did not achieve antifungal inhibitory effect due to low chalcone concentration. In conclusion, chalcone was successfully isolated from SC crude extract through column chromatography and antifungal assay of AB treated with crude extract was tested positive for inhibition.

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LIST OF ABBREVIATIONS

ABPA	Allergic bronchopulmonary aspergillosis
AB	*Aspergillus brasiliensis*
ATCC	American Type Culture Collection
CDC	Center for Disease Control
CFU	Colony forming units
CLSI M27 A3	Clinical Laboratory Standards Institute guidebook
EA	Ethyl acetate
EtOH	Ethanol
IC_{50}	50% half maximal inhibitory concentration
MeOH	Methanol
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
mL	mililitre
PDB	Potato Dextrose Broth
RPM	Revolutions per minute
SC	*Syzygium campanulatum*
TLC	Thin Layer Chromatography
VEGF	Vascular Endothelial Growth Factor
µL	microlitre
UV	ultra-violet
(w/v)	weight/volume

CHAPTER 1

INTRODUCTION

Approximately 5.1 million species of fungi are present on Earth and many of them are undiscovered and undocumented yet (Blackwell, 2011). Some fungi species like *Saccharomyces cerevisiae* are useful and contribute in the brewery industry while others like certain *Aspergillus* species may cause aspergillosis (Latgé, 1999). Currently, there are many synthetic antifungal compounds available in the market such as voriconazole which has an recommended dose treatment of 200 mg every 12 hours for adults against invasive aspergillosis (Medscape, 2017).

However, these drugs albeit effective may still cause adverse effects to certain patients ranging from hypoglycemia, electrolyte disturbance, confusion and pneumonitis (Boyd et al., 2004). Complicating matters are the rise of antifungal resistance which are fungal diseases that are able to nullify the effects of the antifungal treatment. Some variants of *Candida* are already becoming increasingly resistant to first-line and second-line antifungal medications, namely fluconazole and echinocandin (CDC, 2017a). Hence, the need for antifungal compounds derived from natural, easy to access resources that were safe to be consumed by patients is increasing.

Syzygium campanulatum (SC) is a hardy and prolific plant which can be found throughout the South-east Asian region. They can be easily differentiated by their young bright red leaves and usually planted as ornamental plants and they belonged to the same species as *Syzygium aromaticum*, the common clove spice. Previous studies had determined that SC contained antitumor and angiogenesis inhibitor compounds within the extracts of its leaves but no research has yet been done on possible antifungal action (Aisha et al., 2013).

Previous study by Yap (2017) and Lim (2016) have recently isolated terpenoids present in SC crude extract and thus, this is a great opportunity to follow up and determine any other biochemicals present in it. Many other studies however had been conducted on similar *Syzygium* species such as *Syzygium samaragense* of the wax jambu plant that contain cytotoxic chalcone

which had antifungal properties that suggests strongly that SC may have them as well (Simirgiotisa, 2008).

This preliminary evaluation was crucial as it was the first attempt to shed light on the antifungal capability of SC crude extract and to glean the presence of chalcone in SC crude extract using chromatographic methods. We also intended to discover the appropriate concentration of SC crude extract needed to exhibit inhibitory effect on the AB suspension.

The main objectives for this study were

1. Isolate polar compounds in SC crude extract through Amberlite column chromatography using three solvent systems, namely MeOH, MeOH:EA and EA.
2. Study the antifungal effect of both SC crude extract and SC fractions against the growth of AB.

CHAPTER 2

LITERATURE REVIEW

2.1 THE *Syzygium* SPECIES OF PLANTS

Syzygium is a genus of flowering plants that belongs to the family Myrtaceae. The genus comprises about 1200–1800 species, and is typically found in tropical and subtropical regions around the world. The *Syzygium* family includes *S. aromaticum* (clove spice) and *S. cumini* (jambolan). Most species are evergreen trees and shrubs. Several species are grown as ornamental plants for their attractive glossy foliage, and a few produce edible fruit that are eaten fresh or used in jams and jellies. The most economically important species is *S. aromaticum* in which its unopened flower buds are used as spice. Many studies have been conducted on their phytochemical activity including anticancer and antihypertensive properties.

S. cumini extract at 40% concentration has been proven to exhibit 14.4% (HeLa) and 11.8% (SiHa) cervical cell line growth inhibition (Barh & Viswanathan, 2008). Another study that applied *S. cumini* extract to both microbial and fungal species has shown significant bactericidal and fungicidal effects. In that case, the extract applied against various yeasts which yielded MIC values of 125 µg/mL for *Candida albicans*. Similar studies on CHO cell lines to test cyotoxicity show that concentrations approaching 400 µg/mL will show significantly high cytotoxicity (Migliato et al., 2010). This shows significant evidence that many plants in this family possess many bioactive compounds and potentially can be safe to use by humans as well.

2.2 SC: TAXONOMY AND PRIOR STUDIES

Figure 2.1. SC tree

S. campanulatum (SC), commonly referred as the "kelat paya" originates from South East Asia and is popularly used as an urban landscaping plant due to its hardiness and adaptability. SC produces attractive reddish leaf shoots all year round and flushes exceptionally after pruning as can be seen in Figure 3.1 (FRIM, 2014). It can grow naturally up to 20 meters and produce mature spherical blue or red fruits.

Prior studies done by Aisha and et al (2013) have found that the methanolic extract of the plant has valuable angiogenesis inhibitors and inhibit tumor growth in nude mice. They have also found a large assortment of bioactive compounds including betulinic acid and quercetin. Their research also suggests that SC extract may be involved in suppression of Vascular Endothelial Growth Factor (VEGF) signaling pathway which contributes to angiogenesis. Another study found out on the optimum extraction of compounds in SC using hexanol maceration and yielded 15.8% dimethylchalcone (Memon et al., 2016). However, very little data or studies have been done on other potential antifungal or antimicrobial properties of SC.

2.3 CHALCONES

Chalcones also known as 1,3-diaryl-2-propen-1-ones, belong to the chemical family of flavonoids. Chemically they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon a,b-unsaturated carbonyl system as shown in Figure 2.3. They have a molecular weight of 208.26 g/mol and have a melting point of 57.5°C (PubChem, 2017). The largest numbers of natural chalcones have been isolated from families of the *Leguminosae, Asteraceae* and *Moraceae* plants.

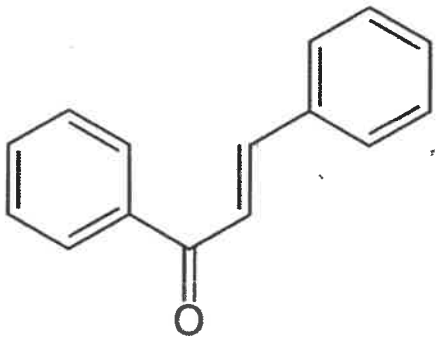

Figure 2.2. Skeletal structure of chalcone

Previous research has been done on its wide range of bioactive properties including antibacterial, antifungal and anti-inflammatory effects. The reason for their bioactive properties are due to the presence of reactive α, β-unsaturated keto group in chalcones is found to be responsible for their biological activity (Gupta & Jain, 2015). Compounds 114 (isobavachalcone) and 168 isolated from *Maclura tinctoria* showed inhibitory activity against *Candida albicans* (with IC_{50} of 3 and 15 µg/mL, respectively).

Another study has also described in detail the mode of action of chalcones against yeasts. It was revealed that DNA of yeast strains tested was not targeted by chalcone. The antifungal activity of chalcones was examined against nine laboratory employed haploid strains of *S. cerevisiae* and four artificially constructed or naturally occurring diploids. No clear relationship was observed between the ploidy level and the strain sensitivity. The yeast survival depended on the chalcone concentration and the strain cell density (Lahtchev, Batovska, Parushev, Ubiyvovk, & Sibirny, 2008).

2.4 *Aspergillus brasiliensis* (AB)

Aspergillus brasiliensis (AB) is a member of the genus *Aspergillus* and ubiquitously found throughout in nature. They produce colonies of yellow and white hyphae and turn black upon maturation and formation of conidia. They are extremely thermotolerant especially in colder climates and tend grow rapidly. The fungal species first gained prominence to its industrial potential in producing citric acid. Since then, the fungus has been exploited successfully to produce other important enzymes and compounds (Schuster, Dunn-Coleman, Frisvad & Van Dijck, 2002).

AB is generally regarded as a non-pathogenic fungus widely distributed in nature. Humans are exposed to its spores every day without disease becoming apparent. Only in few cases has AB colonize the human body as an opportunistic invader and in almost all these cases the patients have a history of severe illness or immunosuppressive treatment (Schuster et al., 2002). Rarely, AB causes symptom among healthy humans and they usually prefer forming colonies in spoiled food (Encyclopedia of Life, 2017). Humans who suffer food poisoning after consuming spoiled food colonized by *Aspergillus* species are due to the consumption of their mycotoxin Ochratoxin A (Abarca, Bragulat, Castella, & Cabanes, 1994).

Germination of a conidium of *Aspergillus* results in a network of hyphae known as mycelium or colony. The colony extends at its periphery. Its center thus represents the oldest part, while the periphery is newly formed (Wang, 2014). Current effective treatments to similar *Aspergillus* species (like the more lethal *A. fumigatus*) include voriconazole. However, adverse effects have been observed in humans including visual abnormalities, skin rashes and rarely hepatic failure (Lat & Thompson, 2011). However, common *Aspergillus* species like AB pose almost no threat to human health under normal conditions. It should be noted that aspergillosis cases are rarely reported and thus accurate statistics are hard to determine. Currently, there are an estimated 1 to 2 cases of aspergillosis occur out of a population of 100000 yearly. Allergic bronchopulmonary aspergillosis (ABPA) likely affects between 1 and 15% of cystic fibrosis patients. One study calculated that 2.5% of adults who have asthma also have ABPA, which is approximately 4.8 million people worldwide (CDC, 2017b).