FINAL REPORT

Study of Open Wound Healing Properties of Thai Propolis in Rabbits (Family Leporidae, Order Lagomorpha)

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PREFACE

Propolis, also called "bee glue," is a resinous substance bees use to construct and maintain their hives. Propolis contains many of the same polyphenols found in bee pollen, but in much higher quantities. A broad analysis reveals approximately 55 percent resinous compounds and balsam, 30 percent beeswax, 10 percent ethereal and aromatic oils, and 5 percent bee pollen. Many flavonols contribute to propolis. The flavonoids account for much of the biological activity in propolis. Due to high level of flavonoids in propolis, the propolis products have shown an inhibitory affect on wide range of gram positive and gram negative bacteria including MRSA, fungi, protozoa and a wide range of herpes and influenza. The propolis has a wide range of other therapeutic properties such as anticancer, antioxidant effects, wound healing, tissue repair effects, gastro-intestinal effects, skin infection effects, anti-inflammatory effects, anaesthetic effects, effects on the immune system, cardiovascular effects and dental care effects. Propolis has shown to stimulate various enzyme systems, cell metabolism, circulation and collagen formation, as well as improve the healing of burn wounds. The propolis has also used in hospital patients with infected wounds. The propolis improved wound healing rates, while at the same time reducing infection. A study of topical application of propolis on wounds, burns and ulcers showed up to an eighty percentages increase in healing rate compared to controls using routine healing regimes. However, studies on wound healing properties of Thai propolis in vivo have never been conducted. Therefore, it is urgently important to know wound healing properties of Thai propolis products. So that, in near future, the products of Thai propolis can be applied for therapeutic purposes. Last but not least, the finding of this research may provide information on wound healing in small animals and help to cure those secondary intention wounds in small animals.

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Ratna Thapa

EXECUTIVE SUMMARY

The main objective of this research were to study the effect of propolis on movement of fibroblast cell using *in vitro*, and wound healing properties of propolis in small animals

Samples were collected from the European honeybee, *A. mellifera* and extracted with 70% of aqueous ethanol. The cytotoxicity of propolis was tested by varying propolis concentrations on human gingival fibroblast *in vitro*. The proliferation activity and cell migration properties of Thai propolis were determined. In vivo tests, seven New Zealand rabbits age two months were used in this experiment. All the rabbits were in small animal husbandry under the room temperature. The experimental rabbits anaesthetized and four experimental wounds size 2 cm were made on the dorsal surface of each rabbit. Out of four wounds, the wounds on right of the spinal cord were considered as control and left wounds were treatment. The control wounds were treated with ethanolic alcohol and medicine whereas left wounds treated with 30% of ethanolic extract of propolis. Ethanolic extract of propolis were topically applied one time per day till wounds head.

The results showed that Thai propolis has cytotoxic and activate cell migration properties *in vitro* tests. The in vivo results showed that the wounds were healed within 10 days. This indicates that Thai propolis has wound healing properties. HPLC analysis results showed that Thai propolis has one biologically active compound of phenols so called "5-caffeoylquinic acid" (5-CQA) which has antioxydative properties.

In conclusion Thai propolis has main biological active compound is "5-caffeoylquinic acid" (5-CQA). The Thai propolis has shown cytotoxicity, cell proliferation and cell migration in *in vitro* tests. In vivo experiment showed that Thai propolis has wound healing properties.

ABSTRACT

Propolis is a resinous substance collected and used by honeybees; *Apis mellifera* in to protect their larvae from virus and bacterial infections. Due to various chemical composition of the propolis, it was hypothesized that Thai propolis may have the action on the movement of the fibroblasts. This research has been divided into two parts; (a) in *in-vitro* tests of propolis and (b) in vivo tests of propolis. In this six months study, fibroblasts were cultured and made incision in the plate. Then, the movement of fibroblast cells to the incision activated by the propolis was compared with experimental group and a control groups. The results showed that Thai propolis has cytotoxic and activate cell migration properties *in in vitro* tests. The in vivo results showed that the wounds were healed within 10 days. This indicates that Thai propolis has wound healing properties. HPLC analysis results showed that Thai propolis has one biologically active compound of phenols so called "5-caffeoylquinic acid" (5-CQA) which has antioxydative properties. The most possible explanation is that the Thai propolis has chlorogenic acid (CGA), biologically active compound of phenols so called "5-caffeoylquinic acid" (5-CQA) which has antioxydative properties. In conclusion Thai propolis has main biological active compound is "5-caffeoylquinic acid" (5-CQA). The Thai propolis has shown cytotoxicity, cell proliferation and cell migration in i*n vitro* tests. In vivo experiment showed that Thai propolis has wound healing properties.

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ABBREVIATIONS AND SYMBOLS

EEP	Ethanolic extracts of propolis
MTT solution	
DMEM	Dulbeco Modified Eagle's Medium
ND	Not Detectable
CGA	Chlorogenic acid
5-CQA	5-caffeoylquinic acid
mg	milligram

CHAPTER-1
INTRODUCTION

1.1 Propolis

The word propolis is reputed to have been coined by Aristotle, from the Greek words pro (before) and polis (city) or defender of the city. In his writings, Aristotle showed a remarkably accurate and detailed knowledge of bee propolis. The name, "Defender of The City", is a very appropriate term to describe the role of propolis in the beehive. Honey bees also use propolis to encase the inside of their hive. It is used to seal every crack, and a very thin coat of propolis is spread over the surfaces of the honeycomb cells, inside and out. Propolis, also called "bee glue," is a resinous substance bees use to construct and maintain their hives. Propolis contains many of the same polyphenols found in bee pollen, but in much higher quantities. However, while bees eat pollen for food, they do not eat propolis. While bees benefit from collecting tree resins for propolis, trees do not benefit from the bees collection of propolis in return. The beneficial properties of tree resins have evolved for the trees, although the bees were quick to discover and use tree resins for their own uses. Therefore, to understand propolis, one must first understand tree resin, and what tree resin does as its primary function. It is not surprising, then, that tree resin functions as the immune system of the trees. When physical damage occurs to trees, then tree resin, or sap, floods into the area and seals it off. In this way, the damaged tissues are protected from infection from bacteria or fungi. Similarly, when parasites, such as bark beetles, attack a tree, tree resin flows into the wounded area and kills the insects and their larvae. Tree resins and waxes are secreted onto the surfaces of sensitive tissues, such as delicate new buds, to protect against harmful ultraviolet radiation. Tree resin screens out harmful radiation, and protects the buds from damage. Ultraviolet radiation also causes free radicals, and tree resin acts as antioxidants to smother the free radicals before they can cause damage. Tree resins typically have high concentrations of polyphenols. Many polyphenols have been shown to be anti microbial against bacteria, fungi and viruses. These actions are essential to the immune defense of the tree. Polyphenols have also been shown to play important roles in the trees biochemical response to stress, disease and physical damage. Polyphenols are also present in high concentrations in developing leaves and buds. Their presence there indicates that in addition to

their protective roles, they may also play a role in the development and maturation of plant tissues.

Honey bees collect tree resin for propolis. Previous research indicates that the chemical structure of resin is altered by the bee secretions during the collection process. Bees work the resin with their front legs, and beeswax to the mixture. The saliva and other secretions are catalysts for biochemical changes within the propolis. The resin is passed to their back legs for storage in their pollen baskets. Bees then transport the resin back to the hive, where it is stored or used. Bees show definite preferences for certain species of tree resins in their collection of propolis.

1.2 Significant of this research

Propolis is a natural bee product comes from different species of trees so it is a natural product does not have any side effects. Secondly, propolis collected from different flowers has distinctly different biochemical properties, has tremendous antibiotic, antiviral and antifungal effects are used in different ways to cure for different diseases. Third propolis is affordable, and does not cost very much like western conventional medicines. Finally, it is easily available in villages, and can be used as medicines with local materials.

1.3 Objectives

The objective of this research were to study :-

- the effect of propolis on movement of fibroblast cell using *in vitro*, and
- wound healing properties of propolis in small animals.

1.4 Scope of this study

Propolis has attracted public interest since it is a natural product with many biological properties. The propolis has been used since the ancient times in many parts of the world. The ancient Egyptians used to embalmed their dead body and propolis is still frequently used medications today (Cohen, 1966; Volpert, and Elstner, 1993). Ethanolic and aqueous propolis extractions have shown to inhibit several oxidative reactions with significant antioxidantive property. More than 180 propolis constitutes have been identified (Greenaway, et al., 1988). Although a very

few propolis have (0.001%) allergic (Hausen, *et al.,* 1992). Frankiewicz and Scheller (1984) reported after treating with elderly patients with propolis capsules observed normal concentration of glucose, urea, and cholestrol and amino-transferase activities. Kedzia *et al.,* (1988) found normal serum glucose at physiological levels after treating with propolis to rabbits. Kedzia *et al.,* (1988) also observed that blood pressures reduced in rats after propolis ingestion. Cohen *et al.* (1966) first reported that the damaged organs mainly in cardiac damaged was normal after treating with propolis.

1.5 Benefits of this research

Increasingly, flavonoids are becoming the subject of medical research. They have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity, antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity (Harborne and Williams, 2000). For a group of compounds of relatively homogeneous structure, the flavonoids inhibit a perplexing number and variety of eukaryotic enzymes and have a tremendously wide range of activities. In the case of enzyme inhibition, this has been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, for example, carbohydrate, phenyl ring, phenol and benzopyrone ring. Several reviews have been written on the interaction between flavonoids and mammalian cells, including comprehensive articles by Harborne and Williams (2000). An extensive review on the biochemistry and medical significance of flavonoids has also recently been produced by Gange and Davey (1990).

CHAPTER-2

LITERATURE REVIEW

2.1 Concept, theories and relevant works

Flavonoids account for much of the biological activity in propolis. A large number of studies have shown propolis to be highly antimicrobial. Propolis has been found to have an inhibitory affect on at least 21 species of bacteria including MRSA, 9 species of fungi including the causative organisms of thrush, ringworm and athlete's foot, 3 species of protozoa including Giardia and a wide range of viruses (including herpes and influenza). Propolis has been shown to have a range of other therapeutic properties, including anticancer effects, antioxidant effects1 wound healing and tissue repair effects, gastro-intestinal effects, skin infection effects, anti-inflammatory effects, anesthetic effects, effects on the immune system, cardiovascular effects and dental care effects. Because of the high levels of flavonoids found in propolis, the product has high value as an antioxidantifree radical scavenger in humans. Of particular interest is its ability to protect vitamin C from being oxidized or destroyed. Propolis is known to cause contact dermatitis in a small percentage of humans. The dermatitis has been shown to be relieved once the skin is no longer in contact with the propolis product. It is therefore recommended that usage is ceased whenever there is an allergic reaction.

2.2 Propolis description

Propolis is a resinous yellow-brown to dark brown substance collected by worker honey bees from the growing parts of trees and shrubs (e.g. leaf buds, trunk wounds). The bees pack the propolis on their hind legs, and carry it back to their colony, where it is combined with beeswax and used by worker "hive" bees as a sealant and sterilant in the colony nest. The uses take advantage of the antibacterial and antifungal effects of propolis in protecting the colony against disease. Propolis has also been shown to kill Bacillus larvae, the most important bacterial disease of bees. Propolis changes consistency with temperature. At temperatures below 150°C it is hard and brittle, but becomes more pliable and sticky at higher temperatures (25-450°C). Propolis generally melts at 60-700°C, although some samples have been found to have a melting point as

high as 1000°C (Krell, 1998). Propolis is collected by commercial beekeepers, either by scraping the substance from wooden hive parts, or by using specially constructed collection mats. The raw product undergoes secondary processing to remove beeswax and other impurities before being used in a variety of natural health care products (e.g. lozenges, tinctures, ointments, drinks).

2.3 Composition of propolis

Chemically, propolis is a very complex mixture. Its chemical elements vary according to its source. Colors range from golden brown to brownish green to reddish brown to blackish brown. A broad analysis reveals approximately 55 percent resinous compounds and balsam, 30 percent beeswax, 10 percent ethereal and aromatic oils, and 5 percent bee pollen. Many flavonols contribute to propolis. Other components include cinnamic acid, cinnamyl alcohol, vanillin, caffeic acid, tetochrysin, isalpinin, pinocembrin, chrysin, galangin, and ferulic acid. Propolis consists of more than 100 substances (Krell, 1996) and microelements such as iron, copper manganese, zinc, aminocids, phytoncides, and antibiotics, plus a high content of vitamins B, E, C, H and P, as well as pro-vitamin.

Table 1. Chemical composition of propolis

Class of Compound	Group of Components	Amount
Resins	Flavonoids, phenolic acids and esters	45-55%
Waxes and Fatty	Acids Beeswax and Plant Origin	23-35%
Essential Oils	Volatiles	10%
Pollen	Proteins (16 free amino acids >1%) arginine and proline together 46% of total	 5%
Other Organics and Minerals	14 trace minerals, iron and zinc most common; ketones, lactones, quinones, steroids, benzoic acid, vitamins and sugars	5% 5%

The most important pharmacologically active constituents in propolis are the flavones, flavonols, and flavanones (collectively called flavonoids), and various phenolics and aromatics. Flavonoids play a major role in plant pigmentation. However, the flavonoids present in propolis are different in composition to those normally found in plants, since propolis flavonoids are not glycosides

(that means they do not have sugar molecules attached to their chemical structure). The majority of flavonoids found in plants are glycosides. Flavonoids are thought to account for much of the biological activity in propolis (Grange and Davey, 1990), although other phenolic compounds are also involved. At least 38 flavonoids have been found in propolis, including galangin, kaempferol, quercetin, pinocembrin, pinostrobin and pinobanksin. Some of the other phenolics include cinnamic alcohol, cinnamic acid, vanillin, benzyl alcohol, benzoic acid, and caffeic and ferulic acid.

2.4 Human nutrition

Propolis has little direct nutritive value, apart from the presence of small amounts of proteins, amino acids, minerals and sugars. Vitamins include small amounts of A, B1, B2, B6, C and E (Ghisalberti, 1979). Propolis is used by humans almost solely as a therapeutic. Propolis and a number of its components exhibit a wide variety of biological and pharmacological activities.

2.5 History of use of propolis

Propolis has been used by man since early times, for various purposes, and especially as a medicine because of its antimicrobial properties. Ancient Greek texts refer to the substance as a "cure for bruises and suppurating sore", and in Rome propolis was used by physicians in making poultices. The Hebrew word for propolis is tzori, and the therapeutic properties of tzori are mentioned throughout the Old Testament. Records from 12th century Europe describe medical preparations using propolis for the treatment of mouth and throat infections, and dental cares (Krell, 1996). One of the non-medicinal uses of propolis is as a varnish, and it has been suggested that the special properties of Stradivarius violins may be partly due to the type of propolis used, although the claim cannot be substantiated.

2.6 Therapeutic properties

2.6.1 Antimicrobial effect

Because of its strong antimicrobial activity, propolis is often known as a "natural antibiotic". A large number of studies have shown an inhibitory effect on a variety of micro-organisms. The antimicrobial effects are summarized in the following table (Krell, 1996).

Table 2. Antimicrobial effects of propolis

Organism	Comment	Reference
Bacteria		
Bacillus larvae	destroyed	Mlagan and Sulimanovic 1982
B. subtillis	destroyed	Meresta and Meresta 1985
Helicobacter pyiori	inhibited	Itoh et al 1994
MRSA	strong inhibition	Grange and Davey, 1990
Mycobacteriumtuberculosis	Tb	Grange and Davey, 1990
Staphylococcus sp.	inhibited	Chernyak, 1973
Staphylococcus aureus	synergistic effect	Kedzia and Holderna. 1986
Streptococcus sp.	inhibited	Rojas and Cuetara, 1990
Streptomyces	inhibited	Simuth et al, 1986
S. sobrinus, mutans, cricetus	dental caries	Ikeno et al, 1991
Saccharomyces cerevisiae	brewer's yeast	Petri et al,1986
Escherichia coli	inhibited	Simuth et al, 1986
Salmonella	potential treatment	Okonenko, 1989
Giardia lambia	positive effect	Olarin et al, 1989
Bacteroides nodosus	reduced foot rot	Munoz, 1989
Klebsiella pneumoniae	positive effect	Dimov et al, 1991
Fungi		
Candida albicans	synergistic effect	Holderna and Kedzia, 1987
Aspergillus niger	positive effect	Petri et al, 1988

Botrytis cinerea	in vitro fungicidal	La Torre et al, 1990
Ascosphaera apis	inhibited	Ross, 1990
Viruses		
Herpes	inhibited in vitro	Sosnowski,1984
Potato	virus effective	Fahmy and Omar, 1989
Influenza (in mice)	reduced mortality	Serkedjieva, 1992 and others
Newcastle Disease	affected virus reproduction	Maksimova-Todorova et al 1985

Active components of propolis showing an antibacterial effect include pinocembrin, galangin, caffeic acid and ferulic acid. Antifungal components include pinocembrin, pinobanksin, caffeic acid. benzy ester, sakuranetin and pterostilbene. Anti-viral components include caffeic acid, lutseolin and quercetin. Propolis has been found to inhibit the synthesis of protein by bacteria, which may account for at least some of its antimicrobial effects.

2.6.2 Synergistic effects

Most studies on the therapeutic properties of propolis have centered on the phenolic constituents (flavonoids and other phenolic compounds such as caffeic acid esters). Research has tended to isolate and test single substances in propolis. However, it is likely that the presence of a large number of compounds in propolis may produce a synergistic effect greater than the sum of the effects of individual components. Studies have shown that the flavonoids in propolis exert significant antibacterial activity, but that isolated flavonoids show reduced activity compared to whole product extracts. Propolis has shown to have a synergistic effect with certain antibiotics, and to increase their effectiveness on some bacteria and yeasts some cases 100 fold. Antibiotic-resistant strains of Staphylococcus were found to become sensitive to antibiotics in combination with propolis.

2.6.3 Anticancer effects

Ethanol extracts of propolis have been found to transform human hepatic and uterine carcinoma cells in in vitro, and to inhibit their growth (Matsuno, 1997). Substances isolated in propolis which produce this cytotoxic effect are quercetin, caffeic acid, and clerodane diterpendoid. Clerodane diterpendoid shows a selective toxicity to tumour cells. Propolis has also a cytotoxic and cytostatic effect in vitro against hamster ovary cancer cells and sarcoma-type tumours in mice (Ross, 1990). The substance has also displayed cytotoxicity on cultures of human and animal tumour cells, including breast carcinoma, melanoma, colon, and renal carcinoma cell lines (Grunberger *et al,* 1988). The component producing these effects was identified as caffeic acid phenethy ester. Caffeic acid esters have been shown to inhibit chemically induced tumour production in mice, as well as having a selective toxic effect on cells affected by genes which promote the development of cancerous cells.

2.6.4 Antioxidant effects

The flavonoids concentrated in propolis are powerful antioxidants. Antioxidants have been shown to be capable of scavenging free radicals and thereby protecting lipids and other compounds such as Vitamin C from being oxidized or destroyed. It is probable that active free radicals, together with other factors. are responsible for cellular aging and degradation in such conditions as cardiovascular diseases, arthritis, cancer, diabetes, Parkinson disease and Alzheimer disease. Oxidatve damage may also result in poor liver function. Studies on rats in vitro show that propolis extracts protect against damage to liver cells.

2.6.5 Gastro-intestinal effects

Propolis has been shown to inhibit the development of externally induced stomach ulcers in rats (Aripov, 1968). Flavonoid components of propolis have also been shown to have this effect (Ciaceri and Attaguile, 1972).

2.6.6 Skin infection effects

Propolis has been shown to be effective in inhibiting the growth of yeasts and fungi responsible for such skin infections as ringworm and athlete's foot (Metzner, et al, 1979). Propolis

compounds showing activity against these organisms are the flavonoids and caffeic acid derivatives.

2.6.7 Anti-inflammatory effects

Studies on mice have shown that extracts of propolis have an anti-inflammatory effect similar to that of indomethacin, a common drug used to treat inflammation. Again, flavonoids and caffeic acid are known to play a role in inhibiting the inflammatory response (Mirozeva and Calder, 1996).

2.6.8 Anaesthetic effects

Propolis and some of its components produce anesthesia, which in some studies has been shown to be 3 times as powerful as cocaine and 52 times that of procaine, when tested in rabbit cornea (Ghisalberti, 1979). The anesthetic effect has been shown to be produced by pinocembrin, pinostrobin, caffeic acid esters components in propolis (Paintz and Metzner, 1979). The anesthetic effect may explain why propolis has been used for centuries in the treatment of sore throats and mouth sores. An anaesthetizing ointment for dentistry using propolis has been patented in Europe.

2.6.9 Effects on immune system

Propolis has been shown to stimulate an immune response in mice. More recently, Japanese researchers have shown an extract of propolis to produce a macrophage activation phenomenon related to the immune function in humans. Propolis activates immune cells which produce cytokines. The results help to explain the ant-tumour effect produced by propolis. Propolis has been shown to stimulate antibody formation in immunized mice. In a joint US-Polish study, spleen cells producing antibodies in mice administered a propolis extract were three times greater than controls. A second dose administered 24 hours later produced an even larger effect, although further doses reduced the effect (Scheller, et al, 1988). Propolis was shown to increase antibody formation between 2-3 times that of controls in pigs vaccinated with "BUK-628" live Aujeszky's disease vaccine with and without addition of propolis. Antibody formation reached its maximum in 14 days, and antibodies could be detected for up to 330 days. Propolis also enhanced production of plasmacytes in the lympholdal tissue of the spleen and lymph nodes.

Propolis has been shown to suppress HIV-1 replication and modulate in vitro immune responses, and, according to the authors, "May constitute a non-toxic natural product with both anti-HIV-1, and immunoregulatory effects".

2.6.10 Cardiovascular effects

In mice1 a concentrated extract of propolis has been shown to reduce blood pressure, produce a sedative effect, and maintain serum glucose (Kedzia, et a1., 1988). Dihydroflavonoids1 as contained in propolis, have been shown to strengthen capillaries and produce antihyperlipidemic activity. Propolis has also been shown to protect the liver against alcohol (ethanol) and tetrachloride in rats.

2.6.11 Dental care effects

In rats inoculated with & sobrinus, about half of their fissures were carious, while dental canes were significantly less in rats given water containing propolis extract. No toxic effects of propolis on the growth of rats were observed under experimental conditions in this study (Ikeno, et al, 1991). Propolis has also been shown to be effective as a subsidiary treatment for gingivitis (gum infections) and plaque. A 50% propolis extract was found to antiseptic against pulp gangrene (Gafar, *et al,* 1986). Propolis has also been shown to inhibit the growth of a range of bacterial organisms found in dental caries. Sixty students were divided into groups to test the effect of propolis on the development of plaque and gingivitis. The results suggest that a propolis preparation can be a useful subsidiary treatment in oral hygiene. A double-blind clinical trial showed that a propolis mouthwash (10% tincture diluted l:5 with water) produced significant improvements in patients with gingivitis and periodontal disease. Patients were evaluated for plaque formation and inflammation of the gums. A clinical study used a paste made from propolis extract and zinc oxide on 150 teeth with indirect capping of deep cavities, and 50 teeth with direct capping. The results showed that the paste with propolis exerted effects similar to those of zinc eugenate, and were superior for healing compared to pastes based on calcium hydroxide. A clinical study found propolis useful for the treatment of gum inflammation and oral mucosa, and also showed anti scarring effects (Gafar, *et al.,* 1989). Another study showed similar results for periodontitis and suggested propolis is used in root canal fillings because of its bone-regeneration and anesthetic properties.

2.6.12 Clinical effects on humans

The diverse use of propolis in clinical trials shows that its therapeutic efficiency lies mainly in diseases caused by microbial contamination (Marcucci, 1995).

2.6.13 Respiratory infections

A total of 260 steel workers suffering from bronchitis were treated for 24 days by various methods including local and systemic regulation of the Immune system and local treatment with an ethanol extract of propolis (EEP) in a physiological salt solution. Best results were obtained with inhalation of the extract, together with propolis tablets (Scheller *et al,* 1989). Propolis has also shown positive effects in other otorhinolaryngologic diseases, such as pharyngitis (Doroshenko, 1975), chronic bronchitis (Scheller, *et al,* 1989), rhinopharyngolaryngitis and pharyngolaryngitis.

2.6.14 Viral infections

A clinical trial has shown a prophylactic effect against influenza infection in humans. Mother clinical trial showed that infections of the common cold were shorter in duration and completely recovered within 3 days for patients treated with propolis, compared to 5 days for recovery for patients not given propolis. (Scheller *et al,* 1989). A clinical trial conducted on dermatology patients showed a propolis cream had significant therapeutic effects against recurrent herpes (Herpes simplex Type 1) and Herpes zona zoster (shingles). The propolis cream reduced duration of lesions and pain, and increased interval between lesion episodes (Giurcaneanu, *et al,* 1988).

2.6.15 Skin infections

Clinical applications of propolis (1-10%) in ether or alcohol were effective against 10 superficial fungi and 9 deep-growing fungi. On oral treatment of 160 psoriasis patients with 0.3g propolis 3 times daily for 3 months, about one-third was cured or greatly improved. Patients (110) Infected with ringworm were treated with 50% propolis as an unguent. In 97 patients it was found to produce excellent results.

2.6.16 Wound healing and tissue repair effects

Propolis has been shown to stimulate various enzyme systems, cell metabolism, circulation and collagen formation, as well as improve the healing of burn wounds (Ghisalberti, 1979; Krell, 1996). These effects have been shown to be the result of the presence of arginine in propolis (Gabrys *et al,* 1986). Propolis and aloe-vera was found to be superior to standard wound treatment products in trials on mice. Sixty four patients with tibial skin ulcers, aged from 23 to 98 years, were treated using propolis tincture in an ointment. The ointment was applied daily to the ulcerated area, which was also treated on the periphery with antibiotic ointments. The treatment lasted for 4-12 weeks. At the end of treatment, 19 of the 84 treated patients exhibited no clinical signs of the condition, 19 an improved condition (Korsun, 1983). Propolis was used in a trial of hospital patients with infected wounds. The propolis improved wound healing rates, while at the same time reducing infection. Over half of infective bacteria were eliminated within 4 days. Propolis did not produce antibio~resistance strains of the bacteria (Damyanliev, 1982). A study of topical application of propolis on wounds, burns and ulcers showed up to an 80% increase in healing rate compared to controls using routine healing regimes. Patients (229) with bums, clean wounds, infected wounds or abscesses / ulcers were treated with cream containing propolis at two concentrations (2% and 8%). The higher concentration caused local intolerance in 18% of patients by day 9, whereas the lower concentration caused symptoms in only 1.8% of patients by day 16. Burns and wounds treated with the low concentration cream healed in 11 days on average, septic wounds in 17.5 days, 67% of ulcers in 38 days (Morales and Garbarino, 1996).

2.6.17 Ear infections

Patients (126) suffering external otitis, chronic mesotypanic otitis and tympan perforation were treated with propolis solutions (5-10%). A positive therapeutic result was reported in most cases (Matel, *et al,* 1973). Propolis has also shown positive results in the treatment of acute inflammations of the ear (Palos *et al,* 1989).

2.6.18 Immune deficiency

A strong immune deficiency was found in 2 patients with alveolitis fibroticans. Treatment with a combination of propolis, Esberitox N and calcium-magnesium resulted in good improvements in the state of the immune system and the clinical condition of both patients (Scheller *et al,* 1989).

2.6.19 Inflammation

Injections of an aqueous solution of propolis were used in the treatment of 22 patients with this hip joint disease caused by aseptic necrosis of the thigh bone. A further 32 patients with the same condition were given different forms of routine treatment. Significant improvement was observed In the patients given propolis. Patients (90) with cases of vagina and uterus cervix inflammation caused by *S. pyogenes* were treated with 3% propolis ethanol extract. Over 50% of the cases responded well to this treatment (Zawadzki and Scheller, 1973).

2.6.20 Adverse effects

Propolis has been shown not to be toxic to humans or mammals unless very large quantities are administered (Ghisalberti, 1979). Some of its constituent flavones, e.g. quercetin, might be mutagenic by the Ames test, but mutagenicity per SE for propolis has not been reported. Contact dermatitis is a well documented allergic reaction to propolis, with approximately 200 cases reported in the literature over the last 70 years (Hausen, *et al,* 1987). Initial reports were made by beekeepers, who came into daily contact with the raw product. Allergic reactions are now also reported in the general population, due to the more wide-spread use of products containing propolis. Dermatitis can be produced by skin contact with raw propolis, as well as propolis extracts and products containing caffeic acid and its derivatives have been identified as the major allergenic agent (Hashimoto, *et al.,* 1988). Cinnamic acid derivatives have also been implicated. Dermatitis is relieved once the skin is no longer in contact with the propolis product. It is therefore recommended that with all preparations intended for human use, usage is ceased whenever there is an allergic reaction. Very few other adverse reactions to propolis have been documented in the literature, and the product is considered generally not to be harmful. Rare cases of oral inflammation and ulceration, mouth oedema (swelling) and stomatitis have been reported. however, as a result of oral ingestion of propolis (Hay and Grieg, 1990).

2.7 Commercial use

Raw propolis is collected by beekeepers and sold in bulk to companies that refine the product and turn it into usable extracts. Main commercial uses of propolis are as a dietary supplement and therapeutic. Propolis is sold in tablets (singularly, or in combination with other substances such as pollen, royal jelly and non-hive products). In Japan, the use of propolis is permitted as a preservative in frozen fish (Krell, 1996). Tinctures and lozenges are popular treatment for sore throats, and tinctures are often used to treat Cuts, mouth sores and skin rashes. For internal use, 1-3mL does three times daily of a 1:10 tincture are typical, but higher doses can use used if necessary. Propolis tincture is normally diluted in water. producing a cloudy liquid. For external use, the 1:10 tincture is diluted in water, and used as a lotion or gargle. Propolis is a stable produce but should nevertheless be stored in airtight containers in the dark, preferably away from excessive and direct heat. Propolis does not lose much of its antibiotic activity, even when stored for 12 months or longer. Propolis and its extract function as a mild preservative due to their antioxidant and antimicrobial activities and thus may actually prolong the shelf life of some products (Krell, 1996).

2.8 Food safety

Because of its antioxidant and antimicrobial activities, microbial contamination is not considered to be a problem with propolis, either in the raw form, or as extracts. Concentrations of lead above maximum allowable levels for food products have been found in propolis studies have shown that lead levels may be reduced by placement of hives away from areas with heavy air pollution and the use of oil based paints on hive parts. Propolis destined for commercial use should be routinely tested for lead concentration. Brazilian propolis is of the highest quality available where Chinese propolis has been noted for excessive lead.

2.9 Bee Propolis : Past to Present

Propolis has been used by man for thousands of years and recently has enjoyed a boom in popularity. Bees have used propolis for millions of years, and humans have used it for thousands. Both species find it immensely useful and beneficial. Much of the bees' success in surviving through the ages may be accredited to propolis. As humans, we may yet discover we've only just scratched the surface to the benefits of this resinous wonder.

The Greek physician, Hippocrates, prescribed the use of propolis to help heal internal and external sores and ulcers. Ancient Egyptians depicted propolis-making bees on vases and other ornaments, and used the resinous substance to alleviate many ailments. Pliny, the Roman scholar, wrote much on the use of resins such as propolis in his massive book, Natural History. He touts the abilities of propolis to reduce swelling, soothe pain, and heal sores, to name a few. In The History of Plants written by John Gerard in 1597 showed that the propolis was noted for its ability to provide swift and effective healing for many conditions. During this era, propolis was used in many different healing ointments.

Propolis is a sticky resin which seeps from the buds of certain trees and oozes from the bark of other trees. Although propolis is vitally important to the colony, there are usually just a few propolis gathering specialists in the hive. The bees gather propolis, sometimes called "bee glue," and carry it home in their pollen baskets. There they are met by one or two other worker bees that help them unload. These workers take the resinous material and add salivary secretions and wax flakes to it then use the new product for numerous protective purposes as bee propolis. The bees use it to coat the inside of the hive, including the passageway and the brood chambers. Propolis protects the hive in two ways: First, it reinforces the hive itself; second, it protects the hive from bacterial and viral infection. And it is these latter properties which man has found so helpful through the centuries. The reason propolis is such an effective protector is related to the diversity of flavonoids. Propolis consists of approximately 55 percent resinous compounds and balsams, 30 percent beeswax, 10 percent aromatic oils, and 5 percent bee pollen. Other constituents include flavonoids, amino acids, B vitamins, and most importantly, antibiotic substances. Often called "nature's penicillin," bee propolis has effective antibacterial, antiviral, antiseptic, antifungal, and antibiotic-properties. These protective and healing properties have been conclusively demonstrated in numerous studies all over the globe.

CHAPTER-3

MATERIAL AND METHODS

3.1 Propolis Sample collection

Samples were collected from the European honeybee, *A. mellifera*. The propolis samples were collected thought out a whole the year. The collected propolis samples were kept plastic bags in deep freeze for future use. Seasonal sample were kept in separate bags.

3.2 Preparation of propolis tincture

Crude samples of propolis were collected from hives of *A. mellifera* from Chiang Rai Province, Thailand (455 m, N:20°04"35.9', E:99°52"31.6'). The dried propolis samples were grounded into fine powder and 5, 20 and 30 grams of propolis powder were mixed 70 mL of 70% of aqueous ethanol (C_2H_5OH, 46.07g/mol) (w/v) respectively in three different flasks and kept in dark room at 24.7±0.2°C and 83.3±1.2% of RH with moderate shaking. After 10 days, the solvents were filtered and the liquid extracts evaporated in a rotary evaporator until of constant weight. This weight was used to determine the yield (% w/w) of EEP for each extraction period (Krell, 1996).

3.3 *In vitro* tests

3.3.1 Cytotoxicity assay

The cytotoxicity of propolis was tested by varying propolis concentrations on human gingival fibroblast *in vitro*. First of all, human gingival fibroblasts were seeded into 96 well plate culture plates at the density of $1x10^3$ cell/well and leave for another 48 hours. Then cells were stained with MTT solution. Viable cells will be stained because they have active enzymes. Then the number of viable cells was determined by dye solution and measure the optical density means cells are viable.

3.3.2 Proliferation assay

Next experiment was to determine the proliferation activity of propolis. In this experiment human gingival fibroblasts were seeded into 24 well culture plates at the density of $2x10^3$ cell/well and leave for 48 hours in CO_2 incubators. After that propolis was added, cells were collected by digestion with trypsin and the numbers of cells were counted under microscope using hemacytometer at day 2, 5 and 7.

3.3.3 Migration assay

Last experiment was to test the effect of propolis on cell migration. Human gingival fibroblasts were plated into 35 mm×10 mm cell culture dish at the density of 1×10^5 cell/ml in DMEM (Dulbeco Modified Eagle's Medium) containing 10% fetal bovine serum and penicillin and streptomycin in 5% of CO_2 incubator at 37°C with 95% humidity for 3 days until cells reached confluency. The wound were made on the surface of cells by scraping cells out from the dish. Then media containing propolis were added and cultures were left incubated under the previous condition for 48 hour. Stained the cells with Toluidine blue-O, and then photographed and measured the distance of migration and counted the numbers of cells migrated. The control solvent was ethanolic alcohol.

3.4 In vivo tests

3.4.1 Wound healing properties of propolis

Seven New Zealand rabbits (Pety bear) age two months were used in this experiment. All the rabbits were in small animal husbandry under the room temperature. The experimental rabbits anaesthetized and 2 cm wound were made on the dorsal surface of each rabbit. Before creating wounds, hair from the body were completely removed and marked with body marker. Four experimental wounds were made on each rabbit. Out of four wounds, the wounds on right of the spinal cord were considered as control and left wounds were treatment. The control wounds were treated with ethanolic alcohol and medicine whereas left wounds treated with 30% of ethanolic extract of propolis. Ethanolic extract of propolis and medicine were topically applied one time per day till wounds healed.

Figure 1. Experimental rabbit (Peaty bear) (A) removing hair from the body, (B) Marking the dorsal side of rabbit for creating wounds, (c) Anesthetizing rabbit, and (D) Fresh wounds.

3.5 Chemical analysis of propolis

To identify and determined the constituents in EEP, HPLC was used. EEP sample was dissolved in ethanol (5mg/ml) at room temperature for 24 hours and then filtered prior to 20 µl injected into the HPLC system. The PHLC system used was a SI-1 (Shiseido, Tokey, Japan) with C18 (Zorbex SB-C-18) column (4.6×150 mm). The mobile phase was consisted of 0.2% of formic acid in water (A) and 0.2% of formic acid in acetonitrile (B) at the flow rate of 0.9ml/min.

CHAPTER - 4

RESULTS

4.1 Cytotoxicity assay

Both Thai and Nepali propolis were toxic to human gingival fibroblast cells at the concentration greater than 0.1 mg/ml. However, 0.05 mg/ml of concentration has no toxicity (Figure 2).

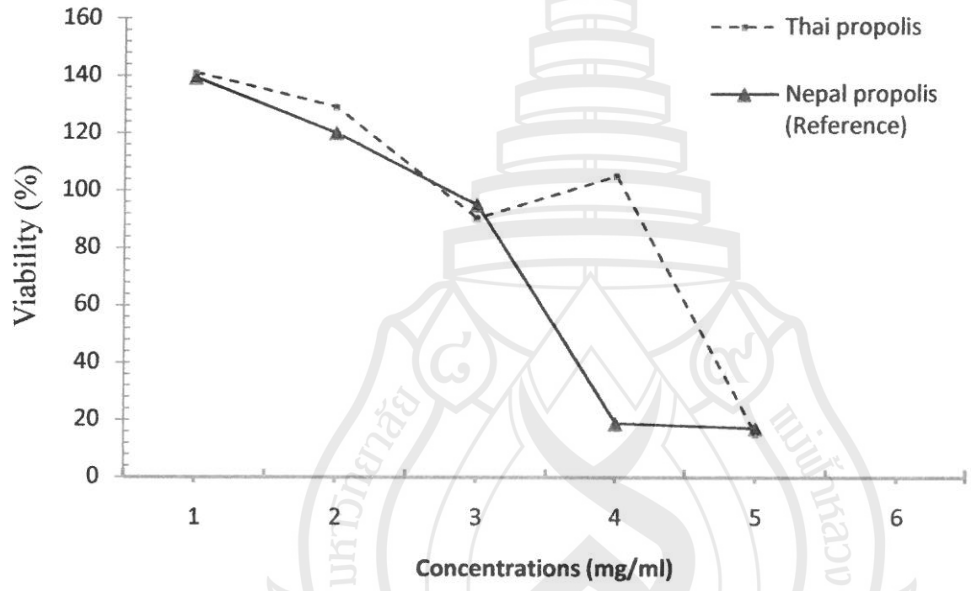

Figure 2. Cytotoxicity of Thai propolis compare with reference propolis.

4.1.1 IC_{50} of propolis

Nepali propolis was found highly toxic (0.163mg/ml) compared to Thai propolis (0.644mg/ml) (Table 1).

Table 3. IC_{50} of propolis

Propolis	IC_{50}
Thai	0.644 mg/ml
Nepal	0.163 mg/ml

4.2 Proliferation assay

Thai propolis and Nepali propolis have proliferative effect on human gingival fibroblasts. The Thai propolis has highest proliferative activity (Figure 3).

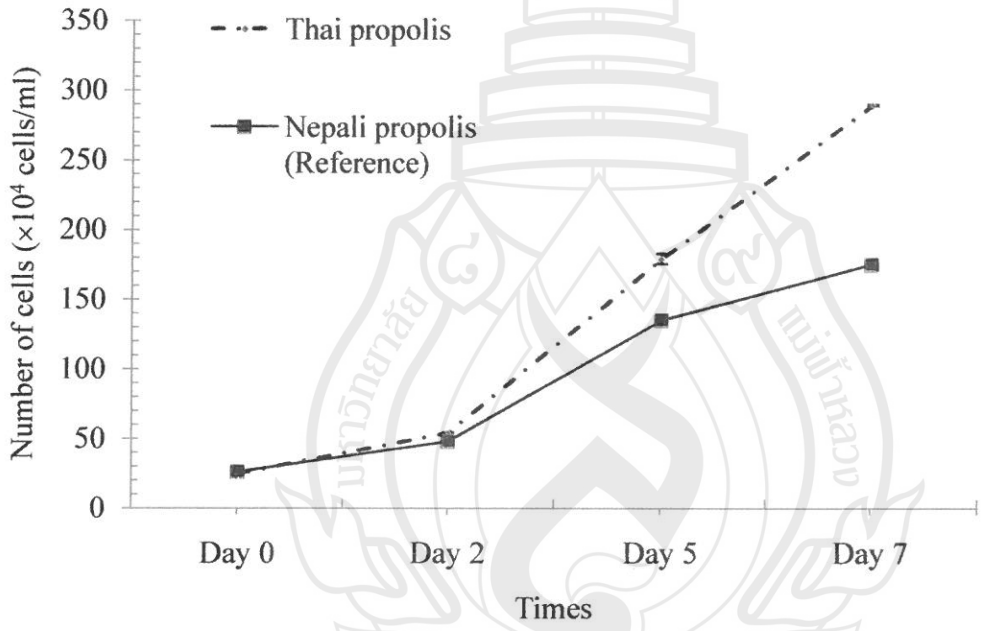

Figure 3. Proliferations of Thai propolis compare with reference Nepal propolis.

4.3 Migration assay

Thai propolis showed tendency of promoting cell migration. The migration of cells rate of Thai propolis was 6.12cm, whereas Nepali propolis had 5.7cm (Figure 4). However, cells migration

percentages of Thai propolis were lower (125.16%) than reference propolis (191.7%) (Figure 5). The control solvent 100% migration (Figure 5).

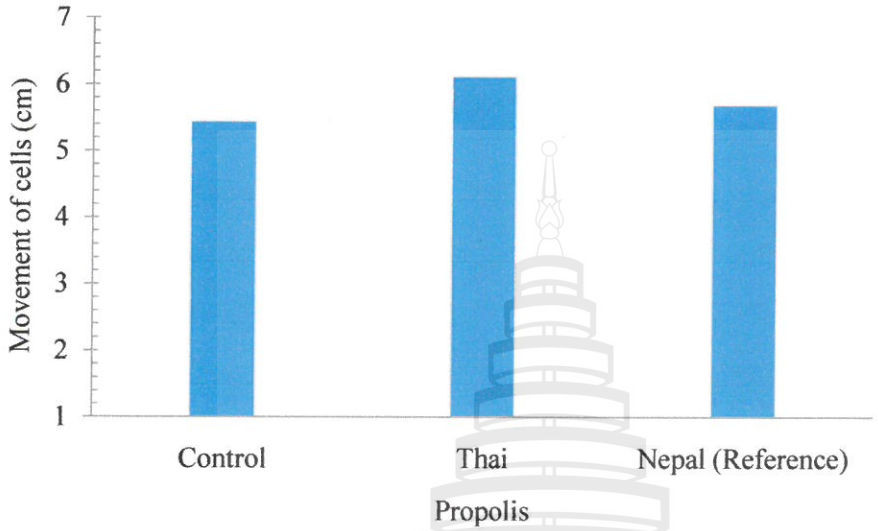

Figure 4. Comparison of cells movement *in in-vitro.*

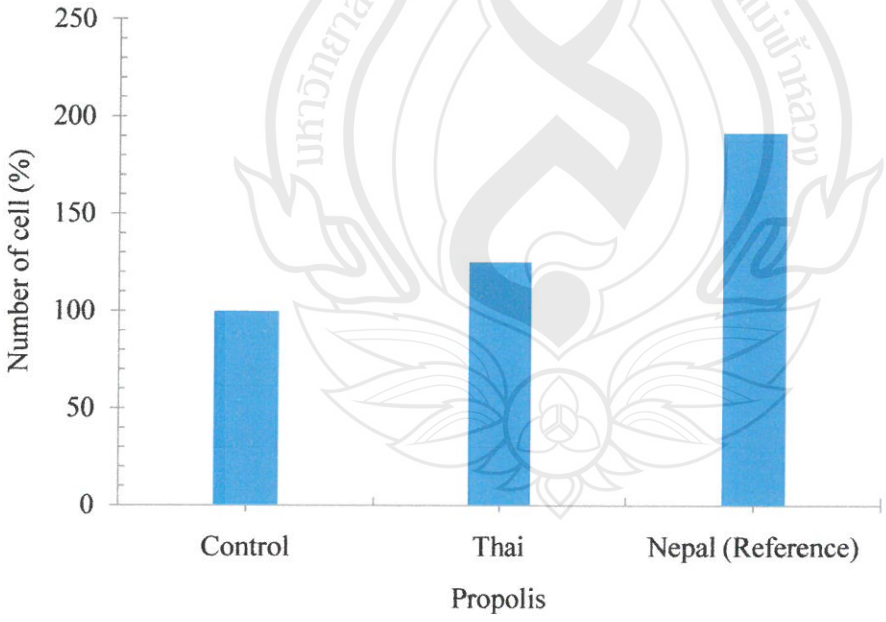

Figure 5. Percentages of migration rate in *in vitro.*

4.4 Wound healing

The freshly created wounds on dorsal sides of the rabbits were recovered within 10 days. The hairs were grown in saving areas (Figure 6).

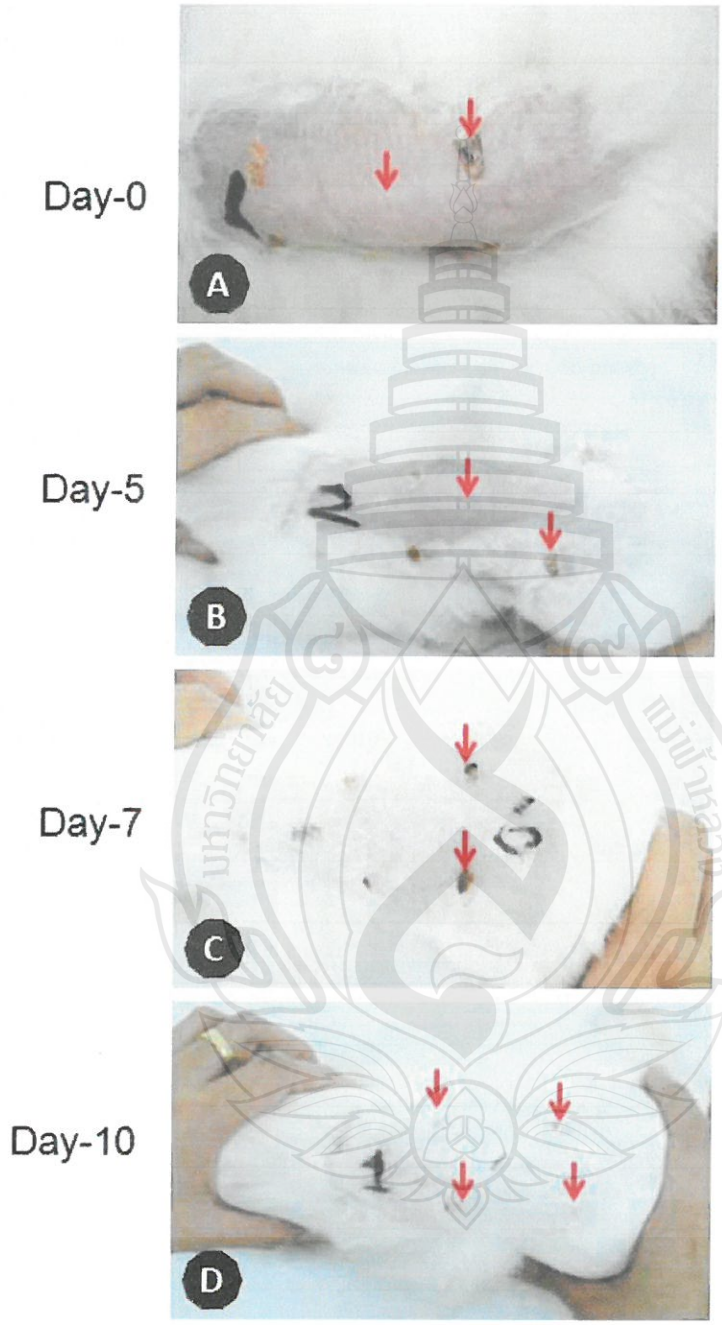

Figure 6. Effect of ethanolic extract of propolis wound healing in rabbit.
The arrows indicate wound location and conditions

4.5 Chemical analysis

The chemical analysis of Thai propolis showed that chlorogenic acid was detected, but other three compounds; hydroquinone, caffeic acid, and quercetin bioactive active compound were not detected (Table 4 and Figure 7).

Table 4. HPLC analysis of propolis

No.	Tested Items	Tested Rank	Unit
1	Hydroquinone	ND*	mg/g
2	Caffeic acid	ND*	mg/g
3	Chlorogenic acid	0.22	mg/g
4	Quercetin	ND*	mg/g

"*" ND = not detectable

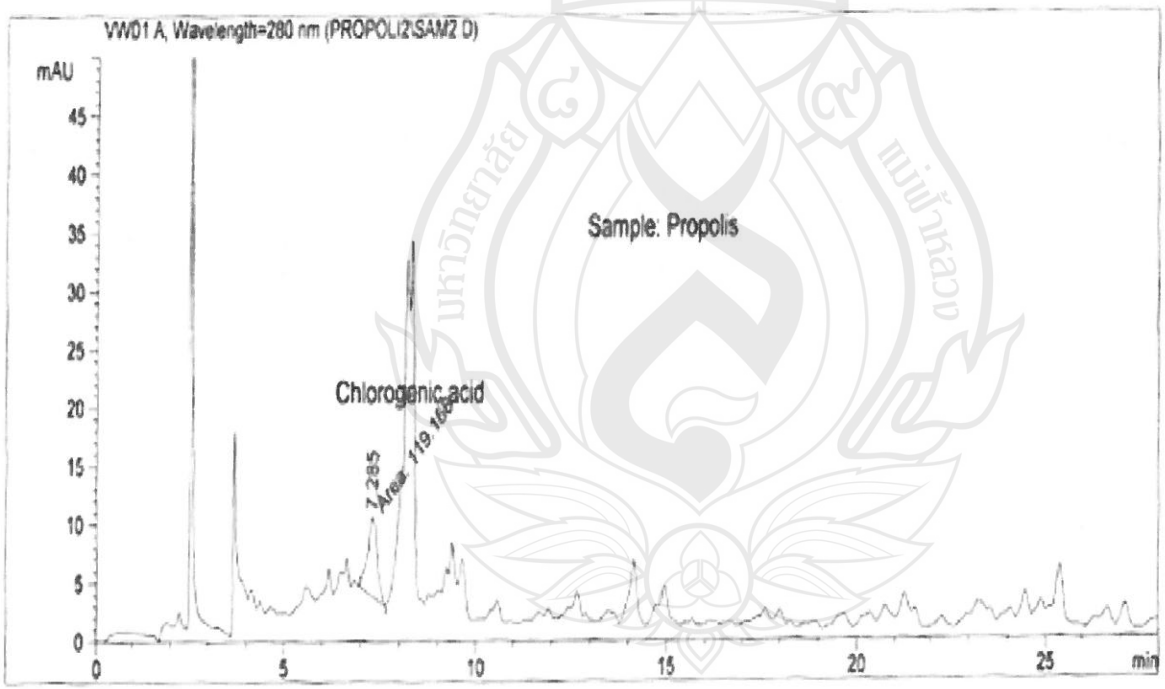

Figure 7. HPLC display of the major phenolic in Thai propolis tincture, Chlorogenic acid.

CHAPTER – 5
DISCUSSION

The results showed that Thai and Nepali propolis were toxic to human gingival fibroblast cells at the concentration greater than 0.1 mg/ml. However, 0.05 mg/ml of concentration has no toxicity. Previous report indicated that the Taiwanese propolis has also shown cytotoxic properties against cancer cells (Che, *et al.,* 2003).

The results showed that Thai propolis and Nepali propolis have anti-proliferative effect on human gingival fibroblasts. The Thai propolis has highest proliferative activity. The results suggest that Thai propolis has slightly high cell proliferative effect than Nepali propolis. This different may due to different locations and different species of trees. can be used to treat cancer cells.

The results showed that Thai propolis and Nepali has potential tendency of promoting cell migration. The in vitro results suggest that Thai and Nepali propolis have chlorogenic acid, which may help in cell migration. The wounds which do not have new cell generation power and cell migration properties, then the wound would not heal. However, the results also showed that percentages of cells migration caused by Thai propolis were lower than Nepali propolis. The reason may that the propolis collected from the different geographical regions has different chemical properties and wound healing rate.

The results showed that the Thai propolis has wound healing properties both in vitro and in vivo. The freshly created wounds were healed within 14 days. The most possible explanation if that Thai propolis has enzyme that stimulate collagen formation in wounds as improving of created wounds. Subsequently, Ghisalberti, (1979) and Krell, (1996) have also observed similar results. These effects have been shown to be the result of the presence of arginine in propolis (Gabrys, et al, 1986).

The results showed that Thai propolis has Chlorogenic acid (CGA) which is belongs to the important group of biologically active compound phenols of which 5-caffeoylquinic acid (5-CQA) is the most important (Johnston et al., 2003). CGA has been shown to have antioxydative properties in the laboratory experiment (Johnston et al., 2003). It is believe that the content of polyphenols in propolis can contribute to cardio protection effect. Ethanolic extract of Thai propolis has shown an in-vitro cytotoxic effect on human cells which means that Thai propolis has anti cancer properties. The component that produces cytotoxic was identified as Chlorogenic acid. This component is a part of caffeic acid phenethy ester. Previous studies have also showed that ethanol extracts of propolis have a cytotoxic and cytostatic effect in vitro against hamster ovary cancer cells and sarcoma-type tumours in mice (Ross, 1990). The substance has also displayed cytotoxicity on cultures of human and animal tumor cells, including breast carcinoma, melanoma, colon, and renal carcinoma cell lines (Grunberger, *et al,* 1988). The component producing these effects was identified as caffeic acid phenethy ester. Caffeic acid esters have been shown to inhibit chemically induced tumor production in mice, as well as having a selective toxic effect on cells affected by genes which promote the development of cancerous cells.

CHAPTER 6
CONCLUSIONS

The Thai propolis has shown cytotoxicity, cell proliferation and cell migration in *vitro* tests. In vivo experiments, the fresh wound created on the experimental rabbits were healed within 10 days. The bioactive compound "5-caffeoylquinic acid" (5-CQA) has identified from Thai propolis. Chlorogenic acid also is a competitive inhibitor of glucose-6-phosphatase (Glc-6-Pase) in intact microsomes (Arion *et al.*, 1997). Hepatic GLC-6-Pase may be a key control site in the homeostatic regulation of blood glucose concentration, and the increase of GLC-6-Pase activity is widely held to be a significant factor in the abnormally high rates of hepatic glucose production observed in the diabetic state (Arion, *et al.*, 1997). The chlorogenic acid presence can be mitigated cellular damage by the simultaneous formation of peroxynitrite and release of myeloperosidase during chronic infection and inflammation (Grace *et al.*, 1998).

REFERENCES

Aripov, K. L. M., Kamilov, I. K., Aliev Kh. U. (1968). Effect of propolis on experimental stomach

ulcers in rate Jr. Medish. Zh. Uzbek. (5): 50-52.

Arion W. J., Canfield W. K., Ramos F. C., Schindler P. W., Burger H. J., Hemmerle H., Schubert G., Below P., and Herling A. W. (1997), Chlorogenic acid and hydroxynitrobenzaldehyde: New inhibitors of hepatic glucose 6-phosphatase. Arch. Biochem. Biophys. 339: 315-322.

Ciaceri, G., Attaguile, G. (1972). Influence della luteolina, dell'apigenina e dell'acacetina sull'ulcer gastric spermentale. Minerva Med. 63(29): 1665-1668

Cohen, L.; Djordjevich, J. and Jacibsen, S. (1966). The contribution of isoenzymes of serum lactic dehydratogenases (LDH) to the diagnosis of specific organ injury. *Med. Clin. North. Am.*, 50: 193-205.

Doroshenko, P. N (1975) Propolis and chronic pharyngitis, in A Value-added bee products: Propolis Researches and views of its composition, properties, and therapeutic value: Apimondia Romania 106 pp

Gabrys, J., Konecki, J., Krol W., Scheller S., Shani, J. (1986) Free amino acids in bee hive product

(propolis) as identified and quantified by gas-liquidchromatography. Pharmacol Res Comm;18: 513–518.

Ghisalberti E L. (1978). Propolis—review. *Bee World,* 60:59–84

Giurcaneanu, F., Crisan I., Esanu V., Cioca, V., Cajal, N. (1988). Treatment de l'herpescutane et du zona zoster a laidia de Nivcrisol-D. Rev. Roun. Med. Virol. 39:21-24

Greenaway W, Scaysbrook, T., Whatley F. R. S (1988). Composition of propolis in Oxfordshire, UK and its relation to propolis bud exudates, *Z. Naturforsch.*, 43c: 301-304.

Grange, J M, Davey, R W (1990). Antibacterial properties of propolis (bee glue), *Jr. Roy. Soc. Med.* 83(3): 159-160.

Gafar, M., Sacalus A., David e., David N. (1986) Treatment of simple pulp gangrene with the apitherapy product propolis. Stomatologie, 33:115-117.

Grace, S. C., Salgo, M. G., and Pryor, W. A. (1998), Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage DNA. FEBS Lett., **426**, 24-28.

Grunberger, D, Banerjee, B., Eisinger K., Oltz E. M. (1988). Preferential cytotoxicity on tumor cells by caffeic acid phenethyl easter isolated from propolis. Experientia 44:230-232.

Hay K. D. Grieg, D. E. (1990).Propolis allergy : a cause of oral mucositis with ulceration. Oral Surg. Oral Med. Oral Pathol 70: 584-586.

Harborne, J. B, Williams, C. (2000) Advances in flavonoid research since 1992. *Phytochem* 55: 481–504

Hashimoto, T., Tori, M., Wollenweber E. (1988). Synthesis of two allergenic constitutes of propolis and poplar bud extraction. Z. Nat. 43c: 470-472

Hausen, B. M.; Evers, P.; Stuwe, H. T.; Konig, W. A.; and Wollenweber, E. (1992). Propolis IV. Studies with further sensitizers from propolis and constitutes common to propolis, poplar buds and balsam of Peru. *Contact Dematitis*. 26: 34-44.

Hausen, B. M., Wollenweber, S., Senff, H., Post, B. (1987). Propolis allergy: origin, properties, usage, and literature review. Conct. Derma. 17:163-170

Ikeno, K., Ikeno T., Miya zawa C. (1991). Effetcs of propolis on dental caries in rats, Caries Res. 25: 347-351

Kedzib, B.; Iwaszkiewicz, J.; and Geppert, B. (1988). Pharmacological investigations on ethanolic extraction of propolis *Herba. Pol.* 34: 243-53.

Korsun, V P. (1983).The use of propolis in treating trophic ulcers. Vestin. Dermatol. Veneol, 11: 46-48

Krell, R (1996). Value-added products from beekeeping. FAO Agricultural Services Bulletin 124 pp

Johnston K. L., Clifford M. N., Morgan L. M. (2003). Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. Am. Jr. Clin. Nutr.78:728-33.

Marcucci M. C. (1995) Propolis—chemical-composition, biological properties and therapeutic activity. *Apidologie*;26:83–99.

Matsuno T, Matsumoto Y, Saito M, Morikawa J. (1997). Isolation and characterization of cytotoxic diterpenoid isomers from propolis. *Zeitschrift Naturforschung C J Biosci*;52:702–4.

Matel, I., Straka J., Cizmark, J., (1973). Results of the use of propolis in the treatment of ear, nose and throat diseases. 1st symposium of propolis 64-77.

Metzner, J., Bekemeire, H., Paintz, M., Schneidewind E. (1979) Zur antimikrobielle wirksamkeit von propolis und propolisinhaltsstoffen, Pharmazie, 34:97-102

Mirozeva O. K., Calder, P. C. (1996). The effect o propolis and its components an eicosanoid production during the inflammatory response. *Prosctaglandins Leukot Essent Fatty Acid*. 55: 441-449.

Paintz, M., Metzner, J. (1979), Zur Lokalanästhetischen Wirkung von Propolis und einigen Inhaltsstoffen, Pharmazie **34**, 839-841.

Palos, E., Popescu, F., Mateescu C (1989). Apitherapeutics in the treatment of several otorhinolaryngologic diseases. Proc. XXXII inter. Conf. Api. Rio de Janeiro, Brazil. 214.

Ross, P. B. (1990). The effects of propolis fractions on cells in tissue culture. MPhil. Thesis. Uni. Wales. Cardiff, UK 193 p.

Scheller, S., Krol, W., Swiacik, W., Owczarek, S., Gabrys, J., and Shani J. (1989). Antitumor property of ethanolic extract of propolis in mice bearing Ehrlich carcino, as compared to bleomycin. Z.Naturforsch 44c: 1063-1065.

Volpert R and Elstner EF (1993). Biochemical activities of propolis extracts. I. Standardisation and antioxidant properties of ethanolic and aqueous derivatives, *Z.Naturforsch.*, 48c: 851-857.