AN *IN VITRO* STUDY ON THE ANTIMICROBIAL PROPERTY OF MALAYSIAN HONEY AND PROPOLIS EXTRACTS AGAINST MICROORGANISMS IMPLICATED IN DENTURE STOMATITIS

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

G	Gram
Mg	*Miligram*
Ml	*Mililitre*
Mm	*Milimetre*
µl	*Microliter*
%	Percentage
°C	Degree Celcius
ANOVA	Analysis of variance
C. albicans	*Candida albicans*
C. tropicalis	*Candida tropicalis*
C. glabarata	*Candida glabrata*
CLSI	Clinical & Laboratory Standards Institute
ELISA	Enzyme-linked Immunosorbent Assay
L. salivarius	*Lactobacillus salivarius*
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
S. aureus	*Staphylococcus aureus*
S. mitis	*Streptococcus mitis*
WEP	Water extraction of propolis

KAJIAN *IN VITRO* SIFAT ANTIMIKROB MADU DAN EKSTRAK PROPOLIS MALAYSIA TERHADAP MIKROORGANISMA BERKAITAN STOMATITIS GIGI PALSU

ABSTRAK

Stomatitis gigi palsu adalah penyakit kronik pada pesakit yang memakai gigi palsu, terutamanya di bawah prostetik maksila. Walaupun terdapat pelbagai agen antibakteria dan antifungus, keberkesanan rawatan adalah tidak menentu. Penyelesaian alternatif adalah untuk mencari bahan yang mempunyai ciri antibakteria dan antifungus yang bersamaan dengan tindakan antimikrob komersial topikal seperti *miconazole* dan *chlorhexidine*. Ia dapat diterima dengan baik oleh badan, tidak menyebabkan kesan sampingan atau gangguan terhadap metabolisme badan. Madu dan propolis dilihat sesuai dengan semua kriteria yang disebutkan di atas. Madu dan propolis mempunyai sifat anti-radang, antimikrobial, antioksidan dan antikanser. Tujuan kajian semasa adalah untuk menyiasat sifat fizikal dan sifat antimicrobial madu dan ekstrak propolis *Apis dorsata*, *Apis mellifera* dan *Trigona* spp terhadap mikroorganisma biasa yang terlibat dalam stomatitis gigi palsu. Sifat fizikal madu diperiksa berdasarkan kandungan warna, keasidan dan kelembapan. Keberkesanan madu dan ekstrak propolis terhadap mikroorganisma telah dinilai dengan menggunakan ujian penyebaran agar. Kepekatan berkesan madu dan ekstrak propolis ditentukan oleh Kepekatan Perencat Minimum (MIC). Dalam ujian fizikal, data dibentangkan sebagai min dan sisihan piawai bagi setiap sifat. Untuk ujian penyebaran agar, ANOVA tiga arah diikuti dengan perbandingan selepas hoc digunakan untuk analisis statistik ($p < 0.05$). Untuk penentuan

kepekatan halangan minimum, bacaan dari pembaca ELISA dan pemerhatian visual dilaporkan pada kepekatan madu dan ekstrak propolis. Hasilnya menunjukkan bahawa madu Malaysia mempamerkan keasidan yang lebih baik serta kandungan kelembapan tertinggi daripada penanda aras, madu *Apis mellifera*. Untuk hasil penyebaran agar, *Trigona* spp honey mempamerkan aktiviti antimikrobial yang baik terhadap beberapa mikroorganisma yang zon perencatan besar terhadap *Actinomyces* spp dan *Candida tropicalis* pada 25.79 ± 0.32 mm dan 12.29 ± 0.23 mm masing-masing. Untuk nilai madu MIC, *Trigona* spp. menunjukkan nilai MIC terendah terhadap *Staphylococcus aureus* dan *Actinomyces* spp. pada 62.5 mg/ml manakala terhadap semua *Candida* spp. pada 250mg/ml.Kegiatan antimikrobial ekstrak propolis dari *Trigona* spp memperlihatkan aktiviti antibakteria yang lebih baik daripada ekstrak propolis dari *Apis mellifera*terutama terhadap *S. aureus* dan *Streptococcus mitis* pada 13.58 ± 0.90 mm dan 14.05 ± 0.13 mm masing-masing. Tiada zon perencatan pada aktiviti antikulat terhadap *Candida* spp. Bagi nilai MIC ekstrak, *Trigona* spp menunjukkan MIC terendah terhadap *S. aureus* dan *S. mitis* pada 62.5 mg/ml manakala 500 mg/ml terhadap semua *Candida* spp. Manakala, nilai MBC madu dan propolis lebih tinggi dari nilai MIC. Oleh itu, dapat disimpulkan bahawa madu dan ekstrak propolis Malaysia terutamanya *Trigona* spp memperlihatkan aktiviti antimikrobial yang lebih baik daripada *Apis mellifera* terhadap mikrorganisma yang berkaitan dengan stomatitis gigi palsu seterusnya berpotensi untuk kegunaan rawatan bagi penyakit mulut.

AN *IN VITRO* STUDY ON THE ANTIMICROBIAL PROPERTY OF MALAYSIAN HONEY AND PROPOLIS EXTRACTS AGAINST MICROORGANISMS IMPLICATED IN DENTURE STOMATITIS

ABSTRACT

Denture stomatitis presents as a chronic disease commonly occurs under maxillary prosthesis. Despite the existence of a great number of antibacterial and antifungal agents, treatment failure is observed frequently. An alternative solution is to find material that has antibacterial and antifungal properties of equivalent nature to the action of miconazole and chlorhexidine. It must be well tolerated by the body, causing no side effects or interference with drug metabolism. Honey and propolis seem to fit all the criteria mentioned above. Honey and propolis have anti-inflammatory, antimicrobial, antioxidant and anticancer properties. The aim of the current study was to investigate the physical andantimicrobial properties of honey and propolis extracts of *Apis dorsata*, *Apis mellifera* and *Trigona* spp. against common microorganisms implicated in denture stomatitis. The physical properties of honey were examined based on colour, acidity and moisture content. The efficacy of honey and propolis extracts towards microorganisms was evaluated by using agar well diffusion assay. The effective concentrations of the honey and propolis extracts were determined by Minimum Inhibitory Concentration (MIC). In physical test, the data are presented as mean and standard deviation of each of the attributes. For agar well diffusion assay, three-way ANOVA followed by post-hoc comparison was used for statistical analysis ($p < 0.05$). For determination of minimum inhibitory concentration, the reading from ELISA reader and visual observation were

reported on the concentration of honey and propolis extracts. The results showed that Malaysian honey exhibited better acidity and highest moisture content than the benchmark, *Apis mellifera* honey. For result of agar well diffusion assay, *Trigona* spp. honey exhibited favourable antimicrobial activity against several microorganisms with large inhibition zones against *Actinomyces sp* and *Candida tropicalis* at 25.79±0.32 mm and 12.29±0.23 mm, respectively. For MIC value of honey, *Trigona spp* showed lowest MIC value against *Staphylococcus aureus* and *Actinomyces* spp. at 62.5mg/ml while against all *Candida* spp. at 250mg/ml. The propolis extract from *Trigona* spp.exhibited better antibacterial activity compared to propolis extract from *Apis mellifera* against *S. aureus* and Streptococcus *mitis* at 13.58±0.90 mm and 14.05±0.13 mm, respectively with no antifungal activity against *Candida* spp. For MIC value of propolis extracts, *Trigona* spp. showed lowest MIC against *S. aureus* and *S. mitis* at 62.5mg/ml while 500mg/ml against all *Candida* spp.The MBC value was higher than MIC value for honey and propolis. Hence, it can be concluded that Malaysian honey and propolis extract mainly *Trigona* spp. exhibited better antimicrobial activity than *Apis mellifera* against microorganisms implicated in denture stomatitisand have potential usage in the treatment of common oral diseases.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Honey is the sweet product of nature, viscous in consistency produced by honeybees using the nectar of flowers (Kivrak *et al.,* 2017). Propolis is the resinous substance collected by honeybees from plant buds and exudates which is employed for construction and repair of the honeycomb (Uzel *et al*., 2005). Honey and propolis are very popular supplement because of their beneficial effect on human health. It is noteworthy that honey and propolis possess therapeutic potentials such as antimicrobial, antioxidant, anti-inflammatory that boost the immune system (Tan *et al*., 2009; Vallianou *et al*., 2014). Honey and propolis extract have been used commercially for treating various ailments, wound care and oral hygiene maintenance such as in mouthwash and oral paste. Various forms of commercial product have been produced either raw or processed in either liquid, tincture or paste forms (Santos *et al*., 2008).

Apart from having sugar and water as main components, honey also contains vitamins, minerals, amino acid, proteins and micronutrients that enhance digestion, metabolism and body functions. Honey contain natural prebiotic that can enhance *Bifidobacteria* populations in the gastrointestinal tract that helps in digestion. Comparative study done by Shamala *et al*. (2000) showed that honey increased the population of lactobacteria in the intestinal of rat, both *in vivo* and *in vitro*. No such effect by sucrose was observed. Honey hassimple form of sugar molecules and can be directly absorbed and digested

into human system in contrast to refined sugar which must undergo further processes into simpler forms. In oral health perspective, honey has the potential to reduce the risk of dental caries, dental plaque, gingivitis and periodontitis due to its high level of antibacterial activity. At present, antifungal agents such as nystatin, miconazole and amphotericin B commonly used to treat denture stomatitis.However there have been side effects reported. Furthermore, amphotericin B and nystatin have an unfavourable taste and may contribute to gastrointestinal upset such as nausea, vomiting and diarrhoea. In a study done by Sajjan *et al*. (2016), 0.2% chlorhexidine gluconate mouth rinse was found to significantly reduce dental plaque. However, the number of *Candida* sppwhich is the main cause for denture induced stomatitis was found not significantly affected. Apart from that, inflammatory oral lesions are frequently encountered and most of these lesionsrequires regulartopical application to alleviate symptoms. One such condition is denture stomatitis.

Denture stomatitis is defined as an inflammatory process of the denture bearing mucosa which is characterised by erythema of the palate and the alveolar ridges (Farlex Medical Dictionary, 2009). The aetiological factors in denture stomatitis appear to be trauma due to ill-fitting denture, chronic candidal infections, microbial infections, poor denture hygiene, allergic reaction to denture materials, continuous denture wear and dietary factors which becomes initiated or aggravated by high carbohydrate intake and iron deficiency. Systemic factors that contribute to this disease includes immunodeficiency states, diabetes mellitus, anemia,chronicmultiple antibiotic drugs usage and oral

contraceptives are also known as a predisposing factor (Gendreau and Loewy, 2011; Cubera, 2013).

The microorganisms implicated in denture stomatitis lesion proved to be poly microbial (Pereira *et al*., 2013). It was reported that a combination of *Candida albicans* and other microorganisms which include staphylococci, streptococci, *Neisseria* sp. and *Actinomyces* sp. are more likely to be responsible for denture stomatitis (Kulak *et al*., 1997; Barnabe *et al*., 2004). Based on study done by Salerno *et al*. (2011), the two species of fungi most frequently isolated from oral mucosa of patients infected by denture stomatitis includes *C. albicans* and *C. glabrata*. Other *Candida* spp, such as *C. tropicalis, C. krusei* and *C.parapsilosis* were occasionally isolated. The most common type of bacteria isolated from similar cases were *Neisseria* sp.and *Staphylococcus aureus*. Gram-positive cocci including streptococci, pneumococciand staphylococciwere also predominantly isolated from the palatal mucosa of patient with denture stomatitis (Barnabe *et al*., 2004). In this study, the selection of microorganisms namely *S. aurues, S. mitis, Group B Streptococcus, L. salivarius*and *Candida* sppwere based on the previous isolation studies that reported these microorganisms in denture stomatitis lesions.

Denture stomatitis is a chronic disease that affects the denture-bearing area, especially under maxillary prosthesis. Despite the existence of a various types of antifungal agents, treatment failure is observed frequently (Parolia *et al*., 2010). The current antifungal treatments used are miconazole and fluconazole. These antifungal agents were shown to

have potential in inhibiting cytochrome P-450 enzyme system in the liver, thereby reducing the clearance of several drugs. Miconazole drug interaction significantly decreased the clearance of warfarin, subsequently raising the International Normalized Ratio (INR) andelevate the risk of bleeding (Pemberton *et al*., 2004; Minno *et al*., 2017). Treatment with topical antifungal agents such as nystatin and amphotericin B are effective in the treatment of most cases of denture stomatitis but it has been shown to produce side effects in some patients. Both antifungal agents also have unpleasant taste and oral use may lead to gastrointestinal upset such as nausea and vomiting. Furthermore, when the antifungal therapy is stopped, the condition can recur therefore prolong prescription usually necessary.However, the usage of these drugs in long-term is not favourable due to the side effects (Salerno *et al*., 2011).

Apart from the antifungal agents, the usage of chlorhexidine also proves to be clinically effective for denture stomatitis (Budtz-Jorgensen and Loe, 1972). Study done by Lal *et al*. (1992) has shown eradication of *Candida albicans* on acrylic resin denture surface and significant reduction of palatal inflammation when chlorhexidine is used. Nonetheless, after the treatment was terminated, *C. albicans* was found to recolonise the denture surface and palatal inflammation recurred. Prolong usage of chlorhexidine on oral mucosa is biologically not acceptable because it can cause discoloration of teeth and fillings and give rise to the unpleasant taste with chronic use (Flotra *et al*., 1971).

Honey and propolis extracts might prove to be the organic solution to the denture stomatitis problem. Santos *et al*. (2008) had carried out a study on clinical efficacy of a new Brazilian propolis gel formulation in patients diagnosed with denture

stomatitis.Fifteen patients prescribed with miconazole gel and other fifteen prescribed with Brazilian propolis gel. All patients were recommended to apply the product four times per day in one week. All patients using either Brazilian propolis gel or miconazole gel were reported to have complete clinical remission of palatal edema and erythema. The author concluded this new Brazilian propolis gel formulation had an efficacy comparable to oral miconazole gel. This finding suggests that propolis extract or its honey counterpart could be an alternative topical choice for the treatment of denture stomatitis. In the light of this finding, this study will investigate whether Malaysian honey and propolis extract have the same property in term of antimicrobial activity against the microorganisms in denture stomatitis.

Besides that, there is limited availability of antifungal gel for use in the mouth. One such example mentioned previously was Daktarin®, with miconazole as its main active ingredient. Alternatively, topical application of honey and propolis in gel form does shows benefit and may prove to be more tolerable, less side effects to oral mucosa tissues. It does not affect liver or kidney metabolism and widely available. According to Ajibola *et al*., (2012), honey contains enzymes such as glucose oxidase which enhance the digestion. Unlike the refined sugar, sugar molecules in honey are in pre-digested form and can be directly absorbed into the human system. As for propolis, it has shown to have a positive effect on phosphocalcic metabolism and helps maintain an appropriate level of magnesium metabolism (Haro *et al*., 2000). Propolis has potential application in oral health such as wound healing, pulp capping agent, mouth rinse, cariostatic agent and in the treatment of denture stomatitis. These are due to its flavonoids component

(Parolia *et al*., 2010). In addition, honey generates hydrogen peroxide when diluted with distilled waterbecause of the oxidation of its glucose content, which is the major contributor to the antimicrobial activity of honey, apart from its active compounds (Molan, 2012).

There are many types of honey and propolis being researched internationally. This includes Manuka honey, Brazilian propolis, other honey and propolis from different geographical origin. Both are mostly used for various types of treatments either for systemic conditions or oral health-related diseases. Manuka honey has been recommended for the treatment of ailments such as leg ulcers, pilonidal sinus disease and gastrointestinal infection (Thomas *et al*., 2011; Lin *et al*., 2010). Periodontal diseases have been shown to respond to the treatment of Brazilian green propolis (Amaral, 2006). For Malaysian honey and propolis, there were studies done previously to evaluate the antimicrobial properties of both. Tumin *et al*. (2005) investigated the antibacterial properties of Tualang honey and four other local Malaysian honeys against six bacterial species and reported that Tualang honey and the other two honeys showed antibacterial effect against *Escherichiacoli*, *Salmonella Typhi* and *Streptococcuspyogenes.* Tualang honey can be used as an alternative therapeutic agent against certain microorganisms particularly *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*, with its antibacterial activity within the same range as those of Manuka honey (Tan *et al*., 2009). The antimicrobial activity of propolis was reported higher against the Gram-positive than the Gram-negative bacteria with relatively stronger activity against *C. albicans* (Majiene *et al*., 2007).

Despite that, the application of Malaysian honey mainly Tualang and Kelulut has not yet been established for oral treatment. The present study was designed to investigate the antimicrobial properties of honey and propolis extracts against those microorganismsimplicated in denture stomatitis. Physical properties of these honey and propolis werealso recorded to help evaluate whether any difference in their physical properties could influence their antimicrobial susceptibility.

1.2 Problem Statement

At present, there is no satisfactory treatment for denture stomatitis. Most patients with denture stomatitis are elderly individuals with multiple systemic problems such as diabetes mellitus, hypertension, kidney disease and cardiac problems which contribute to the chronicity and recurrences of the lesion. It is also noted that the prevalence of oral mucosal lesions was significantly higher in diabetic patients (Saini *et al*., 2010).

The general management includes providing oral hygiene instruction pertaining to denture care, application of antifungal medication and provisioning of new dentures. Problem with current oral medications includes interference with several drugs taken by patients, too costly for long term usage for resistant cases and most are not available locally.

1.3 Justification of the Study

An alternative solution to this problem is to find material that has antibacterial and antifungal properties of equivalent nature to the action of miconazole, chlorhexidine and at the same time, do not inhibit the growth of probiotics, well tolerated by the body, do not cause side effects or interference with drug metabolism. Most importantly, the material should be widely available at an affordable price. Honey and propolis seem to fit all the criteria mentioned above plus it is proven to be good for general well-being. In addition, there is limited data on the antimicrobial properties of honey and propolis available in Malaysia that are derived from *Apis dorsata* and *Trigona* spp against oral microorganisms. As for honey and propolis of *Apis mellifera*, it will be used as a benchmark because of its extensive and well-established data on antimicrobial capabilities. In summary, the main outcomes of this studyis the determination of antimicrobial strength of Malaysian honey and propolis against microorganisms implicated in denture stomatitis.

1.4 Objectives of the Study

1.4.1 General objective

To investigate the physical properties and antimicrobial properties of honey and propolis extracts of *Apis dorsata*, *Apis mellifera* and *Trigona* spp against common microorganisms implicated in denture stomatitis.

1.4.2 Specific Objectives

1) To examine the physical properties of honey and propolis based on colour, acidity (pH) and moisture content.

2) To assess the sensitivity of honey of *Apis dorsata*, *Apis mellifera* and *Trigona* sppandpropolis extracts of *Apis mellifera* and *Trigona* spp against *Streptococcus mitis*, *Group BStreptococcus*, *Staphylococcus aureus*, *Lactobacillus salivarius*, *Actinomyces* spp, *Candida albicans*, *Candida glabrata* and *Candida tropicalis.*

3) To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of honey of *Apis dorsata*, *Apis mellifera* and *Trigona* sppandpropolis extracts of *Apis mellifera* and *Trigona* spp against *Streptococcus mitis*, *Group BStreptococcus*, *Staphylococcus aureus*, *Lactobacillus salivarius*, *Actinomyces* spp, *Candida albicans*, *Candida glabrata* and *Candida tropicalis.*

1.5 Research Hypothesis

Honey and propolis exhibit significance antimicrobial activity against microorganisms implicated in denture stomatitis.

CHAPTER 2

LITERATURE REVIEW

2.1 Denture stomatitis

2.1.1 Definition

Denture stomatitis is defined as an inflammatory process of the mucosa thatoccurs under a complete or partial removable dental appliance. It is characterized by chronic erythema and edema. It has been associated with *Candida* spp especially *C.albicans*. It is common in area covered by a denture as it is easily colonise by fungus and usually occurs in the maxilla. Several terms have been used to define this condition which includes chronic denture palatitis, stomatitis prothetica, denture-induced stomatitis, denture related candidiasis, denture sore mouth, inflammatory hyperplasia and chronic atrophic candidiasis (Dos Santos *et al*., 2009). Denture stomatitis has a multifactorial etiology and significant predisposing factors which includes trauma due to ill-fitting denture, *Candida* spp infections, prolonged use of denture, improper denture cleanliness, dietary factors and underlying systemic conditions. The main principle causative agent of denture stomatitis is the *Candida* spp, especially *Candida albicans*, although the denture plaque bacteria and predisposing factors may also be involved (Salerno *et al*., 2011). Diagnosis is usually established after positive observation of inflammation on the palatal mucosa and the presence of *C. albicans* on the denture or underlying mucosa following oral culture swab (Barbeau *et al*., 2003). Generally, denture stomatitis can be effectively managed by strict denture hygiene measures and the use of antifungal agents. Nocturnal

wearing of dentures should be discontinued and overnight soaking of dentures in an antiseptic solution should be encouraged.

2.1.2 Prevalence

Most patients with denture stomatitis are elderly individuals with multiple systemic problems such as diabetes mellitus, hypertension, kidney disease and cardiac problems. As stated by Sadeq *et al*., (2013), the prevalence of denture stomatitis in patient associated with type 2 diabetes attending Hospital Universiti Sains Malaysia is higher than control subjects. Study done by Jainkittivong *et al*., (2010), the prevalence of denture-related mucosal lesion was found to be 45% in denture wearers and the most common is denture-induced stomatitis (18.1%). It affects one in every three complete denture wearers (Emami *et al*., 2014). The frequency of its development is 25–67%, frequently seen among female patientsand prevalence increases with age (Naik & Pai, 2011).

2.1.3 Classification

Denture stomatitis is classified based on Newton (1962) who divided it into three groups. A diagnosis of denture stomatitis was made based on the changes in the inflammatory findings beneath removable denture which are categorized into three types: Newton's type I is the pin-point hyperemic lesions which localized as simple inflammation. Newton's type II is the diffuse hyperemia mucosa including all the entire denture-bearing area, with a clear surface and Newton's type III is hyperemic mucosa with a papillary surface appearance which usually localized the central of the hard palate

(Newton, 1962). However, Budtz–Jorgensen & Bertram (1970) has modified the terminology of denture stomatitis as: (I) simple localized inflammation (involving a limited area), (II) simple diffuse inflammation (involving the whole area covered by the denture) and (III) granular inflammation (often localized to the central part of the hard palate).Bergendal and Isacson (1983) proposed another classification of denture stomatitiswhichfollowed Ostlund's classification.

Local inflammation to describe red spots usually found around the small palatal minor salivary glands; was thought to be associated with trauma from the dentures. Diffuse reddening was referred as diffuse hyperaemic, smooth and atrophic mucosa extending over the entire denture area and was associated with increased growth of yeasts. The third type of denture stomatitis was described as granulated and was characterized by hyperaemic mucosa with a nodular appearance in the central part of the palate. Both trauma and candida infections have been linked with this lesion (Salerno *et al*., 2011).

2.1.4 Symptoms

For a lesion categorized as denture stomatitis, there must be an inflammatory change under the denture. If the patients report discomfort with absence of inflammation, it will not be categorized as denture stomatitis. In most patients, the symptoms seem to show redness of the tissue, mucosal bleeding, burning or painful sensation, halitosis and unpleasant taste and dryness in the mouth (Gendreau and Loewy *et al*., 2011).

2.1.5 Aetiology

The etiology factors causing denture stomatitis is of multifactorial in nature. These factors include traumatic occlusion, poor oral and denture hygiene, microbial factors, dietary factors, candidal infections and predisposing systemic conditions.

2.1.5(a) Trauma

Trauma being one of the major independent causes in denture stomatitis. The trauma may occur due to the ill-fitting dentures, continuously worn dentures or dentures that do not have correct vertical and horizontal arch relations. According to Nyquist (1953), trauma caused by dentures accounted for most cases of denture stomatitis. While, Cawson (1965) concluded that the trauma and candidal infection are significant causes of denture stomatitis. The latest study pointed out that trauma alone does not induce pictures of generalized denture stomatitis, but it could be the cause of localized forms. In the generalized forms, the principal pathogenic role is played by *C.albicans.* In this case, trauma act as a co-factor that favours the adhesion and the penetration of the yeast, sustains phlogosis of the palate and increases the permeability of the epithelium to toxins and soluble agents produced by *Candida* yeast (Emami *et al*., 2008).

2.1.5(b) Candidal infection

Recolonisation of the *Candida* spp is essential for the development denture stomatitis. Mechanism related to candidal infections causing denture stomatitis mainly involves an inflammatory response following release of yeast antigens, toxins and irritants from the denture plaque. Smoking was also associated with denture stomatitis as effects of

tobacco increases the susceptibility to oral candidal infections. This resulted in combination of factors contributing to the development of denture stomatitis (Coco *et al*., 2008).

2.1.5(c) Microbial infection

Although *Candida* species mainly *C.albicans* are the predominant pathogen in denture stomatitis, other equally pathogenic microorganisms may be present. Bacteria load also essential for the development of denture stomatitis. According to Lakshmi Prabha (2015), the predominant bacterial species present among ten patients was *Alpha-haemolytic streptococcus* and *Coagulase negative staphylococcus*. Small number of patient had enterococcaland rarely micrococcal present. The main cause for the existence of microorganisms was the continuous irritation caused by the denture.

2.1.6 Management of denture stomatitis

The management of denture stomatitis includes adequate history taking, thorough examination of the mouth especially the soft and hard palate, dorsum of tongue and buccal mucosaeof denture wearers. Without this examination, subsequent treatment using antifungal therapy will only result in the temporary relief of the infection. The right diagnosis is usually made based on the finding of the characteristic lesion, ruling out other possibilities and also based on the response to antifungal treatment (Akpan & Morgan, 2002; Sherman et al., 2002; McCullough & Savage, 2005; Williams et al., 2011). Nutritional deficiency states such as vitamin B12, iron and folate, diabetes

mellitus and immunodeficiencies must be excluded as well (Farah et al., 2010). Any pharmacologic agents used by patients that may contribute to denture stomatitis should be identified and substituted for an alternative drug. For example, use of corticosteroid inhalers for asthma by asthmatics should include advice on rinsing the mouth with water after each use. Additionally, antifungal therapy is highly recommended in patients who have chronic and severe underlying disease such as HIV infection or had undergone immunosuppressive therapy following bone marrow or organ transplant (Gumru & Ozbayrak, 2010).

Antifungal drug choice is determined by several factors including the medical history of the patient, severity of infection, oral symptoms and feasibility of its delivery to the affected areas (Farah *et al*., 2010). There are two types of antifungal agents used totreat oral mucosa candidal infection; systemic antifungals and topical antifungals. Topical antifungals agents available in many forms like creams, oral suspensions, pastilles, troches and ointments. It is the basic treatment in mild and localized cases of candidiasis (Najla *et al*., 2012). On the other hand, systemic antifungals have been recommended for treatmentof patients with poor compliance, special needs and for immunocompromised patients whose immune response had been overwhelmed by systemic candidiasis (Grant, 2001).

For topical antifungal therapy, nystatin has been recommended to treat denture stomatitis. It is available in oral suspension (100000 IU/ml) as a mouthrinse and pastilles (100000 IU) used four times a day for the minimum duration of 2 minutes then

swallowed (Farah *et al*., 2010). After using nystatin, patients should avoid eating or drinking for 20 minutes. In addition, for the topically medication to work effectively,it must be in contact with the tissue, therefore any intraoral appliances should be removed (Salerno *et al*., 2011). Nevertheless, a study done by McCullough and Savage (2005) concluded that nystatin is ineffective for candidal lesions in cancer patients. This is due the fact that oral suspension of nystatin contains abundant sucrose, which helps the *Candida* spp to thrive in the oral environment.It is also contraindicated in the treatment of oral candidiasis in patients with diabetes mellitus (Akpan and Morgan, 2002).

A wide variety of mouthwashes, including chlorhexidine gluconate, trichlosan and essential oils, exhibits anticandidal activity (Webb *et al*., 2005). Studies have shown that 0.2% chlorhexidine gluconate mouth rinses present clinical benefit in the treatment of oral candidiasis. However, there are reports of reduced efficacy of nystatin when used in combination with chlorhexidine gluconate, and therefore it is often advised to delay nystatin treatment for 30 min after the use of chlorhexidine mouthwash (Dagistan *et al*., 2008; Webb *et al*., 2005). Failure to respond clinically to the topical therapy might be an initial sign of underlying immunosuppression that might arise from undiagnosed systemic diseases. If this case, the use of systemic antifungal agents may be warranted.

2.2 Honey

2.2.1 History

Honey is the sweet, viscous, yellow to brown coloured fluid produced by honeybees using the nectar of flowers. Different types of honey are produced depending on the different variety of flowers. In general, the composition of honey contains approximately 79.7% carbohydrate mainly from fructose, glucose and sucrose. Water, protein, ash, enzymes, free amino acids, minerals, vitamins and antioxidants make up the remainder (Ahuja, 2010).

Honey is a natural product that has been used since ancient time and now it has been recently introduced in modern medical practice. It has been used by ancient Romans, Greeks, Chinese, Assyrians and Egyptians to treat wounds, ulcers and diseases of the gut (Stomfay-Stitz, 1960). In Islam, the benefits of honey that can heal a variety of medical problems were stated in the Quran about 1400 years ago in surah Al-Nahl. Besides, the Quran also promotes honey as a nutritious and healthy food. According to hadith, the application of honey in the treatment of diarrhea was recommended by the Prophet Muhammad (Purbafrani *et al*., 2014). Honey was proven to have antimicrobial, anti-inflammatory and antifungal effects (Mandal and Mandal, 2011; Israili, 2014).

2.2.2 The antimicrobial components of honey

2.2.2(a) High Sugar Content

As the main composition, sugar contributes to the osmolarity of the honey. It was assumed that the high sugar content of honey was responsible to prevent the growth of

most bacteria as it makes water become unavailable for microorganisms. However, the high-water content of honey does impair its longevity and also contributes to the growth of yeast colony. Nevertheless, no fermentation occurs if the water content is below 17.1% (Olaitan *et al.,* 2007). The water content of honey is usually within the range of 15-21% (Malika *et al*., 2005). According to Molan (2012), bacteria are less tolerant to the high levels of sugar than fungi because the cell wall of bacteria is thinner than fungi. The osmolarity of honey diluted to about 10% honey will not allow many species of bacteria to survive except for *S. aureus* which can survive at concentration of up to 30% (Molan, 2009). This is due to its hygroscopic property, based on the high osmotic pressure where it draws water out of bacterial cells and causes them to die. In general, honey and other saturated sugar syrups have an osmolarity sufficient to inhibit microbial growth (Altalibi, 2012). However, fungi can survive in sugary environment as yeasts require organic compounds such as sugar derivatives to obtain energy and therefore its concentration in the culture medium or in the environment will affect the rate of its growth (Arroyo-Lopez *et al.*, 2009). Therefore, fungi are more resistant than bacteria strains in sugary environment.

2.2.2(b) Acidity

Another antimicrobial component of honey is its acidity. Honey is characteristically quite acidic, its pH range between 3.2 and 4.5 which are low enough to inhibit bacterial growth such as *S. aureus* and *Streptococcus*spp (Altalibi, 2012). The acidity of honey is formed by the action of enzyme glucose oxidase that oxidize glucose to gluconic acid which bees add to the nectar they collected to make honey (Malika *et al*., 2005).

However, it is shown that when honey is mixed with saliva, neutralisation occurs as the concentration acid in honey is low. This implies that the acidity of honey makes a minor contribution to antibacterial activitywhen honey gets diluted by body fluid (Molan, 2012).

2.2.2(c) Hydrogen peroxide

Most types of honey generate hydrogen peroxide which is the major antimicrobial factor produced enzymatically in the honey (Ahuja, 2010). Honey generates hydrogen peroxide when diluted because of the activation of the enzyme glucose oxidase which oxidizes glucose to gluconic acid and hydrogen peroxide. Oxygen needs to be available for the reaction:

β-D-glucose + oxygen→ δ-gluconolactone + hydrogen peroxide

(Malika *et al*., 2005; Molan, 2012)

The level of hydrogen peroxide produced in diluted honey varies based on the types of honey.According to Bang *et al* (2003), common level of hydrogen peroxide production in diluted honey have been reported to be within the range of 0–0.6mmol/l/h.

The association of high antibacterial activity with floral sources suggests that the non-peroxide antibacterial activity is of floral origin. Non-peroxide antibacterial activity in *Apis mellifera* honey is derived from flower of *Leptospermum* species (Henriques *et al*., 2005; Cabrera *et al*., 2006). The compound primarily responsible for non-peroxide activity in New Zealand manuka honey has recently been identified as methylglyoxal

(Adams *et al*., 2008; Mavric *et al*., 2008). Methylglyoxal derived from dihydroxyacetone, a compound present in high levels in manuka nectar (Adams *et al*., 2009). In a clinical application where honey is used as a topical antimicrobial and wound dressing, non-peroxide activity may be advantageous as it is not destroyed by catalase present in body fluids, and is unaffected by gamma irradiation (Irish *et al*., 2011).

2.2.3 Types of honey

2.2.3(a) *Apis dorsata* (Tualang honey)

Tualang honey is produced by the rock bee (*Apis dorsata*), which builds hives high up in the branches of Tualang tree (*Kompassia excelsa*). It is commonly known as 'Mengaris'is mainly found in the tropical rain forests of the north eastern region of Kedah. It can reach up to about 250 feet with honeycombs can be up to 6 feet across that can contain as many as 30 000 bees. More than 100 nests may be found on one Tualang tree that yields some 450 kg of honey (Tan *et al*., 2009; Mohamed *et al*., 2010; Fauzi *et al*., 2011; Ahmed and Othman, 2013).

Tualang honey appears to be dark brown in colour. It has pH of 3.55-4.00 with a specific gravity of 1.34. It is more acidic in comparison to other Malaysians honey such as Kelulut Hitam, Kelulut Putih and Gelam (Ghazali, 2009). The concentration of 5-(hydroxymethyl) furfural (HMF) in Tualang honey is greater than in other Malaysian honey (Khalil *et al*., 2010). These properties contribute to the antibacterial activity of Tualang honey on *S. aureus, S. epidermidis, E. coli, E. faecium, E. faecalis, S. enteric, S. Typhimurium* and *K. pneumonia* as reported by Ng *et al*. (2014). In addition, Tualang

honey also showed antifungal effect on *C. albicans* and *C. neoformans* (Shehu *et al*., 2016) who reported the total phenolic acids and flavonoids in Tualang honey as 275.6 mg/kg and 71.8 mg/kg, respectively.

2.1.3(b) *Apis mellifera* (Manuka honey)

Manuka honey is derived from the nectar of manuka shrub *Leptospermum scoparium* that is native to New Zealand and Australia. Its antimicrobial activity has been attributed to a property referred to as Unique Manuka Factor (UMF) that is absent in other types of honey (Hammond and Donkor, 2013). Some years ago, UMF was introduced for marketing purpose and based on microbiological assays, UMF was described as having antibacterial activity equivalent to 10% solution of phenol (Allen *et al*., 1991).

In comparison to Tualang honey, the colour of Manuka honey varies from light to dark brown depending on the species of bees. The pH is 3.2-4.21 with a specific gravity of 1.39. The moisture content is 18.7%, lower than Tualang honey which was reported at 23.30 % (Ahmed and Othman, 2013). Other than hydrogen peroxide, studies have shown that the active ingredient in Manuka honey is methylglyoxal (Atrott *et al*.,2012, Mavric *et al*., 2008),a compound known to have synergistic effect with some antibiotics such as piperacillin (Mukherjee *et al*., 2011).Among other therapeutic properties of Manuka honey includes its activity against a wide range of pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Hemolytic streptococci*, *Actinomyces viscous*, *Streptococcus mutan* and *Candidaalbicans* (Taormina *et al*., 2001; Sherlock *et al*., 2010; Ahmed and Othman, 2013; Siti Aisyah Sayadi *et al*., 2015). Manuka honey

has been recommended for the treatment of ailments such as gastrointestinal infection, leg ulcers and pilonidal sinus disease (Thomas *et al*., 2011, Lin *et al*., 2010).

2.2.3(c) *Trigona* spp (Kelulut honey)

Kelulut honey is produced by the stingless bee (*Trigona* spp.) commonly known as '*Kelulut'* in Malaysia. Both *Apis* and *Trigona* spp were classified under the family *Apidae* and subfamily *Apinae* which have tribe *Bombini* (Bombal, Euglosa and Psithyus), tribe *Apini (Apis)* and tribe *Meliponini* (*Melipona, Scaptotrigona* and *Trigona*). Nowadays, Kelulut honey is widely used and is believed to have better performance in preventing microbial infections compared to honey of honeybees (Shahjahan *et al*., 2007). Stingless bee commonly builds their hives at the root of tree or tree stump on the grounds.

Based on colour, Kelulut honey is lighter, clearer, less viscous than others and has a distinct sweet-sour taste. The quantity of honey produced is usually less than other honeys because stingless bee is smaller in sizecompared to common honeybee. Among the active ingredients found in Kelulut honey are phenolic acid, cinnamic acid and stillbene. With regards to phenolic compounds seven types of free phenolic substances have been identified in stingless honey. The presence of the various types of phenolic acids in Kelulut honey may indicate that it has various properties (Yaacob *et al*., 2017).

In Malaysia, current study has revealed five species of stingless bees and one unidentified *meliponia* species. The five species included *Trigona itama, Trigona*

thoracica, Trigona terminate, Trigona laeviceps, Hypotrigona Scintillans and the one with unidentified species at a bee farm located in the state of Kelantan, Malaysia as stated in Table 2.1(Kelly *et al*., 2014).

Table 2.1 Diversity of stingless bees in the farm

Species	**Colonies (n) (%)**
Trigona (Geniotrigona) thoracica	18 (11.2)
Trigona (Heterotrigona) itama	134 (83.2)
Trigona (Lepidotrigona) terminate	4 (2.5)
Hyporigona (Lisotrigona) scintillans	1 (0.6)
Trigona (Tetragonula) laeviceps	3 (1.9)
Unidentified	1 (0.6)
Total	161 (100)

2.3 Propolis

2.3.1 History

Propolis is well known as a healing agent and is used as such since the time of Egyptian and Greek civilizations. Propolis or Russian Penicillin is derived from the Greek word "*pro*" meaning before and "*polis*" implies city or defender of the city. The action against microorganisms is an essential characteristic of propolis.It has been used in oral application as anti-inflammatory, antimicrobial, anti-cancer and antioxidant agents (Miguel and Antures, 2011; Santos, 2012; Vijay, 2013).Propolis is lipophilic in nature, and it is a hard and brittle material. When heated, it becomes gummy, soft, pliable and very sticky. It has pleasant, aromatic aroma with colour thatrange from yellow green to red and dark brown, based on its source and storage age (Alfahdawi, 2017).

Propolis (bee glue) is a sticky dark-colored sap that honeybees collect from living plants, mixed with wax and used in the construction and adaptation of their nests (Bankova *et al.* 2000). Bees use propolis not only as a building material, but also as a means of maintaining low levels of bacterial and fungal concentrations in the hive. Propolis possesses antibacterial, antifungal, antiviral properties, and many other beneficial biological activities such as anti-inflammatory, antiulcer, local anesthetic, hepatic-protective, antitumor, and immune-stimulating (Vijay, 2013). For this reason, propolis is widely used as a popular remedy in folk medicine, in apitherapy, as a constituent of 'biocosmetics', 'health food' and for numerous purposes (Bankova *et al.* 2000).