EFFECTS OF *Trigona apicalis* PROPOLIS ETHANOLIC EXTRACT ON ANTIOXIDANT ACTIVITIES AND WOUND HEALING USING HUMAN GINGIVAL FIBROBLAST CELLS

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by

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TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHOD

CHAPTER 4: RESULTS

CHAPTER 5: DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
EEP	Extract ethanolic propolis
FRAP	Ferric ion reducing antioxidant power
PDL	Periodontal ligament
HGF	Human gingival fibroblast cell
HPLC	High-performance liquid chromatography
DMEM	Dulbecco’s Modified Eagle Medium
PBS	Phosphate buffer saline
FBS	Fetal bovine serum
FBM	Fibroblast basal medium
rhFGF	Human fibroblast growth factor
TPC	Total phenolic content
TFC	Total flavonoid content
TEAC	Trolox equivalent antioxidant capacity

LIST OF SYMBOLS

°C	degree celcius
μm	micrometer
nm	nanometer
μg	microgram
g	gram
v/v	volume per volume
w/v	weight per volume

KAJIAN KESAN ETANOLIK PROPOLIS *Trigona apicalis* KE ATAS AKTIVITI ANTIOKSIDAN DAN PROSES PENYEMBUHAN LUKA DENGAN MENGGUNAKAN SEL FIBROBLAS GINGIVA MANUSIA

ABSTRAK

Propolis adalah bahan resin yang terdapat di dalam sarang lebah. Ia memberikan kesan menguntungkan kepada kesihatan manusia dan telah digunakan untuk merawat banyak penyakit sejak zaman dahulu. Objektif kajian ini adalah untuk menganalisis profil kimia ekstrak propolis dan mengira sebatian kimia utama ekstrak propolis dengan menggunakan jumlah kandungan fenolik dan jumlah kandungan flavonoid, untuk menentukan aktiviti antioksidan ekstrak propolis dengan menggunakan ujian DPPH dan ABTS kolorimetri, dan untuk menilai sifat penyembuhan luka ekstrak propolis dengan menggunakan ujian calar. Ekstrak etanol propolis tertakluk kepada analisis HPLC untuk menganalisis profil fitokimianya. Ekstrak propolis kemudiannya diuji untuk keupayaan antioksidan dengan menggunakan ujian DPPH radikal dan ABTS dan ujian FRAP. TPC dan TFC dilakukan untuk menentukan korelasi dengan aktiviti antioksidannya di mana ianya menunjukkan korelasi yang kuat iaitu $r^2 > 0.9$. Ujian calar kemudian diuji untuk sifat penyembuhan luka dalam garisan sel fibroblast gingiva berdasarkan dos bergantung kepada dan bergantung masa. Hasilnya menunjukkan bahawa aktiviti antioksidan ekstrak propolis bergantung kepada dos. IC_{50} untuk ujian DPPH dan ABTS masing-masing adalah 1.90 mg / ml dan 0.57 mg / ml. Nilai korelasi TPC dan TFC terhadap DPPH, ABTS dan FRAP menunjukkan bahawa aktiviti antioksidan ekstrak propolis yang digunakan dalam kajian ini boleh dipengaruhi terutamanya oleh kandungan fenolik dan flavonoid. Kesan ekstrak propolis pada pemulihan penyembuhan luka

dipengaruhi oleh dos (5, 10, 15, 20, 25 dan 30 μg / mL) dan masa (0, 6, 12, 24 dan 48 jam) seperti pada 6 jam selepas rawatan dengan ekstrak propolis berbanding dengan kawalan, yang hanya bermula pada 12 jam. Secara keseluruhan, kajian ini menunjukkan bahawa ekstrak propolis *T. apicalis* mempunyai potensi untuk dikembangkan menjadi agen antioksidan, serta sesuai untuk penyembuhan luka permohonan topikal, contohnya dalam prosedur cabutan gigi.

EFFECTS OF *Trigona apicalis* PROPOLIS ETHANOLIC EXTRACT ON ANTIOXIDANT ACTIVITIES AND WOUND HEALING USING HUMAN GINGIVAL FIBROBLAST CELLS

ABSTRACT

Propolis is a resinous substance found in beehives. It provides beneficial effects on human health and has been used to treat many diseases since ancient times. The objectives of this study were to analyse the chemical profile of the propolis extract and to quantify the major chemical compounds of the propolis extract using total phenolic and total flavonoid content methods, to determine the antioxidant activities of the propolis extract using DPPH and ABTS colourimetric assays, and to assess the wound healing properties of propolis extract using scratch assay. The ethanolic extract of propolis was subjected to HPLC analysis to analyse its phytochemical profile. The propolis extract was later tested for antioxidant capacities by using DPPH and ABTS radical scavenging assay and FRAP assay. TPC and TFC were performed to determine the correlation with its antioxidant activities which show the strong correlation, $r^2 > 0.9$. Scratch assay was later tested for wound healing properties in the gingival fibroblast cell lines based on dose dependent and time dependent manner. The results demonstrated that the antioxidant activities of propolis extract were dose dependent. The IC_{50} for DPPH and ABTS assay were 1.90 mg/ml and 0.57 mg/ml, respectively. Correlation values of TPC and TFC against DPPH, ABTS and FRAP indicated that the antioxidant activities of propolis extract used in this study could be mainly influenced by the phenolic and flavonoid content. The effects of propolis extract on

wound healing initiation were influenced by dose (5, 10, 15, 20, 25 and 30 μg/mL) and time (0, 6, 12, 24 and 48 hour). The wound healing initiation started as early as at 6 hour of post-treatment with propolis extract compared to the control, which only started at 12 hour. Overall, this study has showed that propolis extract of *T. apicalis* has a potential to be developed into an antioxidant agent, as well as suitable for wound healing topical application, for example, in dental extraction procedure.

CHAPTER 1

INTRODUCTION

1.1 Research background

Caries and periodontal disease are major oral diseases caused by biofilms (Costerton et al., 1999), which can cause loss of alveolar bone in periodontal disease accompanied by inflammation of the gingiva. In chronic periodontal disease, *Porphyromonas gingivalis* (*P. gingivalis*) is periodontopathic bacteria isolated from the periodontal pockets of patients (Holt et al., 1988). Periodontopathic bacteria produce virulence factors that would result in the production of pro-inflammatory cytokines that destroyed periodontal tissue (Tamura et al., 1993). The most abundant cells in periodontal tissue are human gingival fibroblasts are and has an important role in host defense against pathogens in the oral cavity (Ara et al., 2009).

Oral mucosal lesions in the mouth are injuries caused by various disorders (Gonsalves et al., 2007), where the wound is not only a physical barrier but can cause infections and sepsis due to the invasion of microorganisms or contaminants. Wound healing is common and important in surgeries. Although wound healing is improved with sound surgical principle, but it still can be resolved by biological processes which is beyond the control of the surgeon (Markiewicz et al., 2007). Typically, oral mucosal healing occurred within 5 to 7 days (Gordon, 1987), by means of a rapid and robust inflammatory response, with the recruitment of neutrophils, macrophages and lymphocytes to the wound site, followed by fibroplasia, reorganisation and extra cellular matrix (ECM) synthesis (Enoch and Stephens, 2009).

In the case of dental avulsion, an immediate replantation of the tooth into the socket causes the alveolar part of the periodontal ligament (PDL) to be separated from the cementum by a blood clot (Andreasen, 1980). Blood clots can serve as granulation tissue that regenerate in the ideal condition (Pohl et al., 2005).

Propolis has been known to have antimicrobial, antivirus, anti-fungus, local anaesthetic, antiulcers, immunostimulating, hypotensive and cytostatic properties (Ikeno et al., 1991), and been used for treating the root surface of teeth for replantation (Gulinelli et al., 2008).

Stingless bees are divided into five different genera which are *Trigona, Melipona*, *Dectylurina* , *Meliponula*, and *Lestrimelitta* (Heard, 1999), where all these genera produce propolis, a mixture of bees wax, pollen and resin of plants that are used for sealing their nests. *Trigona* is the largest genus in the Neotropics, from Mexico to Argentina and Australia in the region of India Indo-Sri Lanka to Taiwan, the Solomon Islands, East Indonesia, New Guinea and Australia (Michener, 2000). Propolis from *Trigona* spp. is popular in the Western Maharashtra region of India to treat various diseases, but there is no scientific research on the chemical composition or bioactive compounds, and it has been used based on empirical knowledge only (Choudhari et al., 2012).

Stingless bee is a large group of eusocial insects that pollinate plant in tropical regions (da Cunha et al., 2013). Stingless bee propolis in Thailand and India is more popular for treatment of diseases such as acne, diabetes, and inflammatory conditions (Choudhari et al., 2012). Besides, the stingless bee propolis from Thailand

(*Lepidotrigona terminata Smith, Lepidotrigona ventralis Smith* and *Tetragonula pagdeni Schwarz* (Apidae)) are grown in artificial nests in fruit gardens (Vongsak et al., 2015). Stingless bee propolis is more popular as it produces propolis in higher quantity than other bees, where their propolis and honey are different from the one produced by honey bees. Propolis that is produced from stingless bees is more potent than the one produced by honey bee (Ibrahim et al., 2016(a)).

Propolis is also called bee glue, produced by worker bees from collected plant resins or secretion from phloem-feeding insects that is used for assembling, protecting or repairing the beehive (Marcucci et al., 2001). Raw propolis need to be extracted because it contains impurities such as wood, wax, pollen and even dead bees. Among the solvents that are used to extract the propolis are methanol and ethanol. Ethanol is more commonly used compared to other solvent because 70% ethanol was found to remove most of the active components of propolis (Bankova et al., 1992).

Flavonoids and cinnamic acid derivatives are phenolic compounds found in propolis (Marcucci, 1995). Additionally, a report stated that the properties of the propolis by using different chemical assays such as scavenging of DPPH radical (Izuta et al., 2009) and superoxide anion (Russo et al., 2004), are varied due to differences in the chemical composition of the propolis which depends on its origin (Bankova et al., 1989).

Natural antioxidants can be phenolic compounds (phenolic acids, flavonoids and tocopherols), nitrogen compounds (amino acids, derivatives of chlorophyll, alkaloids, and amines), or carotenoids and ascorbic acid compounds (Halliwell, 2011).

Chemical compounds of propolis depends on the resin from the plants in the bees foraging area because bees prefer targeting certain plants in their beehives as a source of propolis (Falcão et al., 2013).

Propolis and its extracts has been used for prevention and treatment of a variety of diseases due to its antiviral, antibacterial, antioxidant, antifungal, anaesthetic, cytostatic, immune strengthening, anti-inflammatory, and hepatoprotective effect (de Castro et al., 2012). Ethanolic extract of propolis is effective against a variety of bacteria, mainly against gram-positive bacteria (Jorge et al., 2008).

Propolis has more than 150 constituents and is rich in biochemical constituents, including a mixture of polyphenols, flavonoids aglycones, phenolic, and ketones (Marcucci, 1995). The most widely studied components were flavonoids and phenolic compounds and reported as type of antioxidants that has strong inhibitory effect against lipid oxidation by radical-scavenging. In addition, the contents of flavonoid and other phenolic substances may also prevent the development of cancer and heart disease (Bankova et al., 2000).

In Malaysia, research on propolis is limited and only a few preliminary investigations on the composition and biological activity of propolis derived from Malaysian stingless bees are currently available (Ibrahim et al., 2016(a)). Although studies have been carried out on the properties of propolis, only slight evidence of its effectiveness was seen in the mechanism of fibroplasia in the ulcerated lesion (Benderli and Deniz, 2011). Therefore, this study was aimed to compare the normal

oral wound healing process and the healing process with addition of propolis extract derived from *Trigona apicalis* stingless bee, on the gingival fibroblast cells.

1.2 Objectives

1.2.1 General objectives

This study was aimed to evaluate the effects of *Trigona apicalis* propolis on wound healing by using human gingival fibroblast (HGF) cells.

1.2.2 Specific objectives

1. To analyse the chemical profile of the propolis extract of *Trigona apicalis.*
2. To quantify the major chemical compounds of the propolis extract using total phenolic and total flavonoid content methods.
3. To determine the antioxidant activities of the propolis extract using DPPH and ABTS colourimetric assays.
4. To assess the effects of the propolis extract on wound healing in gingival fibroblast cell lines using scratch assay.

1.3 Significance of study

The results from this study would be used to formulate topical propolis for use in dentistry, specifically for wound healing after tooth extraction or surgery. The benefit of having a quicker wound healing would help to speed up the stages of restorative or other dental procedures. This would benefit the patients as treatment can be completed in a shorter time frame.

1.4 Hypothesis

1. Propolis extracts derived from *Trigona apicalis* stingless bees has a unique chemical profile.
2. Propolis has potent antioxidant activities against DPPH and ABTS assays.
3. Local propolis extract is not toxic to HGFC.
4. Propolis may speed up the creation of granulation tissue and epithelialisation compared to normal wound healing.

CHAPTER 2

LITERATURE REVIEW

2.1 Propolis

Propolis is a non-toxic and wax-cum-resin substance that is produced by bees. Bees use it to protect and reinforce their hives, repair their structure and cover honeycombs. Besides, it kills pathogens, protects against rain and prevents unwanted guests from entering the hive (Wilson-Rich et al., 2009). Raw propolis requires an important process which is extraction because it contains impurities such as wood, wax, pollen and even dead bees.

Propolis is a balsamic resinous material of viscous consistency and different colours falsified by the bee *Apis mellifera* L. (Sonmez et al., 2005). The colour of propolis depends on the botanical source and age, which are yellowish green to dark brown (Ghisalberti, 1979). The chemicals that make up the propolis have a biological effect, such as flavonoids have antioxidant and antimicrobial properties; coumaric acid, lignans and diterpene has anti-bacterial and cytotoxic, and caffeic ester and their derivatives are cytotoxic to tumour cells (Song et al., 2008). Propolis contains phenolic compounds including flavonoids and cinnamic acid (Marcucci, 1995). Propolis has also been marketed in various forms such as tablets, toothpaste, capsules, face creams, medicine preparations, ointments, lotions, and solutions (Kartal et al., 2003).

Composition of propolis are resins (40–55%), bee wax and fatty acids (20–35%), aromatic oils (about 10%), pollen (about 5%) and other components like minerals and vitamins. However, their presence and percentage of content in propolis changes and depends on their origin, the type of plant pollen and the species of bees that produced it (Khalil, 2006). Propolis contains different components that are present in many samples from different places which the components are present from specific plants (Ghisalberti, 1979).

Propolis is soft, pliable and sticky at 25°C to 45°C, and becomes hard and brittle at freezing conditions. It becomes increasingly sticky at temperatures above 45°C. Propolis will melt at temperatures of 60°C to 70°C, and can achieve 100°C melting point for some sample (Wagh, 2013). Propolis cannot be used directly because it has a complex structure and should be extracted with a suitable solvent. The common solvents that can be used are ethanol, methanol, water, chloroform, dichloromethane, ether, and acetone to remove an inert material and maintain the desired material (Kumar et al., 2008). The composition of propolis depends on the geographical area and the extraction method (Marcucci, 1995).

Propolis extracts have received new attention around the world as a beneficial effect of an effective antioxidant activity. Propolis usually contains wax, resins, water, inorganics, phenolics and essential oils (Dobrowolski et al., 1991). Besides, more than 180 separate compounds in which the materials is different based on different plants and geographical areas accessed by propolis making bees, the main active components are considered to be present in all forms of propolis (Wagh, 2013). Among the

biological activity of propolis extracts, antimicrobial effect has been widely reported, that ethanolic extract of propolis found to be effective against a variety of bacteria, particularly against gram-positive bacterial species (Jorge et al., 2008). Ethanol extraction is the most popular technique for the production of propolis extract, which is a suitable method for low-wax propolis extract and rich in biologically active compounds (Pietta et al., 2002).

2.2 Stingless bee

Stingless bee belongs to the Apidae family that is found in tropical and sub-tropical regions. Their size ranges from 2 mm but not more than 5 mm and they have no stinger (Kumar et al., 2012). Stingless bee is the most natural pollinators herbal plants because it only collects nectar and elected pollen to medicine. Due to the shortage of defence organs, it protects the hive for honey with a unique build, has a multilayer structure, provide separate provisions for the storage of pollen, honey storage and brood rearing with a single entrance (Sommeijer, 1999). *Trigona* spp. is a stingless bee that produce less honey and more propolis (Fatoni et al., 2008). Propolis from *Trigona* spp. was proven effective against several types of bacteria, such as *Bacillus subtilis*, *Staphilococcus aureus,* and *Escherichia coli* (Hasan, 2006). In addition, (Sabir, 2005) stated that the flavonoids found in propolis of *Trigona* spp. can inhibit the growth of *Streptococcus mutans*.

The chemical composition found in stingless bee propolis depends on geography, climate, types of bees and plants found in the area that caused the

differences in smell, colour and pharmacological activities such as anti-bacterial (Mărghitaş et al., 2013), antioxidant, antimicrobial (Kujumgiev et al., 1999), anti-ulcer, anti-herpes, anti-inflammatory, anti-hypertensive and also the properties of anti-cancer (Choudhari et al., 2013). Extensive research on propolis has been made worldwide (Rebiai et al., 2014), where in India, a few studies have been reported in Maharashtra, Karnataka, Gujarat and Uttar Pradesh (Surendra et al., 2012).

Ethanolic extract of bee pollen from Brazillian stingless bees show high antioxidant capacity (Silva et al., 2009). However, studies of wild bee pollen of stingless bee in Malaysia are still very low in numbers. Domesticated species used by the industry that involved in the commercialisation of stingless bee products in Malaysia are *Trigona thoracica*, *Trigona itama* and *Trigona apicalis*. The antioxidant activity of these three species of stingless bees in Malaysia was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and high-performance liquid chromatography (HPLC) method, and the results showed that *Trigona apicalis* had the highest inhibition activity (Nurdianah et al., 2016).

Stingless bee (*Tetragonula pagdeni* Schwarz, Apoidea) is a species of bees that is grown in artificial nests in fruit garden, where *T. pagdeni* propolis is used as natural medicines and has been marketed in Thailand (Thummajitsakul et al., 2010).

Indian stingless bee propolis has a very complex chemical nature and there are various medicinal properties. A study of anti-cancer activity of ethanolic extracts of propolis (EEP) was carried out by testing the cytotoxic and apoptotic effects against

four different cancer cell lines, namely MCF-7 (human breast cancer), HT-29 (adenocarcinoma of human colon), Caco-2 (human epithelial colorectal adenocarcinoma), and B16F1 (murine melanoma), where cytotoxicity was evaluated by MTT assay and trypan blue dye exclusion assay (Choudhari et al., 2013).

The chemical composition of propolis depends on the geographical location where the source of the plants is different from the local flora at the site collection and season (Salatino et al., 2005). In addition, the bee species equally contribute to the diversity of chemical content, because of their flora priority. Two common stingless bees (known as kelulut) species; *Heterotrigona itama* and *Geniotrigona thoracica* are the main polinators in this region (Ibrahim, 2016(b)).

2.2.1 *Trigona apicalis* stingless bee

There are 500 species of stingless bee that are classified into five genera namely *Trigona, Meliponula, Melipona, Dectylurina and Lestrimelitta*. *Trigona* is the largest genus of stingless bees and has many subgenera found in tropical regions extending from Mexico to Argentina, India, Sri Lanka to Taiwan, the Solomon Islands, South Indonesia and New Guinea. In Thailand, two new species of stingless bee has been reported which are *Trigona binghami* and *T. minor*, which have recently been added to the list of 30 species previously known (Schwarz, 1939). *Trigona's* diversity and their resins and gum collecting depend on the environment. *T. apicalis* is the largest resin and gum collector for propolis production compared to other bee species (Garedew et al., 2003).

However, the diversity of stingless bees is less documented throughout Peninsular Malaysia (Salim et al., 2012). The most common stingless bee species found in Malaysia are *Trigona itama* (83.2%) and *Trigona thoracica* (11.2%), followed by *Trigona terminate* (2.5%), *Trigona laeviceps* (1.9%), and *Hypotrigona scintillans* (0.6%) and one unidentified meliponid (0.6%), respectively (Kelly et al., 2014).

Trigona spp. produces propolis by mixing saliva with food ingredients such as pollen, tree shoots, bark, and flowers. Among the contents contained in the propolis are amino acids, glucose, vitamin A, B, C, D and E, bioflavonoids and minerals that are good for health (Ngoi, 2016). Figure 2.1 shows the side view of a *Trigona apicalis* stingless bee.

Figure 2.1. Side view of *Trigona apicalis* stingless bee (Cockerell, 1918).

2.3 Phytochemical Analysis of Stingless Bee Propolis Extracts

Propolis is known to have a high antioxidant activity and is important for topical formulations. The antioxidant activity should also be properly evaluated by several methods, to choose the most sufficient method. So, the formulation should be emphasised as the most challenging task in assessing the topical formulations when dealing with the presence of formulated compounds that cause interference if using methods that are not specific (Marquele et al., 2005). Propolis extract has received attention worldwide as an effective antioxidant activity and generally "return to nature trend" in which propolis consists of wax, resin, water, inorganic materials, phenolic and essential oils (Dobrowolski et al., 1991).

Propolis is produced from a mixture of resin collected by bees from plants used in medicine for centuries. It is a complex chemical composition, which comprises of 300 compounds including phenolic aldehydes, polyphenols, amino

acids, quinines sequiterpene, coumarins, steroids, and inorganic compounds, have been recognised in different propolis samples (Lustosa et al., 2008). A variety of biological characteristics and therapeutic found in propolis has attracted the attention of researchers (de Castro et al., 2013). In general, the phenolic compound is divided into at least 10 different types depending on the basic structure which is simple phenols, coumarins and isocoumarins, phenolic acids, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids and lignins which polyphenolic flavonoids are the most important (Wollgast and Anklam, 2000).

Natural antioxidants might be phenolic compounds such as flavonoids and phenolic acids, tocopherols, or nitrogen compounds which are amino acids, alkaloids, chlorophyll derivatives, and amines or carotenoids and ascorbic acid (Halliwell, 2011). Their molecular structure include an aromatic ring with a hydroxyl group containing mobile hydrogen makes phenols are highly efficient in scavengers of peroxyl radicals. Besides, the action of phenolic compounds can be attributed to their capability to decrease and chelate ferric ion which catalyse lipid peroxidation (Uttara et al., 2009). A research conducted by Krishnasree and Ukkuru, (2015), concentrated on enhancing component in honey namely polyphenols, flavonoids and flavonols, where the effectiveness of these phytochemicals in improving the therapeutic function of honey is determined by *in vitro* assays antioxidants.

Flavonoids, with a variety of biological activities are considered as the main compound in propolis. Thus, the flavonoid content is an important index to assess the quality of propolis (Chang et al., 2002). The chemical composition of propolis is

complex and more than 150 components have been identified (Marcucci, 1995), where among these compounds, flavonoids are responsible for biological activities (Bosio et al., 2000). Flavonoids (flavones and isoflavones) are the most effective classes of polyphenolic compounds (Hämäläinen et al., 2007), in which several flavonoids can stimulate macrophages to stop further production of eicosanoids and destroy excess oxidant (Havsteen, 2002).

Flavonoids and derivatives of hydroxycinnamic acids are often associated in the biological activity of propolis. Flavonoid is a group of polyphenolic conjugated aromatic compounds, a variety of chemical structures and features, strong antioxidants, free radical scavengers and metal chelators (Harborne and Williams, 2000). Besides that, quercetin and flavonoids also act as anti-inflammatory agents, for the prevention of atherosclerotic plaque formation and platelet aggregation, to promote relaxation of cardiovascular smooth muscle and displaying anti-viral, carcinostatic, anti-ulcer and anaesthetic activity. Colour and high visibility generated by flavonoids are important to attract pollinators such as insects and birds, the need for plant breeding and are present in large quantities in food and beverages (Cook and Samman, 1996).

Phytochemical study has shown that propolis contains a complex phenolic compounds, which are caffeic acid phenethyl ester and prenylflavanone group that are responsible for its activities (Athikomkulchai et al., 2013). Some analytical methods that are used to standardise the European honey bee propolis extract (*Apis mellifera*) are capillary electrophoresis, near infrared spectroscopy and high performance liquid

chromatography coupled with different detectors, have been developed for the evaluation of the quality (Sun et al., 2014). However, chemical constituents and its related bioactivities of propolis depend on the species and their different priorities for resin and food plants, geographical area, changes in the composition of the resin plant and accessible plant species (Ayaad et al., 2012).

Propolis extracts have an antioxidant capacity in *in vitro* conditions (Rebiai et al., 2011) . Propolis also shows high antioxidant activity by inhibiting oxidation reactions, increased from β-carotene and linoleic acid (Chaillou and Nazareno, 2009). Besides, propolis also inhibit hyaluronidase, activities that contribute to the effects of anti-inflammatory, which propolis has strong antioxidant activity also has high scavenging activity and contains many antioxidant compounds, such as caffeic acid, caffeic acid phenethyl ester, kaempferol and ferulic acid (Ahn et al., 2007).

2.4 Benefits of propolis

Natural propolis is a lipophilic, hard and brittle material and it becomes soft, easy to be formed, gummy and very sticky when heated (Hausen et al., 1987). Propolis is a naturally occurring anti-inflammatory bee derived protectant resin which is used to reduce inflammation and promote healing of skin ulcers in diabetic rats (Henshaw et al., 2014). Based on a previous research that was conducted on premature diabetic mice models having full skin wound thickness, topical propolis was applied on normal ulcer and it was seen to reduce the ongoing closure of neutrophil infiltration and elastin activity (McLennan et al., 2008).

Previous research has indicated that bee propolis is an effective ingredient for wound healing (De Castro, 2001). Propolis is a resinous substance some bees collect from the buds of trees and flowers to avoid their nests, and prevent disease and parasites from getting into their hives (Castaldo and Capasso, 2002). Propolis has also been used in the permanent teeth replantation and helps intraoral wound healing. Maintenance of periodontal living cells is one of the important factors that smooth the successful replantation of the avulsed permanent teeth (Goswami et al., 2011).

Propolis has many uses in dentistry such as a direct pulp capping agent, cariostatic agent, intracanal irrigant, intracanal medicament and also been used in treatment of periodontitis (Więckiewicz et al., 2013). Local application of propolis has been shown to help heal wounds after surgery within the oral cavity, reduce inflammation and has analgesic effect (Magro-Filho and de Carvalho, 1994). In addition, a beneficial effect of bee glue on healing of surgical wounds within the oral

cavity has been recognised. Propolis reduces inflammation and speeds up creation of granulation tissue and epithelialisation (Lopes-Rocha et al., 2012).

Research conducted by Tanasiewicz et al. (2012) demonstrated the effectiveness of 3% ethanolic extract of propolis in toothpaste for patients with a greater risk of gingivitis caused by dental plaque. However, the effectiveness of propolis in reducing pathogenic organisms for periodontal disease is cytotoxic to gingival fibroblasts (Sonmez et al., 2005). In addition, they are used as an ingredient in mouthwash or toothpaste to limit the accumulation of dental plaque due to its effects of slowing the formation of calcium phosphate precipitate (Hidaka et al., 2008).

Bruschi et al. (2007) found that the hydrophilic mucoadhesive gel containing propolis, when applied to the gingival pockets, could be useful in the treatment of periodontitis. The study conducted by Coutinho, (2012) concluded that additional subgingival irrigations with propolis extract during periodontal treatment allowed for better results in scaling and root planning. The use of oral propolis-based preparations may be considered in periodontital treatment algorithm in the future.

Research by Oršolić et al. (2006) showed that the activity of chemopreventive propolis in animal models and cell cultures might result from their ability to inhibit DNA synthesis in tumour cells, their ability to induce apoptosis of tumour cells, and their property to activate macrophages to produce factors that are capable of regulating the function of B, T and NK cells, respectively. Besides that, the flavonoids present in propolis also play a role to provide protection against the toxicity of

chemotherapeutic agents or radiation in mice, giving hope that they may have a similar protective action in humans. Combination with antioxidant therapy can increase the effectiveness of chemotherapy by ameliorating the side effects on leukocytes, liver, and kidneys (Oršolić, 2010).

The effect of Chinese and Brazilian propolis on streptozotocin-induced type 1 diabetes mellitus in Sprague Dawley rats was studied and the results showed that both propolis prevented weight loss and blood glucose increase in diabetic mice. Furthermore, mice that were treated with Chinese propolis showed 8.4% reduction in glycated hemoglobin levels compared to the untreated mice (Zhu et al., 2011). The *in vitro* antibacterial activity has been validated against several Gram positive and Gram negative bacteria and results from the synergy between the compounds and flavonoids propolis are pinocembrin and galangin. Other flavonoids, such as chrysin and kaempferol, indicate a reduction in intracellular proliferation of some viruses such as herpes simplex virus (HSV) (Marcucci, 1995).

HSV is one of the most popular human pathogens (virus) that can cause oral mucosa lesion (Jamali et al., 2007). Even though propolis has been known to have a high antivirus property, Schnitzler et al. (2010) found that not a single component of propolis had the same antivirus effects as those in the form of a mixture of propolis. On the other hand, propolis can slow the growth and development of skin changes in the early stages of infection and does not cause cytotoxic effects.

2.5 Effects of propolis on wound healing

The presence of fibroblasts is critical in supporting normal wound healing. They key processes that involved are breaking down the fibrin clot, creating new extra cellular matrix (ECM) and collagen structures to support the other cells associated with effective wound healing, as well as contracting the wound (Bainbridge, 2013). The phenotypic differences between oral and dermal fibroblasts lead to the differences in healing outcome between oral mucosa and skin (Shannon et al., 2006). The oral mucosa heals faster than the skin and with less scar formation (Lee and Eun, 1999). Tissue healing occurs with biological response in which the organism repair damaged tissue. In the early stages, inflammatory cells will migrate to the damaged tissue and debris, and then fibroblast and endothelial cells proliferate and become scars (Ramos and Miranda, 2007).

The biological effects of propolis is widely used in dermatology for injuries healing, external ulcer therapy and heat damage, that include a reduction in healing time, increased wound shrinkage and accelerate tissue repair (Ramos and Miranda, 2007). The active compounds in propolis which is responsible for biological activity are flavonoids (chrysine, galangine, pinocembrine and pinobanxine), phenolic acids (caffeic acid, p-cumaric acid and ferulic acid), and esters (phenylethyl and 1.1-dimethylallyl) (Fokt et al., 2010). The use of propolis has gained attention in various fields of research in medicine and dentistry. The action of propolis in subcutis, in experimental mice with skin wounds and in the repair of dental alveoli, and concluded that in both cases propolis had accelerated the development of scars has been analysed (Magro and de Carvalho, 1990).

Wound healing is a dynamic process that is characterised by the existence of inflammatory, proliferative and remodeling events (Espinosa et al., 2010). Propolis and dexamethasone are among other materials that might accelerate or modulate the healing of surgical wounds. Propolis has been given either topically or systemically in different vehicles, such as alcohol, propylene glycol and water, with a view to modulate the healing of wounds. A study has shown the propolis ability to penetrate the wound by using photoacoustic spectroscopy (Sehn et al., 2009). Song et al. (2008) stated that caffeic acid was a compound that act as an anti-inflammatory and could accelerate wound healing, which was significantly inhibited acid hydrolysis of arachidonic acid and prostaglandin E2 production as well as releasing histamine by mast cells in cell culture.

Fibroplasia is fibroblast on the edge of the surgical wound induced to synthesise collagen during the healing process (Abreu et al., 2012). Fibroblasts and endothelial cells are activated in response to chemotactic factors produced during the inflammatory phase to produce collagen fibres and blood vessels, leading to the formation of granulation tissue (Greiling and Clark, 1997). Deposition and maturation of collagen fibres in the granulation tissue matrix encourage formation of conjunctive scar tissue in denominations fibroplasia process (Moura et al., 2011).

In dental traumatology, there is only one study that assessed *in vitro* use of propolis as a storage medium for avulsed teeth (Martin and Pileggi, 2004). A study performed by Gulinelli et al. (2008) showed propolis had a high ability in maintaining

the vitality of PDL after tooth avulsion compared to Hank's balanced solution, milk and saline.

2.6 Scratch assay

Scratch assay is an inexpensive and economical method of studying cell migration (Todaro et al., 1965). This method is based on the creation of a new artificial gap that is called "scratch". In the confluent cell monolayer, cells on the edge of the newly formed gap will move towards the opening to close the "scratch" until the new cells are established again. Basic steps are involved; the creation of scratch on monolayer cells, capturing images at the beginning and the interval of time during cell migration to close scratches, and comparison of images to determine the rate of cell migration (Liang et al., 2007).

The main advantage of this method is that it imitates a few migration cells *in vivo*. For example, removal of the endothelium part in the blood vessels will induce endothelial cells (ECs) migration to the released area to close the wound (Haudenschild and Schwartz, 1979). Another advantage is the suitability of evaluating the cell migration rules by cell interactions with extracellular matrix (ECM) and cells interactions. Other method such as Boyden chamber assays; the preparation of cells in suspension before testing interfere with cells and cell-ECM interactions.

Furthermore, the scratch assay also corresponds to the microscope including live cell imaging, which allows for analysis of intracellular signal events during cell migration. For example, with the visualisation of green fluorescent proteins (GFP)-tagged protein for subcellular subordination or fluorescent resonant energy transfer for protein interactions. In addition, this method might also be the easiest way to do as it uses only the usual and inexpensive supply available in most laboratories capable of culture cells.

However, this method takes a relatively longer time to perform compared to other methods. One to two days are needed for the formation of cell monolayer and 8–18 h for cell migration to close the scratch. Even though this method has some limitations, overall, scratch assay is still an option for analysing cell migration in the laboratory because it is easy to set up, no special equipment is required and all materials needed for the assay are simply available in any laboratory that conducts cell culture (Liang et al., 2007).

Hence, scratch assay is considered as a very easy tool to estimate the potential of wound healing by using different plant-based extracts (Fronza et al., 2009). Humans have used plants to accelerate the wound healing process from the ancient time (Schmidt et al., 2009). The usage is only based on traditional method without knowledge and scientific evidence of active compounds or their mode of action. Wound healing is a complex biological process and with scratch assay has been proven to be a valuable and inexpensive tool for obtaining a first insight of how plant

preparations or their isolated compounds positively affect the formation of new tissues (Liang et al., 2007).

A previous study aimed to optimise initial tests for quantitative determination of fibroblast migration to and proliferation into wound monolayer, and evaluate its suitability for the test of medicinal plant extracts, isolated compounds and pharmaceutical preparations, Swiss 3T3 fibroblasts were used, and platelet derived growth factor (PDGF) served as positive control. Based form this previous study, manual counting was replaced by the calculation method for quick assessment (Fronza et al., 2009).

Three herbal preparations were used externally in traditional medicine; hexane and ethanolic extracts of *Calendula officinalis*, and *Matricaria recutita* which were applied to scratch assay, as well as a commercially available oil from *Hypericum perforatum*. *Calendula*, is used as a wound healing remedy, but with a largely unknown mode of action (Leach, 2008). Meanwhile, extracts of *Matricaria*, also known as chamomile, are used for their anti-inflammatory effects (Reuter et al., 2009), and *Hypericum* oil is used as a traditional wound healing remedy which is supposed to reduce scar formation (Fronza et al., 2009).