INTERACTION OF COMBINED ESSENTIAL OILS FROM *SYZYGIUM AROMATICUM* AND *CINNAMOMUM VERUM* ON ORAL PATHOGENIC BACTERIA

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Specially dedicated to my beloved parents, my late father Zainol Bin Hassan and Samehah Binti Ahmad, my siblings and friends for their continuous support, prayers, encouragement and also understanding during my master programmes.

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ABSTRACT

Syzygium aromaticum (*S. aromaticum*) and *Cinnamomum verum* (*C. verum*) essential oils offer great potential against the pathogens of the oral cavity. Chemical constituents for both essential oils have been reported to have antibacterial agent. This study investigates the interaction effect of *S. aromaticum a*nd *C. verum* oils against known resistant bacteria in oral infection of *Enterococcus faecalis, Aggregatibacter actinomycetemcomitans, Streptococcus mutan* and *Streptococcus salivarius*. The analysis of oils was done using GC and GC-MS methods to quantify the chemical constituents. Most of the constituents found in *S. aromaticum* and *C.verum* oils contained terpenes and oxygenated compounds which contributed to antibacterial activity. The combination effects of *S. aromaticum* and *C. verum* were evaluated using checkerboard assays. Amoxicillin at concentration of 0.1mg/mL was used as positive control while each bacteria suspension of 10^5 CFU/ml was used as negative control. The minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FIC) were calculated to characterize the interaction between the oil combinations. Both *S. aromaticum* and *C. verum* oils possess antimicrobial activity against the four bacteria when used on its own or in combination. In combination, the MIC values were reduced for all bacteria compared when in single form. Both of these essential oils have maximum antibacterial potential at combination ratio of 1:1. At other ratio 9:1, 3:7 and 6:4 the combinations showed poor antibacterial activity against the four selected bacteria. The combinations of these essential oils within the acceptable range for synergistic of 0.02 to 0.31 mg/mL with the FIC value $\leq$0.5 have shown good antibacterial activity and directly can substitute the common use antibiotics like Amoxicillin. This finding suggests a potential therapeutic benefit using the combination of *S. aromaticum* and *C. verum* oils to overcome oral infection in future.

ABSTRAK

Minyak pati *Syzygium aromaticum* (*S. aromaticum*) dan *Cinnamomum verum* (*C. verum*) berupaya menawarkan rawatan yang baik terhadap patogen di dalam mulut. Kandungan kimia bagi kedua-dua minyak pati ini dilaporkan mempunyai ejen antibakteria. Kajian ini menyiasat kesan interaksi kombinasi antara minyak pati *S. aromaticum* dan *C. verum* terhadap bakteria berdaya tahan yang sering dijumpai di dalam jangkitan mulut seperti *Enterococcus faecalis*, *Actinomycetemcomitans Aggregatibacter*, *Streptococcus Mutan* dan *Streptococcus salivarius.* Analisis terhadap minyak pati ini telah dilakukan dengan menggunakan kaedah GC dan GC-MS untuk mengenalpasti jumlah kandungan kimia. Sebahagian besar kimia yang dijumpai di dalam minyak pati *S. aromaticum* dan *C. verum* mengandungi kompaun terpenes dan kompaun teroksigen yang menyumbang kepada aktiviti antibakteria. Kesan gabungan *S. aromatium* dan *C. verum* di uji menggunakan kaedah papan cerakin. Amoxicillin pada kepekatan 0. 1mg/mL telah digunakan sebagai kawalan positif manakala bakteria dengan kepekatan 10^5 CFU / ml telah digunakan sebagai kawalan negatif. Kepekatan perencatan minimum (MIC) dan indeks kepekatan pecahan perencatan (FIC) telah digunakan untuk mengklasifikasikan interaksi antara kombinasi. Kedua-dua minyak pati *S. aromaticum* dan *C. verum* mempunyai aktiviti antimikrob terhadap semua empat bakteria apabila digunakan secara individu ataupun dalam bentuk kombinasi. Secara kombinasi, nilai MIC untuk ke semua bakteria dapat dikurangkan apabila dibandingkan dalam bentuk individu. Kedua-dua minyak pati ini mempunyai potensi antibakteria apabila dalam kombinasi pada nisbah 1: 1, dengan itu objektif kajian ini telah dicapai. Aktiviti antibakteria pada nisbah kombinasi yang lain iaitu 9:1, 3:7 dan 6:4 telah menunjukkan aktiviti yang lemah terhadap empat bakteria tersebut. Kombinasi minyak pati dalam julat yang boleh diterima untuk sinergi iaitu 0.02-0.31 mg / mL dengan nilai FIC $\leq$0.5 telah menunjukkan aktiviti antibakteria yang baik dan seterusnya dapat menggantikan penggunaan antibiotik biasa seperti Amoxicillin. Penemuan ini menunjukkan manfaat terapeutik yang berpotensi menggunakan kombinasi minyak pati *S. aromaticum* dan *C. verum* dalam mengatasi jangkitan mulut pada masa akan datang.

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LIST OF ABBREVIATIONS

NCDs	-	Non-communicable diseases
MIC	-	Minimum inhibition concentration
FIC	-	Fractional inhibition concentration
GC	-	Gas chromatography
GC-MS	-	Gas chromatography- Mass spectrometer
MS	-	Mutans streptococci
TSBV	-	tryptic soy-serum bacitracin vancomycin
BHI	-	brain heart infusion
mg	-	milligram
CAM	-	Complementary and Alternative Medicines
NIH	-	The National Institute of Health
TCM	-	Traditional Chinese Medicine
WHO	-	World Health Organization
MBC	-	Minimum bacteria concentration
ATCC	-	America Type Culture Collection
DMSO	-	Dimenthysulfoxide
MS	-	Mitis salivarius
µm	-	micrometer
µg/mL	-	Microgram per millilitre
mg	-	milligram
mg/mL		Milligram per millilitre
CFU	-	colony forming units
CO_2	-	Carbon dioxide
OD	-	Optimal Density
FID	-	flame ionization detector
C	-	carbon

KI	-	Kovats Index
t_x		retention time of sample compound
t_m	-	mobile phase R_t (e.g. 1.376 min; first peak for solvent)
t_n	-	retention time of standard hydrocarbon containing n carbon(e.g.C8)
t_{n+1}	-	retention time of standard hydrocarbon containing n+1 carbon (e.g. C9)
n	-	lowest carbon number
n.d	-	Not detected
Amp B	-	amphotericin B
SEM	-	Scanning Electron Mircoscopes

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CHAPTER 1

INTRODUCTION

1.1 Study Background

Oral health is one of the integral element of general health. Sufficient practices and prevention are needed to be applied continuously to boost the application towards oral health. Poor dietary habits, unhealthy life style practices (poor oral hygiene, alcohol consumption), and lack of awareness on the importance of oral health and non-dental attendance are some of the factors contributed to non-communicable diseases (NCDs), including oral infection (Pau, 2012; Poul Erik Petersen, 2004). Oral diseases including dental caries and periodontal disease are major health problem in both developed and developing countries (Poul Erik Petersen, 2004). General health conditions and health care system in developed and developing countries are greatly different based on their economic development. Studies have suggested that people living in the low income countries usually have high morbidity and mortality rates because of their poor health care system. It is not surprising that there are about 90% of oral diseases are still not being untreated in these countries (Corbet *et al*., 2002).

'Tooth decay and, to a lesser extent periodontal infections are perhaps the most expensive infections that most individuals have to contend with, during a lifetime' (Walter J. Loesche, 1986). Dental caries occurs when demineralization process takes place on tooth surface (Shay, 2002), meanwhile periodontal disease (periodontitis) is caused by inflammatory process to the supporting tissues around the teeth as a result of bacteria infection and eventually leading to destruction of these tissues. In addition

to this, penetration of bacteria into the tooth root canal system can also occur and resulting in inflammation pulpal tissues in the tooth (endodontic lesion) (Scannapieco, 2013; Dahlen, 2009; Moynihan and Petersen, 2004).

Microorganisms in the oral cavity are usually found in the form of biofilm. Facultative anaerobes bacteria such as *Streptococci spp.*, is an important bacteria causing dental caries (Botha and Lall, 2013). Streptococci bacteria produces organic acids from sugars and its growth is dependent on the presence of fermentable monosaccharides (Moynihan and Petersen, 2004). Another facultative anaerobe bacteria responsible for dental caries formation is enterococci and they cause infections in the root canal (Wade, 2013b). Meanwhile, other facultative anaerobic bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* are the prime pathogens in the development of chronic periodontitis (Curtis *et al*., 2011).

Nowadays, plant-based medicines and treatments attract much attention especially in developed countries such as Europe, United States and Japan (Pandita e*t al*., 2014). These medicines have been used intensively over the past decades (F. C. Groppo *et al*., 2008). In dentistry, there are several herbal medicine such as *Azadirachta indica*, aloe vera, neem, garlic, tea tree oil, meswak, clove, *cinnamon zeylanicum*, turmeric and curry leaf tree, have been used for the treatment of oral infections (Pandita *et al*., 2014). These herbs are being used as ingredients in tooth pastes and mouth washes.

Essential oils is a natural product produced by plants containing complex mixtures of volatile compounds (Bassolé & Juliani, 2012) such as terpene, terpenoids and aromatic constituents. These volatiles compounds exhibit antimicrobial, anti-inflammatory and analgesic effects (Francisco *et al*., 2008). Natural antimicrobial agent has been used in recent years as a strategies to control the resistant food-borne bacteria and other pathogenic microorganism (Bassolé & Juliani, 2012; Yap et al., 2014). In addition, the interest in the use of natural antimicrobial agents in combinations as a strategies to control food-borne bacteria and other pathogenic microorganisms has been increased. Recent studies have shown interaction between

essential oils may lead to synergistic, additive, indifferent (non-interaction) or antagonistic effect. Synergistic effect is observed when the effect of combined substances is greater than the sum of the individual effects. An additive action occurs when the combined effect of agents is equal to the sum of the effects of individual agents, while the absence of interaction is defined as indifference. Antagonistic action is defined when the effect of one or both agents is lesser than when they are applied together compared to when individually applied (Bassolé and Juliani, 2012).

Syzygium aromaticum (clove) oil has a pleasant aroma and has many useful purposes as food flavour, drugs in pharmaceutical industry and in dentistry. The clove oil has been used for a long time by dentists because of its main active ingredients i.e. eugenol, primarily responsible either as a bacteriocidal or bacteriostatic (Bankar, Kumar, Puri, & Kanpur-, 2011; Nuñez & D'Aquino, 2012). It is used traditionally in dental care for relieving toothache, sore gums and oral ulcers beside as a dressing in dentistry for minor wounds (Aneja & Joshi, 2010).

Cinnamomum verum (cinnamon) oil has high antioxidant activity and antimicrobial properties (Jakhetia *et al*., 2010; Li *et al*., 2013; Wang *et al*., 2009). It has also been used in toothpastes, mouthwashes and chewing gums for dental caries prevention (Pandita *et al.*, 2014).

Some of the earlier studies demonstrated the antimicrobial effect when *S. aromaticum* and *C. verum* oils combined with other essential oils, but not together as combined oil include one study of antimicrobial effect the combination of *S. aromaticum* with rosemary oils on food-borne bacteria and fungi. Both oils contained components that exhibited antibacterial and antifungal activity including additive and synergistic effects on almost all strains of microorganism tested. With this results, the combination of *S. aromaticum* with rosemary oils was suggested as a useful agent in medicine and food industry (Y. Fu *et al*., 2007). Another study showed the effect of combined *C. verum* with *Lavandula angustifolis* oil as a synergistic antibacterial agents against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* (Rapper *et al*., 2013).

The antimicrobial effect of *S. aromaticum* and *C. verum* oil has been intensively being studied, but there are limited studies on the antimicrobial effect either in single or combination of these oils when used against oral pathogens. Therefore, in this study, *Syzygium aromaticum* and *Cinnamomum verum* essential oils has been selected to explore these potential activity in managing oral infection.

1.2 Problem Statement

Malaysia is one of the developing countries that provide oral health care service in both private and public sector. As compared to other developed and developing countries, prevalence of periodontal diseases in Malaysia presents a serious pattern with 20% periodontitis occurring in adults. The numbers of patients with periodontitis has increased every ten years mainly because of the low awareness on oral healthcare (Mohd-Dom *et al*., 2013).

The current treatment used by dentists to eliminate microorganisms is through prescription of oral antibiotics (e.g penicillins and cephalosporins, erythromycin, tetracycline and derivatives and metronidazole, cetylpyridinium chloride, chlorhexidine, amine fluorides). Antibiotic is one of the most common therapies used against infectious diseases and enhanced the human health (Yap *et al*., 2014). Antibiotic are generally active against Gram positive and Gram negative microorganisms. However, there have been reports that bacteria are not only resistant to a single drug but to many drugs. This multi-drug resistant bacteria problems urges the need to find a new therapies to replace antibiotics or enhance the effect antibiotics. On the other hand, antibiotics are known to exhibit undesirable effects like vomiting, diarrhoea, tooth staining and oral cancer (in case of ethanol commonly found in mouthwash) for a long-term used (Palombo, 2011a). The use of herbal medicines in this purpose would be beneficial as they have shown to produce similar of better antibacterial activity than antibiotics scientifically and has been shown to be safe when used traditionally.

With the current increasing evidences of the antibacterial properties of the *S. aromaticum* and *C. verum* oils comparable to antibiotics activities against oral pathogens, our hypothesis is the combination of *S. aromaticum* and *C. verum* oils would produce favourable synergistic activity compared to individual activities of each oils, or to antibiotics when tested against resistant facultative oral bacteria. Therefore, the research findings for this project would answer the following questions:

i- Can both herbal oils inhibit the growth of oral bacteria effectively?

ii- Is the antibacterial is better individually or in combination?

iii- Are the antibacterial activities of the oils better than the common antibiotics used in clinics?

Hence, the aim of this study was to determine the antibacterial activity of *S. aromaticum* and *C. verum* against resistant oral pathogens as individual and combined oil.

1.3 Objectives

The objective of this study was to analyse the chemical constituents in *Syzygium aromaticum* bud and *Cinnamomum verum* bark oils and to investigate the interaction effect of *Syzygium aromaticum* bud and *Cinnamomum verum* bark oils when used against *Enterococcus faecalis, Aggregatibacter actinomycetemcomitans, Streptococcus mutans,* and *Streptococcus salivarius*.

1.4 Scope of the Study

The scope of this study was within the following areas;

1. Quantify the yield of *S. aromaticum* bud and *C. verum* bark oils using hydro-distillation extraction method.
2. Determine the composition of the active ingredients of essential oil using gas chromatography (GC) and gas chromatography-mass spectrometer (GC-MS).

3. Analyze antibacterial activity and combination effect of *S. aromaticum* bud and *C. verum* bark oils using minimal inhibitory concentration (MIC) test and fractional inhibitory concentration (FIC) index method.

1.5 Significant of the Study

Dental caries and periodontal diseases are the major oral health problems and an important global infectious disease to be prevented. Discovery of new treatment therapies for these oral diseases can be a beneficial approach to reduce the problem. The use of these essential oils in clinical management can offer a better treatment than synthetic chemicals and promising approach in the prevention for oral diseases and improve the health of the general population.

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