# Preliminary Determination of Cutaneous Wound Healing and Antibacterial Properties of Thai Propolis

**Ratna Thapa Ph.D.**

**School of Science**

**Mae Fah Laung University**

**Chiang Rai 57100**

**Tel: 053-916778, Fax: 053-916776**

**Email: rthapa@yahoo.com**

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Ratna Thapa
School of Science
Mae Fah Luang University
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## ABSTRACT

Propolis extracts use in folk medicine has still one of the most frequently applied natural antibiotics for treatment of wounds, burns, soar-throat, and stomach ulcer. The main objectives of this research was to determine equine cutaneous wound healing properties of ethanolic extractions of Thai propolis and to determine the antibacterial properties of Thai propolis against pathogenic bacteria isolated from the chronic cutaneous horse wounds. Propolis samples were collected from *Apis mellifera* colonies from Chiang Saen, Chiang Rai. Eleven out of 36 horses (30.5%), 2 horses (5.6%) in Faculty of Veterinary Medicine, Chiang Mai University; 5 (14%) in Lanna Riding Club, and 3 (8.3%) in Pack Squad Army Battalion were used in this experiment. Wounds were evaluated by bed tissue color, measurement of wounds and calculation of wound contraction rate. The results of this study have demonstrated that ethanolic extracts of propolis healed chronic second-infection of the body wounds whereas the wounds on the distal parts of limbs were not cured. Three types of pathogenic bacteria *viz Streptophylococcus saprophyticus, S. haemolytius* and *S. intermedius* were isolated from the wounds. The results demonstrated that all three pathogenic bacteria were susceptible to EEP and that body wounds cured faster than lower limb wounds. Ethanolic extracts of Thai propolis can heal chronic wounds of horses and also wounds caused by IBH. Thai EEP can inhibit the growth of three pathogenic bacteria isolated from the wounds. Thai propolis has also antibacterial and cell generating properties.

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## INTRODUCTION

### Propolis

The word "propolis" is derived from the Greek "*pro*" for, or in defense, and "*polis*" the city, or community. This implies the defense the city (hive) (Nicolas, 1947). Propolis is commonly known as bee-glue (Figure 1). This is a resinous substance produced by the European honeybee, *Apis mellifera* L., obtained from different parts of plants (Ghisalberti, 1979). The Asian honeybees: *A. dorsata, A. laboriosa, A. florea* and *A. andreniformis* neither collect nor use propolis. *A. mellifera* uses propolis in several ways, e.g. to polis the internal part of cell walls, to seal cracks, crevices, and entrances of hives (Alfonsus, 1933; McGregor, 1952). The presence of propolis in the hive is responsible for inhibiting the growth of bacteria, and other microorganisms (Derevici and Petrescu, 1965).

**FIGURE 1.** *Apis mellifera* sealing a hive with propolis

### Chemical composition of propolis

The materials used by honeybees to produce propolis are lipophilic materials deposited on leaves, leaf buds, resins, gums, and substances secreted by plants as well as substances

exuded from plant wounds (Crane, 1990). The chemical composition of propolis depends on the plant origin. Propolis from high elevation has completely different chemical composition than from the lower places, because of the different floras, and seasons (Marletto, 1984). The chemical composition of propolis from the same area in different seasons can have different chemical compositions. Asian propolis has a different chemical composition than European propolis. More than 180 chemicals have been identified from a single sample of propolis by a gas chromatography (Cohen *et al.,* 1966; Greenaway *et al.,* 1990). The most important bioactive compounds in propolis are flavonoids (Grange and Davey, 1990). Around 38 types of flavonoids including galangin, kaempferol, quercetin, pinocembrin, pinostrobin, and pinobanksin have been identified from propolis (Schmidt and Buchmann, 1992). The flavonoids present in propolis are not glycosides, because they do not contain sugar molecules which are widely found in plants. Another important active chemical found in propolis is caffeic acid phenethyl ester (CAPE) (Banskota *et al.,* 2001). In addition, propolis also contains vitamin $B_1$, $B_2$, C, E, nicotinic acid, pantothenic acid, copper, and manganese. The color of propolis is also variable, and ranges from yellowish-green to red-dark brown in color depending upon on the species of plants, and the season (Table 1).

Table 1. Propolis types according to plant origin and chemical compositions.

| Propolis type | Geographic origin | Plant source | Main chemical active substances | References |
|---|---|---|---|---|
| Poplar propolis | Europe, North America, non-tropical regions of Asia | *Populus*, most often *P. nigra* L. | Flavones, cinnamic acids and esters | Nagy *et al.*, (1986), Bankova *et al.,* (2000) |
| Birch propolis | Russia | *Betula verrucosa* Ehrh. | Flavones, flavonols | Popravko and Sokolov (1980) |
| Green propolis | Brazil | *Baccharis* spp., | Prenylated *p*-coumaric acids, diterpenic acids | Marcucci and Bankova (1999) |

| | | | | |
|---|---|---|---|---|
| Red propolis | Cuba, Venezuela | *Clusia* spp. | Polyprenylated benzophenones | Cuesta *et al.* (2002) |
| Pacific propolis | Pacific region (Taiwan) | Unknown | C-prenylflavanones | Chen *et al.*, (2003) |
| Canarian propolis | Canary Islands | Unknown | Furofuran lignans | Christov *et al.*, (1999) |
| Black propolis | Northern parts of Thailand | Unknown | Unknown | per. observations |
| Yellow propolis | Central parts of Nepal | Unknown | Unknown | per. observations |

**Pharmacological properties of propolis**

Numerous studies have already proven propolis versatile pharmacological activities such as antibacterial, antifungal, antiviral, antiinflammatory, hepatoprotective, antioxidant, and antitumor (Banskota *et al.*, 2001). Propolis is used to heal wounds and cartilaginous tissues regeneration (Scheller *et al.*, 1977), bone tissue regeneration (Stojko *et al.*, 1978), dental pulp (Scheller *et al.*, 1978; Magro and Carvolho, 1990), hepatoprotecion activity (Tushevskii *et al.*, 1991), immunomodulatory action (Bankova *et al.*, 1989; Dimov *et al.*, 1992), liver detoxifying and antiulcer action *in vitro* (Kedzia *et al.*, 1988), antioxidant activity (Scheller *et al.*, 1990; Olinescu, 1991), antifungal (Thapa and Wongsiri, 2004), and antitumour use (Scheller *et al.*, 1989). Thapa and Wongsiri (2004) reported that fungal skin disease (psoriasis) in humans is completely cured within 8 weeks after topical application of 300 µg/ml of propolis. Kedzia *et al.*, (1988) also found that a normal glucose level in rabbits after oral administration of propolis. They have also observed reduced of blood pressure in rats. The therapeutic effects of propolis mainly depends on the amount of flavonoids which widely occur in fruits, vegetables, nuts, seeds, stems, and flowers (Havsteen, 1998; Middleton and Chithan, 1993), and honey (Grange and Davey, 1990). Flavonoids are also represented a common constituents of human diets (Harborne and Baxter, 1999). Flavonoids are versatile antioxidants against reactive oxygen species (ROS) that causes cell damage and are implicated in many diseases (Cushnie, and Lamb

2005). The world consumption of propolis is estimated to be around 700-800 tons per year (Nothenberg, 1997).

**Antibacterial effects of propolis**

The sensitivity of ethanolic extracts of propolis (EEP) against several strains of bacteria in *in vitro* tests has been reported by several authors (Pepeljnjak *et al.,* 1981; Toth and Papay, 1987; Petri *et al.,* 1988; Dobrowolski *et al.,* 1991; Kujumgiev *et al.,* 1993; Meresta and Merersta 1985; Woisky *et al.,* 1994). Meresta and Merersta (1985) examined the sensitivity of 75 bacterial strains against EEP. Sixty-nine out of 75 (92 percent) were mostly *Staphylococcus* spp., and *Streptococcus* spp.. All of which are highly sensitive to EEP. *S. aureus* 209P has resistance to several antibiotics, and has shown minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) values 10, and 20 mg/ml, respectively. Grange and Devey (1990) reported that 3 mg/ml of EEP can completely inhibit the growth of *Pseudomonas aeruginosa*, and *Escherichia coli*, but did not show any effects against *Klebsiella pneumonia*. Lavie (1957) reported that EEP has significant antibacterial activity against *B. subtitis*, *B. alvei*, and *Proteus vulgaris*.

**Synergetic effects of propolis against fungus**

Millet-Clerc *et al.,* (1986) demonstrated that EEP has antifungal activity against *Trichophyton* sp., and *Mycrosporum sp.*. They also found that the greatest synergistic effect of propolis against many fungi. Lisa *et al.,* (1989) verified the antifungal activities of 10% of EEP against 17 pathogenic fungi. Fernandes *et al.,* (1995) evaluated antifungal activity of EEP against *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii*. They have demonstrated that 98% of fungi were sensitive to less than 5% of EEP. La Torre *et al.,* (1990) observed that propolis inhibited some plant fungi in *in vitro* tests, but they did not mention which types of plant fungi were inhibited.

**Mammalian skin**

Mammalian skin acts as a barrier to exogenous substances, pathogens, trauma, and has a crucial role in protecting the whole body (Singer and Clark, 1999). Skin is composed of complex tissues which provide important contributions towards regulatory, and protective

functions (Mast, 1992). Skin can histologically be divided into two layers: outer and inner layers. The outermost layer is very thin, the epidermis, and is composed of densely packed keratinocyte cells which continually proliferate, and differentiate (Dover and Wright, 1991). Under the epidermis is the dermis, which is composed of fibroblast cells, matrix, blood capillaries, and structural components (Mast, 1992). Fibroblast cells perform many vital roles in dermal wound healing, and wound contraction (Clark, 1988; Rudolp *et al.*, 1992).

A wound is typically physical trauma where the skin is torn, cut, punctured (an *open* wound), or where blunt force, trauma, causes a contusion (a *closed* wound). In pathology, it specifically refers to a sharp injury, which damages the dermis of the skin. Wounds by etiology are of six types: (i) abrasions, superficial scrapes, or friction burns that may scab as they heal; (ii) contusions, bruises, and swellings that have no external drainage; (iii) incisions, cuts which have clean edges, and often heal with little scarring; (iv) lacerations, cuts with jagged tears, and uneven edges; (v) punctures, wounds that are deeper than wide, and (vi) burns caused by friction, heat, cold (frostbite), chemicals, and/or electric shock.

**Horses**

The horse (Order: Perissodactyla; Genus: *Equus*) is a large odd toed ungulate mammal (Figure 2). Modern horses are economically more important than domesticated animals, cows, pigs, dogs, hens. Horses are prominent in religion, and mythology. The life span of a horse is around 25 years. Horses are prey animals. Their skeletons are like a human's, but their shoulders are not anchored in a socket which allows them to run fast. Horses play a crucial role in human economies, leisure, sport, and work. Horses exhibit a diverse array of coat colors, and distinctive markings. People refer to a horse in the field by its coat color rather than by breed or by sex, and the quality of a horse depend on the skin coat color. It is necessary to keep horses healthy. Horses suffer from two common problems; open wounds, and insect bite hypersensitivity (IBH).

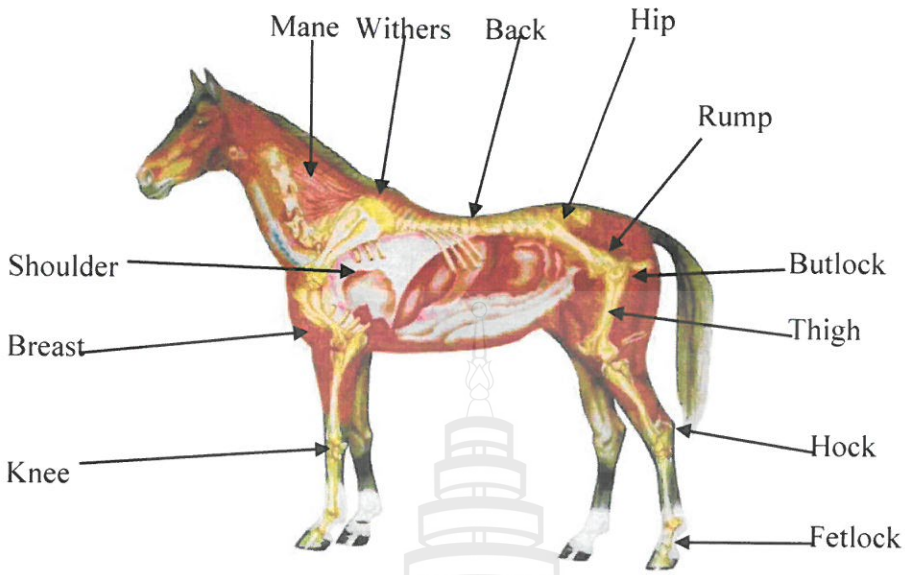


**FIGURE 2.** Morphology of a horse

**Open wounds**

Open wounds are a very common problem in horses. Based on etiological appearance, depth, and degree of contamination, wounds have been classified as partial, or full thickness wounds (Waldron and Trevor, 1993). Partial thickness wounds are surface wounds involving the loss of epidermis, and some dermis, but some epidermal elements are still present in the dermis. Full thickness wounds are deep wounds extending through epidermis, and dermis including the subcutaneous tissue, and other underlying areas. Wounds can heal in two ways: (i) primary intention-healing wounds and (ii) secondary intention-healing wounds. Primary intention healing wounds heal very fast, because they do not depend upon many factors such as wound status, environmental factors, and method of treatments. Second-intention healing wound take a long time to heal, and is often very complicated (Jacobs *et al.*, 1984; Knottenbelt, 1997). Second-intention healing wounds can not be entirely treated (Caron, 1999), because exuberant granulation tissue is formed in horses (Fretz *et al.*, 1983; Bertone *et al.*, 1985; Barber, 1990). The wound healing process in horse, like in humans can be divided into four phases: (a) the inflammatory phase; (b) formation of granulation tissue; (c) wound contraction, and (d)

epithelialization (Stashak, 1991). Wounds normally heal as a result of formation of granulation tissue. When a wound is filled up with granulation tissue, the wound starts to contract by allowing epithelial cells to migrate across the surface, and close it off. This is the normal process of wound healing. In horse leg wounds, the granulation tissue fails to form and as a consequence, the wound can not heal. This can also happen in a human, which is why, leg ulcers and pressure sores are so difficult to heal. Sometimes the granulation tissue grows like a mushroom out of the wound, and prevents the wound from healing. In this situation, a remedy is to simply cut away the granulating tissue. If the wound has developed mushrooming tissue, a tumor commonly known as a sarcoid, is very dangerous for horses. These types of open wounds are usually infected by bacteria. The bacteria enter into the horse's body through wounds. Wounds provide warm, moist, and air-free environment, which are ideal conditions for bacterial growth. Wounds based on bacterial loads can be classified as clean, contaminated, and dirty wounds. Clean wounds are non-traumatic wounds created under aseptic conditions; contaminated wounds include all fresh open traumatic wounds without significant devitalised tissue, and dirty wounds include traumatic wounds including faecal contamination, devitalised tissue, and foreign bodies (Coomer, 2006). Contaminated wounds are called chronic wounds which show considerable diversity in the density, and species of bacteria, especially, *S. aureus, S. intermedius*, and *P. aeruginosa* being the most predominant organisms. *P. aeruginosa* is consistently reported from chronic wounds with a frequency range from 7-33% of ulcers (Cutting and Harding, 1994). Some bacteria such as *Streptococcus* spp., *Proteus* spp., and *P. aeruginosa* are capable of producing destructive proteolytic enzymes, which breakdown fibrinous attachments between the graft, and recipient bed. These bacteria can also produce excessive purulent discharge which forces the graft from the bed (Robson *et al.*, 1970).

**Insect bite hypersensitivity**

Insect bite hypersensitivity (IBH) commonly known as sweet itch (SI), Queensland's itch (QI), *Culicoides* hypersensitivity (CH), summer eczema (SE), allergic eczema (AE), Summer Seasonal Recurrent Dermatitis (SSRD), Mexican itch (MI), Kasen (in Japan), Dhobie itch, or Spanish itch, is a reoccurring seasonal dermatitis disease of horses (Fadok

and Roil, 1990). This disease is usually associated with blood sucking flies (Order: Diptera), and also has been found in various domesticated animals: cows, sheep, pigs, and dogs (Braverman *et al.,* 1983). IBH does not infect all horses, and domesticated animals, but certain horses are genetically very sensitive to the saliva of *Culicoides* sp. flies (Family: Ceratopogonidae) (Riek, 1953; Halldorsdottir and Larsen, 1991). Once a sensitive horse is bitten by *Culicoides* flies, IBH can develop as an allergic reaction at the site of the bite, and cause a localized irritation. As a consequence, the horse may be restless and biting itself on itching sites, or rub against trees, or fences, or keep swinging its tail to keep the flies off. If the itching is persistent for long time, it may cause self-inflicted damage on the itching sites. This type of horse is called IBH, or Equine *Culicoides* Hypersensitivity (ECH). IBH are usually of two types: Type-I, and Type-IV allergic dermatitis. Type-I is an acute hypersensitivity reaction, whereas Type-IV is delayed type.

IBH is a seasonal disease, and occurs from spring to fall parallel with the season of *Culicoides* flies presence. IBH can affect any breed, age, and sex. IBH commonly occurs on the forehead, ears, neck, withers, and hips. In more severe cases, IBH occurs in the mid-line of the belly. Clinical signs of IBH are itching, hair loss, skin thickening, and flaky dandruff (Figure 3A). The exudative dermatitis are weeping sores, sometimes with a yellow crust of dried serum may also occur. As the disease progresses, the skin becomes chronically thickened, blackened, wrinkled, and the hair becomes sparse, and coarse (Figure 3B). The tail takes on a characteristic rat-tailed appearance (Figure 3C). During cool winter in tropical Asia, the horse is totally healed with the absence of *Culicoides* flies, and the disease comes back in summer at the first contact with *Culicoides* flies. IBH is similar to asthma, psoriasis, and atopy involving gene interactions (Kawano *et al.,* 2005). The susceptibility of horses to IBH is due to equine leukocyte antigens such as $Be_1$, $Be_8$, $W_1$, $W_7$ and $W_{23}$ (Halldorsdottir and Larsen, 1991). IBH is most probably a multi-factorial disease (Kawano *et al.,* 2005; Vercelli, 2004). It requires an extensive healing process.

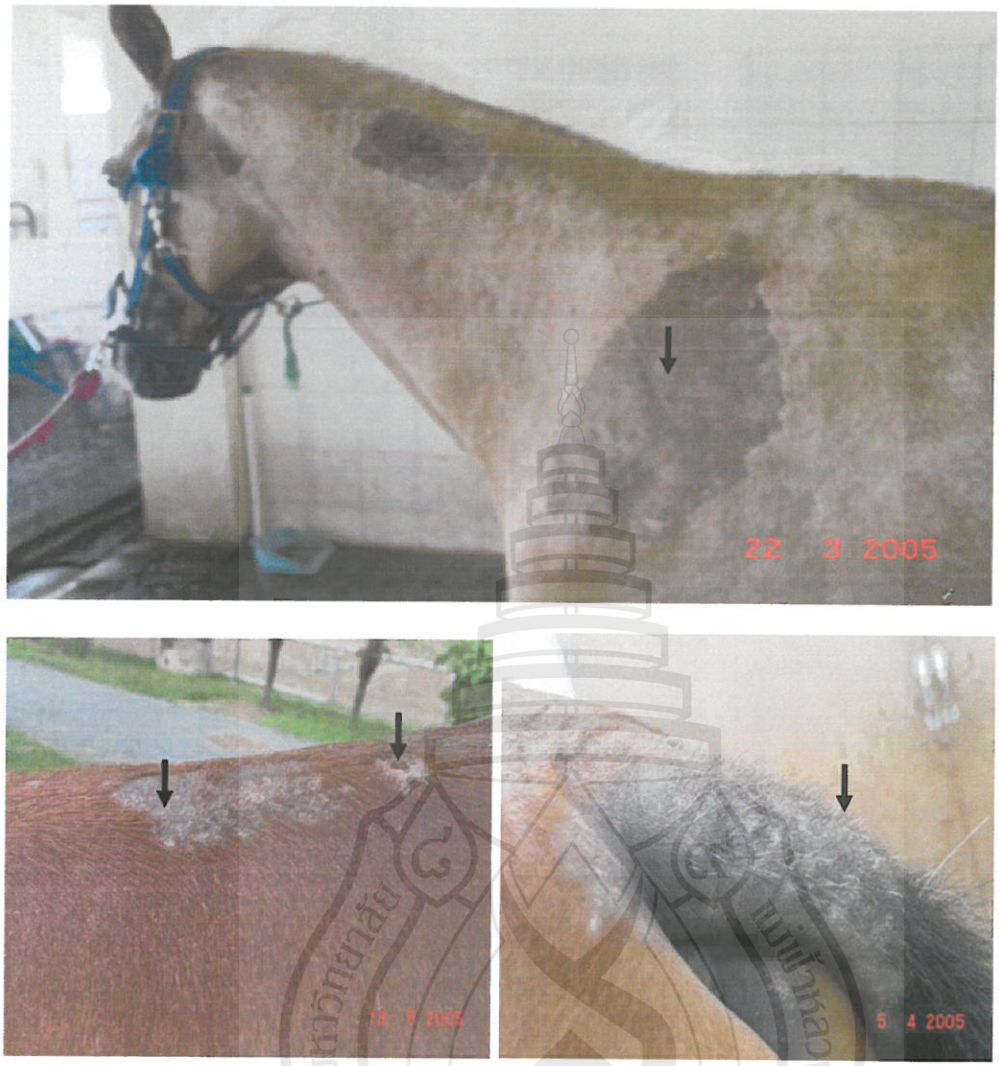


**FIGURE 3.** The photographic prints show symptoms of insect bite hypersensitivity (IBH). (A). IBH on head, neck and shoulder (B) IBH on back and (C) IBH on tail (rat tail appearance) in the Faculty of Veterinary Hospital, Chiang Mai University, and Lanna horse riding club, Changpuek, Chiang Mai in the year 2005.

The literature shows that the Icelandic horse is one of several known horse breeds that frequently suffer from IBH. Thirty percent of those horses, and 6% of the horses born outside Iceland develop summer eczema, this suggests that IBH not only the genes decide the outcome of IBH, but the environment plays a crucial role as well. Recent

studies on IBH in Icelandic horses have shown that it is a heritable disorder skin disease (Hellberg *et al.*, 2006). A strong positive correlation has been demonstrated between histamine or sulidoleukokotriene (sLT) release (Marti *et al.*, 1999). They have suggested that sLT produced by equine peripheral blood leukocytes after incubation with *Culicoides*, or *Simulium* extracts are mostly generated by basophils.

**Rationale**

Once a horse is wounded, and/or has developed IBH, the horse losses its commercial value has lower performance, as well as public interest. Wounded and IBH horses need frequent treatment which is a time consuming, expensive, and painful for horse. The wounds, and IBH have a significantly correlationship, because once the horse has developed IBH, the wounds then are colonized by diverse pathogenic bacteria. These types of wounds are called chronic wounds (second-intension healing wounds). All chronic wounds are contaminated first by gram-positive bacteria, and then followed by gram-negative bacteria. Common pathogenic bacteria are: *Staphylococcus aureus,* Beta-hemolytic *Streptococcus* (*S. pyogenes, S. agalactiae*), *E. coli, Proteus, Klebsiella,* anaerobes, *Pseudomonas, Acinetobacter*, and *Stenotrophomonas* (Dow *et al.,* 1999). Thus, chronic contaminated wounds can not be cured by conventional western medicine, and are full of pathogenic bacteria, *viz S. aureus*, *S. intermedius*, and *P. aeruginosa*, which have already developed resistance to modern antibiotics like penicillin by forming biofilms, which protect them from topical antibiotics.

Wound infections caused by pathogenic bacteria, and previously controlled by antibiotics are now becoming resistant to most of the currently used antibiotics. The increasing incidence of this previously controlled infectious disease is partially due to either overuse, and/or misuse of antibiotics. When veterinarians diagnose an infectious disease, IBH, or chronic wounds, they generally administer antibiotics that have proven to have the highest success rate in curing that infection. The problem with this strategy is that veterinarians do not always know what bacteria they are fighting with antibiotic unless further tests are done in a laboratory. Many reports have suggested that there may be a long-term problem with this approach. In clinical situation, it is often inefficient,

time-consuming, and too expensive to submit samples for microbiological culture, and antibiotic susceptibility testing (Dowling, 2003).

Microbes have developed resistance against antibiotics routinely used to treat domestic animals, and livestock. Antibiotic e.g. Chloramphenicol®, Vancomycin®, and Bacitracin® are relatively ineffective in killing normal horse microbial flora. Both Vancomycin®, and Bacitracin® are human antibiotics, and are used much in the veterinary medicine. Amikacin®, Cefotaxime®, Ciprofloxacin®, and Ceftriaxone® are highly effective with resistance values under 20% whereas Clindamycin®, Ampicillin®, Ticarcillin®, Trimethoprim®, and Tetracycline® have between 20%, and 50%. These numbers are comforting for now, but as resistant organisms become more prevalent the antibiotics will become increasingly ineffective. This can happen at an inexorable rate. Bacteria susceptible to antimicrobials are killed while those bacteria which have the ability to resist the medicine can survive and multiply. The surviving pathogenic bacteria can develop resistant to antibiotics, and as a consequence, the resistant genes are passed from a resistant bacterium to a sensitive one. Antibiotics may increase the prevalence of resistant bacteria among both target pathogens, and normal bacterial flora (WHO, 1998).

A wide range of antibiotics available in the form of topical applications are not always absorbed into the wound bed, and are potentially hazardous to cells. Because of varied barrier, especially necrosis, and pus prevent ointments to diffuse in wounds. Topical antibiotics often do not reach deep in the infected wounds. In addition, there is considerable risk to veterinarians and horses of sensitization, and the development of resistant organisms, especially, when used routinely over prolonged periods in uninfected wounds. Certain topical antibiotics impair proliferation and epithelialization of wounds. For these reasons, topical antibiotics have become controversial, and are no longer recommended.

The cleansing activity of many wound cleansers is dependant on a surfactant that breaks the bonds between foreign bodies, and wound surface (Rodeheaver, 2001). Unfortunately, most ionic, and nonionic surfactants have shown to be toxic to cells, delay wound healing, and inhibit wounds defenses against infection (Edlich *et al.,* 1973). Antiseptics are used early in the wound healing process primarily to reduce bacterial density as well as to reduce the chances of infection. Antiseptic solutions have some toxic

effects that may do more harm than good (Rodeheaver, 2001). For example, hydrogen peroxide (3%) is an effective sporocide with a narrow antimicrobial spectrum, has been shown to be cytotoxic to fibroblasts, and result in thrombosis of microvasculature (Stashak, 1991). The cellular toxicity of hydrogen peroxide to fibroblasts exceeds its bacterial potency, therefore, it is unsuitable as a wound cleansing solution (Swain, 1987). Povidone iodine solution (10%) has a broad range of antimicrobial activity that lasts for 4-6 hours following application. It kills bacteria within 15 seconds, but it is not known about bacterial resistance to this product. In an ex-vivo rat model, betadine has shown to have a negative effect on microcirculation (Stashak, 1991). Povidone iodine solution is inactivated by organic material, and blood (Hugo and Newton, 1964). In this situation, significant bacterial regrowth has been observed with povidone iodine solution. Chlorhexidine diacetate (2%) solution has a wide antimicrobial spectrum, and prolonged residual effect due to its ability to bind proteins in the stratum corneum, and continue activity in the presence of blood, pus, and organic debris with less systemic absorption. A potential drawback of chlorhexidine diacetate is that *Proteus*, and *Pseudomonas* have developed resistance to this drug, and it also has no effect against *Candida* sp. (Prince *et al.*, 1978). Triple antibiotic ointment (Bacitracin, Polymyxin B and Neomycin) has a wide antimicrobial spectrum, but is ineffective against *P. aeruginosa* (Swain, 1987). The zinc component of bacitracin has been shown to stimulate epithelialization (increasing it by 25%), but retard wound contraction. These antimicrobials are poorly absorbed, therefore, toxicity is rare. Silver sulfadiazine (1%) has a wide antimicrobial spectrum including *Pseudomonas* and fungi, but it is slow in epithelialisation, and it may increase wound fragility (Southwood and Baxter, 1996). Nitrofurazone ointment has a good antimicrobial spectrum against gram-positive, and gram-negative bacteria, but has little effect against *P. aeruginosa*, and also delays wound contraction in horses (Stashak, 1991).

A chronic wound fails to heal through prolonged, and incomplete treatment with lack of restoration of integrity and function, frequently occurs, when some factor is disrupted the normal inflammatory phase or the cellular proliferative phase. Generally, the most common cause that stops wound healing is infections: tissue hypoxia (bacteria infection), repeated trauma, presence of debris and necrotic tissue. All wounds are more,

or less contaminated with bacteria with 100,000 ($10^5$) microorganisms per gram of tissue, and can still be healed successfully. Beyond this number, a wound may become chronic.

Arthropod allergies can cause development of skin lesions (wounds) as a result of hypersensitivity to *Culicoids* bites and is very common in horse, and ponies (Riek, 1953; Baker and Quinn, 1978; Braverman *et al.,* 1983; Fadok and Greiner, 1990). IBH is most common in horses, and is seasonally occurring diseases. IBH occurs in April-May (hot dry), and August-September (rainy season) in Thailand. Imported horses in Thailand frequently suffer from IBH, which are often delayed and complicated to heal (Caron, 1999). Usually, the imported horse from Germany, Australia, and New Zealand suffers more frequent IBH because of the hot climate in Thailand and frequent exposure *Culicoids* flies. This is becoming a problematic issue for both horses, and veterinarians for keeping flies off from wounds with an application of insect repellents. The application of corticosteroids, and NASAIDS is not advisable due to there adverse effects on the migration of leukocytes, during the first phase of second-intention wound healing (Proper *et al.,* 1988). Numerous treatments have been advocated for the promotion of wound healing, and prevention of hypergranulation tissue in horses, but the majority of these are of unproven efficacy (Steel *et al.,* 1999, Dart *et al.,* 2002).

Propolis is a natural antibiotic, antibacterial, antifungal, and antiseptic. Propolis is commonly used to treat infections and wounds in humans. Propolis has shown significant antimicrobial activity against gram-positive bacteria, and some gram-negative bacteria (Stepanovic *et al.,* 2003). No research has been conducted to determine the wound healing properties of Thai propolis on wounds of horses. This research can provide a new potential natural product, as a natural antibiotic for wounds, and dermatitis treatment in horses. Considering the discussion above antibiotics administrated to chronic wounds, and IBH of animals, certain basic questions need to be answered such as: (a) how does propolis heal chronic wounds of horses?, (b) what would be the health benefits of topical application of propolis? and (c) how effective is Thai propolis in wound healing and inhibit pathogenic bacteria?

**Usefulness of this study**

1 This study can provide information on wound healing properties of Thai propolis,

2 The findings of this research can help to cure chronic wounds of horses and other domesticated animals,

3 The results of this research can give conclusive suggestions on how propolis can cure chronic wounds of horses to veterinary doctors, veterinary students, horse riding clubs, farmers etc., and

4 The findings of this research can help to enhance income generation of Thai beekeepers.

## OBJECTIVES

- to determine equine cutaneous wound healing properties of ethanolic extraction of Thai propolis, and

- to determine the antibacterial properties of Thai propolis against pathogenic bacteria isolated from the chronic cutaneous wounds of horses.

## HYPOTHESES

### Research Hypothesis

1 The research hypothesis is that Thai propolis contains antibiotic chemicals that heal chronic cutaneous wounds of horses by killing, and balancing the pathogenic bacteria load.

### Statistical Hypothesis

$H_0$ = recovery ratio of cutaneous wound healing after treatment is equal to

1.00 [There is no difference in the size of wounds before, and after treatment, i.e. wound area after treatment will be equal to the initial size]

$$H_0 : \mu_{treatment} = \mu_{control}$$

$H_1$ = recovery ratio of cutaneous wound healing after treatment < 1 .00

$$H_1 : \mu_{treatment} \neq \mu_{control}$$

## MATERIALS AND METHODOLOGY

### Study sites

This study was conducted at the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai; Lanna Riding Club, Changpueak, Chiang Mai, and Pack Squadron, Army Battalion, Mae Rim, Chiang Mai, Thailand.

### Propolis collection

Propolis samples were collected from *A. mellifera* colonies at Chiang Saen, Chiang Rai. The samples from each hive were kept in a separate plastic bag, and stored at -10°C, until it was used.

### Preparation of propolis tincture

EEP samples were extracted with 95.6% ethanol ($C_2H_5OH$; MW. 46.07 g/mol) (Liquid Distillery Organization, Excise Department, Bangkok, Thailand) according to the guidelines of FAO bulletin no. 124 (Krell, 1996), at room temperature (28-30 C), and agitated two times per week. After two weeks, the propolis was extracted using Whatman No.1 filter papers (diameter 110 mm), and stored at 10°C.

**Experimental designs**

**Experimental horses**

A total of 36 horses in three different horse stable (as above mentioned) were surveyed. Eleven out of 36 horses (27.7%), 2 horses (5.6%) in the Faculty of Veterinary Medicine, Chiang Mai University; 5 (14%) in Lanna Riding Club, and 3 (8.3%) in Pack Squad Army Battalion were used in this experiment.

**Selection of horses**

For this experiment, two types of horses, wounded, and IBH based on clinical diagnosis, were selected. All the selected horses had suffered for several years either with wounds, or IBH. The selected horses were classified into two groups: GROUP-A: wounded horses, and GROUP-B: IBH horses. The selections of horses for this experiment included the following criteria:

**Inclusion criteria**

1 wound persistence for several months or years, wounds will heavily contaminate with diverse pathogenic bacteria, which can not be cured by western conventional medicine,
2 horses seasonally had IBH, and
3 bacterial infections were persistent on wounds for a long time

**Exclusion criteria**

1 pregnant, and breast feeding, and
2 wounds, especially primary intension healing wounds, can be cured with western conventional medicine.

**Wounded horses**

In this experiment, 6 (16.7%) wounded horses were used (Table 2). The experimental horses had at least one wound either on the body or a distal limb. No artificial wounds were made in the experimental horses. The Ethnical Committee of Thailand did not approve of making an artificial (puncture) wound on the experimental horses. Another

reason, not to make an artificial wound on the experimental horses was that fresh wounds need a long time to develop full bacterial burden. I decided not to run the experiment on unhealed wounds, and fresh wounds (primary intension-healing wound), can be cured by western conventional medicine.

Table 2. Demography of wounded horses.

| Horse number | Name | Age | Sex | Location | Wound location |
|---|---|---|---|---|---|
| 1 | Brownee | 17 | Female | Lanna Horse Riding Club | neck |
| 2 | Lanna | 18 | Female | Lanna Horse Riding Club | fetlock |
| 3 | Sheela | 14 | Female | Lanna Horse Riding Club | fetlock |
| 4 | Chomtong | 17 | Female | Squad Pack Battalion | hock |
| 5 | Deng | 6 | Female | Squad Pack Battalion | fetlock |
| 6 | Som | 14 | Female | Squad Pack Battalion | fetlock |

**IBH**

Five IBH horses were selected from the Veterinary Hospital, Chiang Mai University, and Lanna Horse Riding Club (Table 3).

Table 3. Demography of IBH horses.

| Horse number | Name | Age | Sex | Location | IBH location |
|---|---|---|---|---|---|
| 1 | Moon Bean | 17 | Female | VT* | head, mane, butlock, belly |
| 2 | Bean | 17 | Female | LHR** | head , mane, butlock |
| 3 | Veejay | 19 | Female | LHR | head , mane, butlock |
| 4 | Shila | 12 | Female | LHR | head, breast |
| 5 | Jasmine | 10 | Female | VH | lower limb |

"*" Veterinary hospital, "**" Lanna horse riding club

**Performance of allergic tests**

First of all, hairs from around the wounds, and IBH infected areas were removed by a clipper. On the first day, 14 March 2005, all selected horses were topically sprayed with 5 µg/ml of EEP directly on the wound for an allergic test. This allergic test technique is very similar to a skin prick test used in humans for allergic tests. After topically spraying of 5 µg/ml of EEP, the experimental horses were examined for clinical allergic symptoms such as itching, swelling, and abnormal behavior (Table 4) for 24 hours under the supervision of veterinary doctors. When no any allergic symptom was observed, EEP was sprayed two times per day on each experimental horse. In this experiment, no horse exhibited any allergic symptoms to propolis.

Table 4. Allergic testing criteria against EEP.

| Symptoms of allergic | Score | Response of allergic |
|---|---|---|
| Itching, swelling and abnormal behavior | +++ | No application of propolis |
| Itching and abnormal behavior | ++ | No application of propolis |
| Itching | + | No application of propolis |
| No itching, swelling and abnormal behavior | - | Application of propolis |

**Topical application of EEP**

First day, each horse received 300 µg/ml of EEP topically two times a day until the wounds healed. Before the application of EEP, wounds were cleansed with saline solution (0.9% NaCl). After the application of EEP, all wounds were covered with a sterile, nonadherent synthetic pad (NASP). Other drugs previously used on these wounds were completely stopped throughout the experimental period.

**Evaluation of wound healing rates**

Cooper (1990) suggested that accurate evaluation of wounds needs to have several criteria have accurate evaluation of wounds, and healing process. In this experiment, the following four criteria were used to accurate evaluate the wound healing process.

- Wound bed tissue color,

- Measurement of wound,
- Calculation of wound healing rate, and
- Calculation of wound contraction

**Wound bed tissue color**

Tissue coloration is widely used to assess open wound status, and wound healing parameters (Copper, 1990). The wound tissue is either viable, or non-viable tissue (necrotic tissue). The viable tissue is granulation, epithelialization, muscle, subcutaneous tissue whereas the non-viable tissue is necrotic tissue. Table 5 shows colors of tissues present on the surface of wounds, which were used to determine the wound beds.

Table 5. Characteristic features and coloration of wound tissue beds.

| Tissue | Color | Healing stages | Indication | Remarks |
|---|---|---|---|---|
| Necrotic tissue | Black | Black wound or hardened eschar dead tissue | Unhealthy granulation | Non-viable tissue is a thick, and hard eschar, the outer layer. It must be removed to commence the healing process. |
| Slough | Yellow | Devitalized tissue | Infection | Non-viable tissue. |
| Granulation tissue | Red | Healthy tissue (bright) | | Viable tissue with small blood vessels and connective tissue. It absorbs excess exudates, maintains a moist environment, and protects wounds |
| Epithelialization tissue | Pink | Final stage of healing | Healthy granulation | Viable tissue is a process of epidermal resurfacing, and appears as pink or red skin. In partial thickness |

| | |
|---|---|
| | wounds, it occurs in throughout the wound bed as well as the wound edges. In full thickness, it occurs only in edges. It protects the delicate tissue, and prevent from drying out. It also acts as insulate. |

Sources: Cuzell, 1988; Carville, 2001; Copper, 1990.

**Measurement of wounds**

Bachand and McNichols (1999) recommended that assessment of wound characteristics occurs at every dressing change. Regular and systematic documentation of wound characteristics can be compared to previous assessments. They have also mentioned that a wound assessment chart can also be a useful tool to assess, and document wound characteristics. Photographs are also becoming excellent for wound documentation instead of an assessment chart in recent years. Photos can provide a degree of detail that cannot be provided by written descriptions, or drawings (Swann, 2000). In this research digitalized photographs of wounds taken by a Sony Digital Camera DSC-S70 were used to document wounds. Before treatment, the wounds were traced on sterilized transparency polyethylene sheets, and photographed. During each visit, each wound photographed to monitor the changing status of wound bed tissue color as it progressed through the healing process. Visits were discontinued once the horse was no longer receiving EEP treatment.

The size of each wound was measured by tracing the outline on digital photograph of wounds using image-J analysis software program at the Radiological Department, Maharaj Hospital, Chiang Mai University, Chiang Mai. The image-J program (NIH-image) is commonly used to measure the size of cells, and tissues of humans at the Department of Radiology, the Faculty of Medicine, Chiang Mai University. First of all, the photograph was digitized in a 16-RGM image. Each digital

image was displayed on the monitor and was calibrated to 1.0 cm distance from the metric scale on the image by the mouse. The mouse was then used to trace a line around the boundary of the wound to define the region of interest (ROI). The area of wound then was calculated (Arngvist *et al.*, 1988). Each wound was measured five times, and the mean±SD of five was calculated by Image-J program.

**Calculation of wound contraction rates**

Contraction is defined as the centripetal movement of wound edges that facilitates closure of a wound. Wound contraction is a process where open wounds close by movement of the wound edges towards the center of wound. The mechanism of contraction, which shrinks wound, is that the generation of forces in the contractile elements of the fibroblasts towards the center of wound. With fibroblast contraction, collagen, and proteoglycan are secreted to cement the new tissue. Wound contraction starts within 5-15 days after injury. The contraction of wound is decrease of wound size. During each visit, the wound size changes were recorded by photographs. Wound closure rate was calculated as percentage. Wound area used to calculate the percentage was the total wound area divided by the number of week and multiplied by 100. These data were used to compare the percentage of wound contraction.

**Calculation of wound healing rates**

Wound healing may be defined as the process where an injured tissue is repaired, resulting in regeneration of cell lining of the tissue with the reorganization of the deep tissue components into scar. The time between initial treatment of the wounds, and the time of each wound to be covered with epithelium was compared to evaluate the time for healing wounds on each horse. The wound area was converted into percentage, and used to calculate wound healing rates by comparing the final wound size with original wound size, and expressed as $mm^2$ per week.

**Microbiological tests**

**Collection of bacteria**

Pathogenic bacteria from cutaneous wounds were collected by rubbing with sterile swabs. Each swab was then returned to it's an original sterile test tube. The swabs were then transported to the laboratory at the Veterinary Hospital, Chiang Mai University. All tubes containing swabs were incubated for bacterial growth at 37°C for 24 hours.

**Isolation and identification of pathogenic bacteria from cutaneous wounds**

The bacteria were cultured on Mullar-Hilton agar according to guidelines formulated by National Committee for Clinical Laboratory Standard (NCCLS, 2002). The plates were incubated at 37°C for 24 hours. Isolated bacteria were identified at the Department of Microbiology, Veterinary Hospital, Chiang Mai University, Chiang Mai.

***In vitro* antibacterial susceptibility tests**

The disc diffusion method was used to determine antibacterial activity of EEP against pathogenic bacteria isolated from the cutaneous wounds of horses. Antibiotic filter paper discs (6 mm diameter) sterilized in an incubator at 37°C for 3 hours were impregnated in three different concentrations; 50, 200, and 300 µg/ml of EEP for 15 minutes. Then, discs were directly put on agar plates. Absolute ethanol (95.6%) and distilled water were used as control solvents. The plates were incubated at 37°C for 24 hours. The zone of inhibition around the each disc was measured using a vernier scale. All tests were done in triplicate.

**Statistical analysis**

**Calculation of wound contraction rates**

The outer margin of wound was traced using tracing paper, and the area was planimetrically determined. The degree of wound contraction (expressed as a percentage) was calculated using the following equation:

$$\text{Wound contraction rate} = \frac{(A_{Day\ 0} - A_{Day\ X})}{A_{Day\ 0}} \times 100$$

where x = 1, 2, 3, 4, 6, 7, 14 weeks after wound creation,

A = wound surface area

**Calculation of wound recovery ratios**

$$\text{Recovery ratio} = \frac{\text{Wound area after treatment}}{\text{Initial wound are}}$$

**Visual scoring of dermatitis condition**

Assessment of deterioration of the IBH skin condition was made by using a visual scoring of dermatitis (Table 6).

Table 6. Evaluation of IBH conditions [scaling for scoring]

| | Score |
|---|---|
| **Lesion** | |
| Normal | 0 |
| very mild lesions | 1 |
| moderate lesions | 2 |
| well defined lesions | 3 |
| severe lesions | 4 |
| **Skin thickening** (Figure 22 A) | |
| no skin thickening | 0 |
| very mild skin thickening | 1 |
| moderate skin thickening | 2 |
| well defined skin thickening | 3 |
| severe skin thickening | 4 |

| | |
|---|---|
| **Hair lost** (Figure 3 A, B, and C) | |
| no hair lost | 0 |
| very little hair lost | 1 |
| moderate hair lost | 2 |
| well defined hair lost | 3 |
| severe hair lost | 4 |
| **Bleeding** (Figure 22 A) | |
| yes | 1 |
| no | 2 |
| **Rat tail appearance** (Figure 3C) | |
| yes | 1 |
| no | 2 |

## RESULTS

### Assessment of wound healing by coloration of wound tissue beds

### EEP against body wounds

First day, the wound was full of pus, flies were feeding, and with exuberant granulation tissue (Figure 4A). On day 3, after spraying 300 µg/ml of EEP, the granulation tissue changed into necrotic tissue, and the wound became completely dry (Figure 4B). On day 14, necrotic tissue fall off, but the wound was still bleeding (Figure 4C). By day 84, the wound was completely healed (Figure 4D). The wound area did not expand during the experimental period.

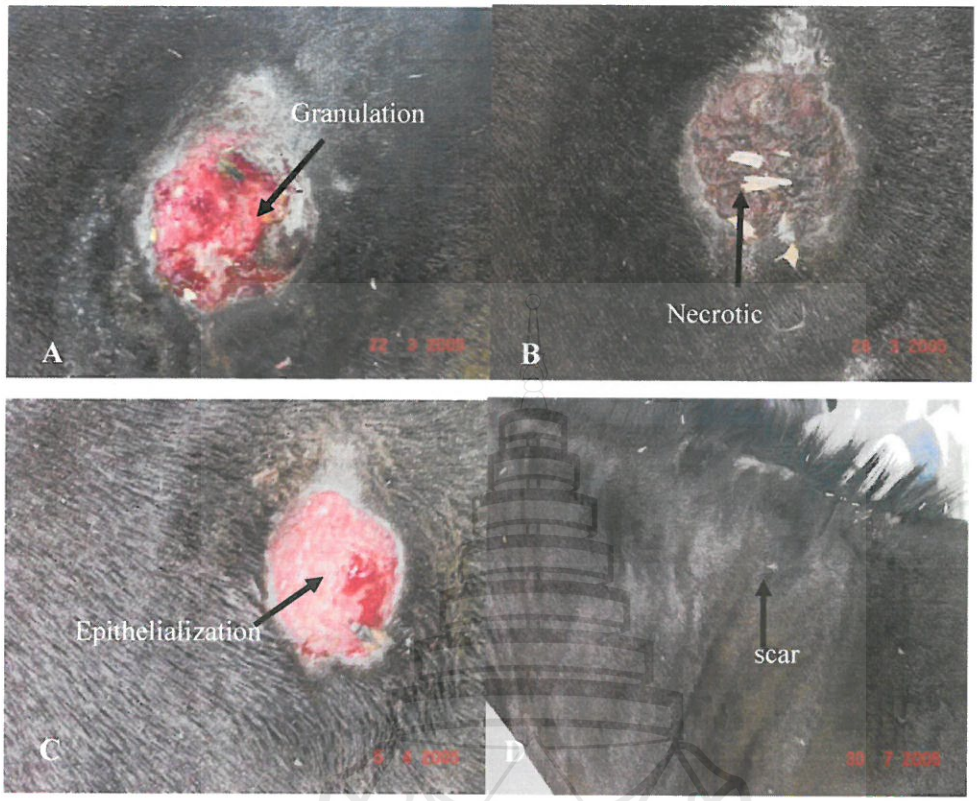


**FIGURE 4.** The photographic prints recorded changes in the surface appearance of wound. A chronic cutaneous wound on left side of the neck (A) first day, bacteria are present on contaminated wound surface, is bleeding and flies are feeding, (B) day 3, necrotic tissue is cover the whole wound, (C) day 14, healthy granulating tissue (red), and epithelial tissue are formed and (D) day 84, wound is completely healed.

**Wound contraction rate**

After the topically application of 300 µg/ml of EEP, wound was contracted from a mean initial size of 35.83±1.87 $mm^2$ to a mean final wound size of 0.02±0.01 $mm^2$ over 12 weeks. The mean initial size of control wound was 11.45  0.18 $mm^2$, and expanded up to a mean size of 15.05±0.56 $mm^2$ over 12 weeks. The final size of the wound was 0.02±0.01 $mm^2$. The wound healing rate was 2.95 $mm^2$ per week (Figure 5).

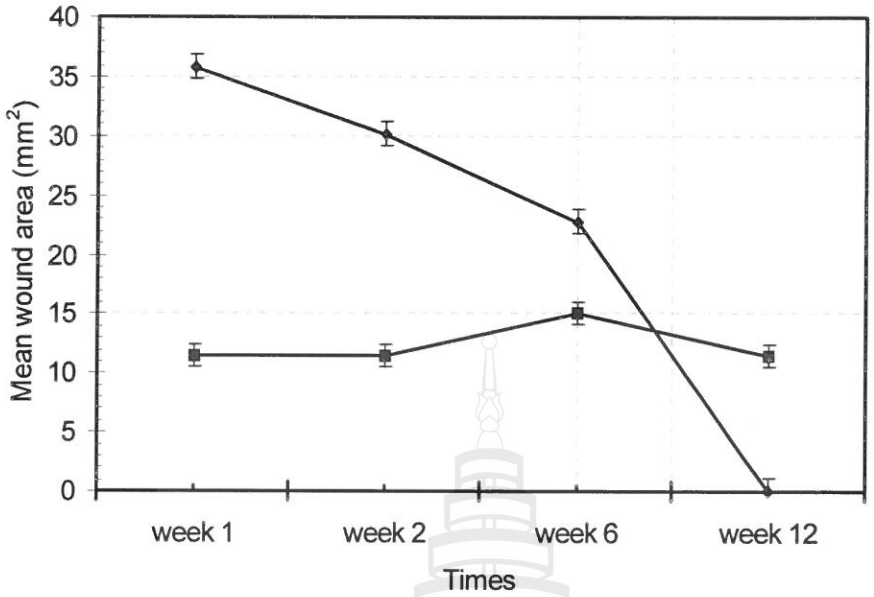


**FIGURE 5.** Mean area of wound contraction after application of 300 µg/ml of EEP. Treatment [♦] and control [■]. Each value is expressed as a mean±SD. Wound size is expressed as $mm^2$.

**Wound healing rate**

On week 2, 15.85% of wound was recovered. Subsequently, on week 5, and week 11, 24.45%, and 99.92% of wound recovered, respectively. Ninety-nine percent of wound was healed in week 12 (Figure 6).

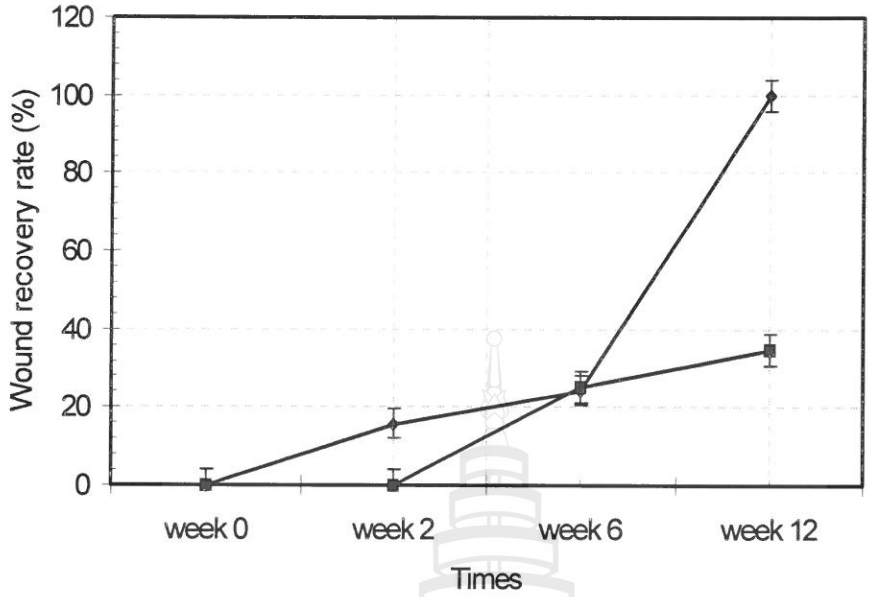


**FIGURE 6.** Wound recovery rate is expressed as a percentage. Treatment [♦] and control wounds [■].

**EEP against hock wounds**

First day, wounds on the hocks of the right, and left legs were slightly bleeding. On day 2, after topically application of 300 µg/ml of EEP, wounds dried. By day 14, wounds completely healed with hair regrowth (Figure 7).

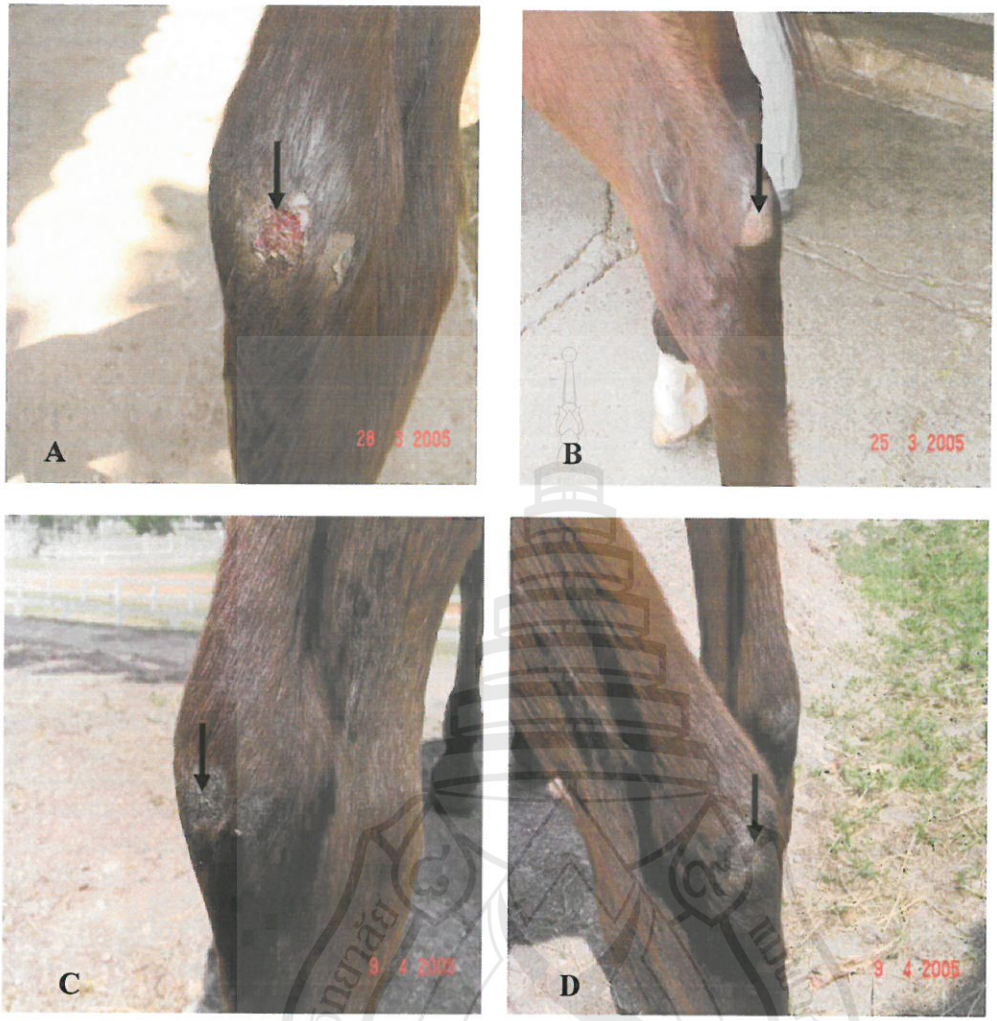


**FIGURE 7.** The photographic prints recorded changes in the surface appearance of wound on the hind legs hocks (A) first day, wound is bleeding, hair is lost (B) day 14, wounds are completely healed from the knee of both hind legs.

**Wound contraction rate**

After topical application of 300 µg/ml of EEP two times per day, wounds contracted from a mean initial size of 5.67±0.20 $mm^2$ to a mean final wound size of 0.02±0.03 $mm^2$ on the right leg, and from 2.98±0.09 to 0.05  0.03 $mm^2$ on the left leg within 3 weeks. The

final wound sizes of the right, and left legs were 0.02±0.01 $mm^2$, and 0.05±0.03 $mm^2$, respectively. The wound healing rate for the right and left legs was 2.46 $mm^2$, and 1.31 $mm^2$ per week, respectively (Figure 8).

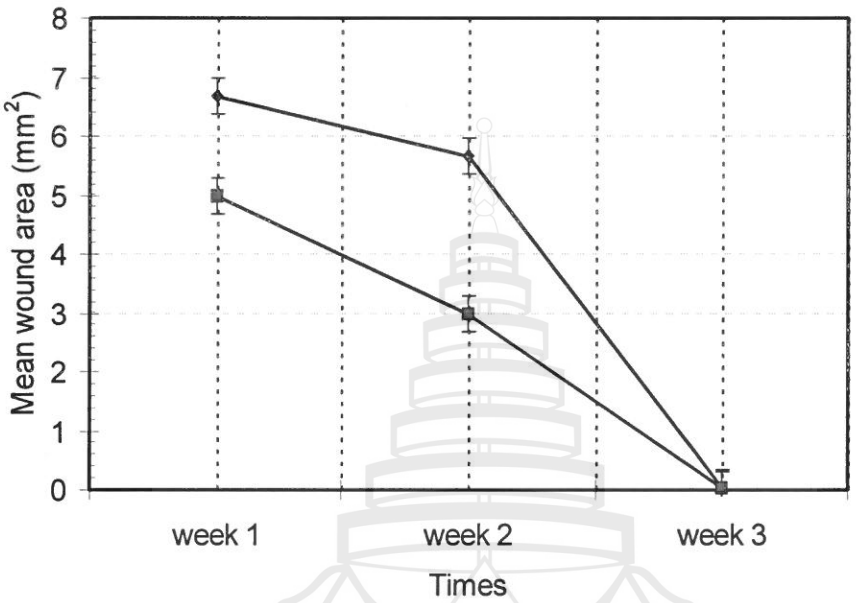


**FIGURE 8.** Mean area of wound contraction after application of 300 µg/ml of EEP. Right leg [♦] and left leg [■]. Each value is expressed as the mean±SD. Wound size is expressed as $mm^2$

**Wound healing rate**

On weeks 2.5, 99.6% of the right and 98.32% of the left leg wounds healed. It took 3 weeks to heal the right and left wounds. The wound contraction rate was 0.42 $mm^2$ per week (Figure 9).

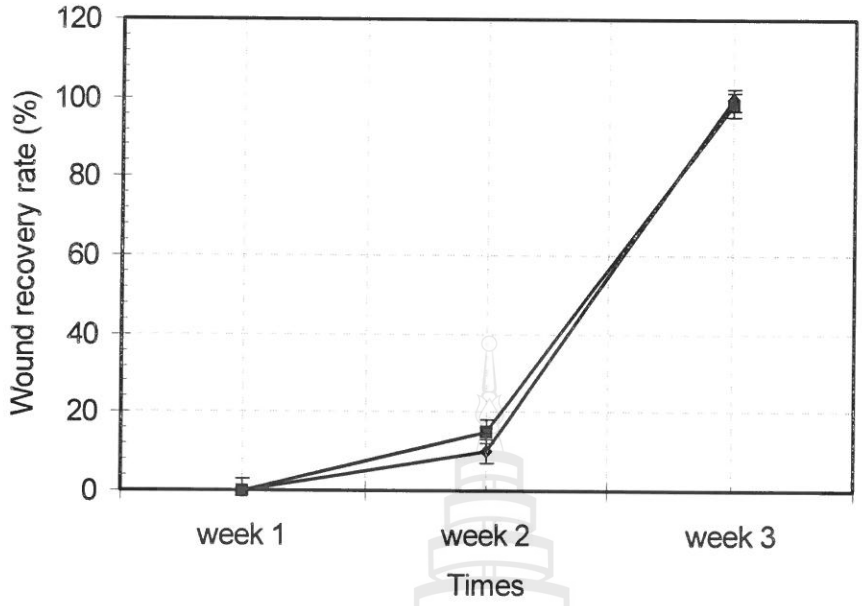


**FIGURE 9.** Wound recovery rate expressed as a percentage. The right leg [♦] and left leg wounds [■].

**EEP against fetlock wounds**

First day, wound on the fetlock was bleeding, and full of pus. After spraying with 300 µg/ml of EEP, necrotic tissue formed on day 3. On day 7, necrotic tissue detached from wound (Figure 10).

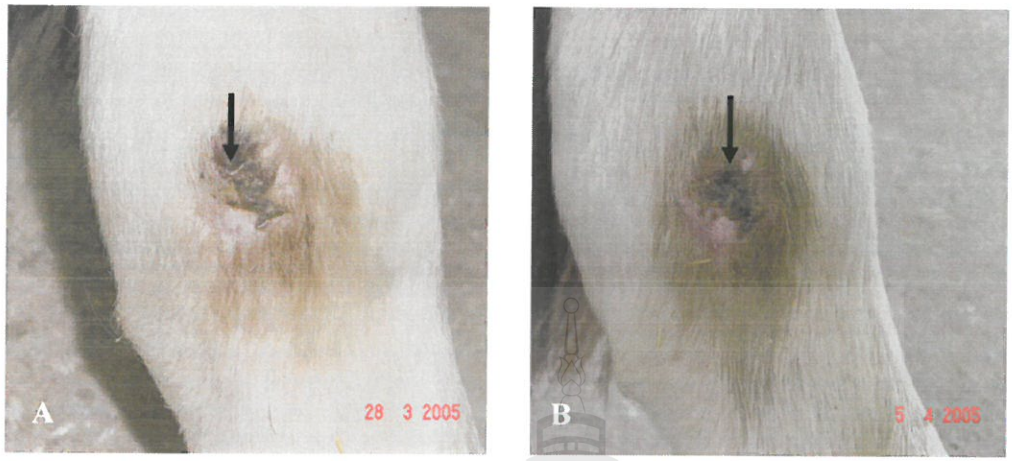


**FIGURE 10.** The photographic prints recorded changes in the surface appearance of wound on the fetlock of the hind leg (A) first day, wound is bleeding, hair lost, infected, and with necrotic tissue, (B) day 7, wound has dried, and necrotic tissue is developed.

**Wound contraction rate**

After topical application of 300 µg/ml of EEP, wounds contracted from a mean initial size of 2.54±1.36 $mm^2$ to a mean final wound size of 0.22±0.21 $mm^2$. The final fetlock wound size was 0.22±0.21 $mm^2$ over 5 weeks (Figure 11).

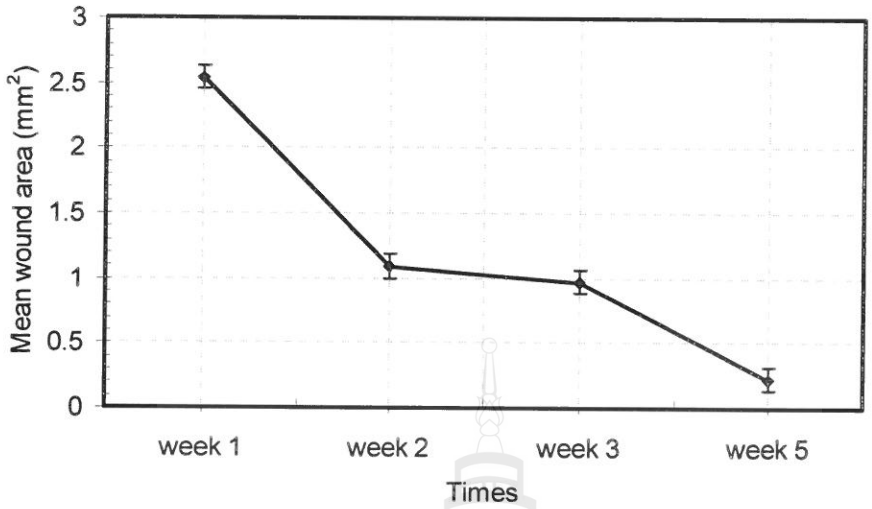


**Figure 11.** Mean area of wound contraction after application of 300 µg/ml of EPP. The right leg wound [♦]. Each value is expressed as a mean±SD. Wound size is expressed as $mm^2$.

**Wound recovery rate**

On weeks 2, 57.1% of wound had healed. By 6.5 weeks, 79.82% of wound had recovered (Figure 12).

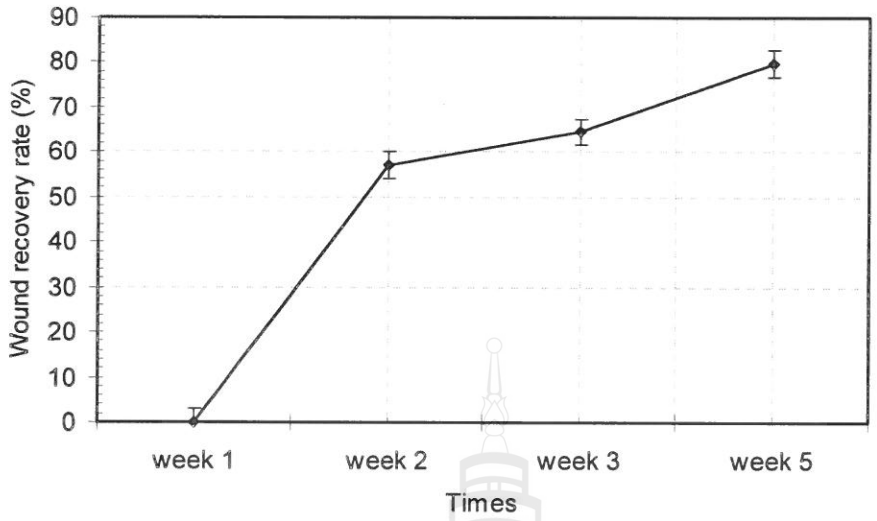


**FIGURE 12.** Wound recovery rate is expressed as a percentage. The right leg fetlock wound [♦].

**EEP against fetlock wounds**

First day, the wound was full of pus, flies were feeding, and had exuberant granulation tissue (Figure 13A). On day-3, after spraying with 300 µg/ml of EEP, granulation tissue developed (Figure 13B). On day 7, epithelial tissue had developed (Figure 13C). On day 14, necrotic tissue fell off, but the wound was still bleeding. After 4 weeks, wound had expanded, and flies were heavily feeding (Figure 13D).

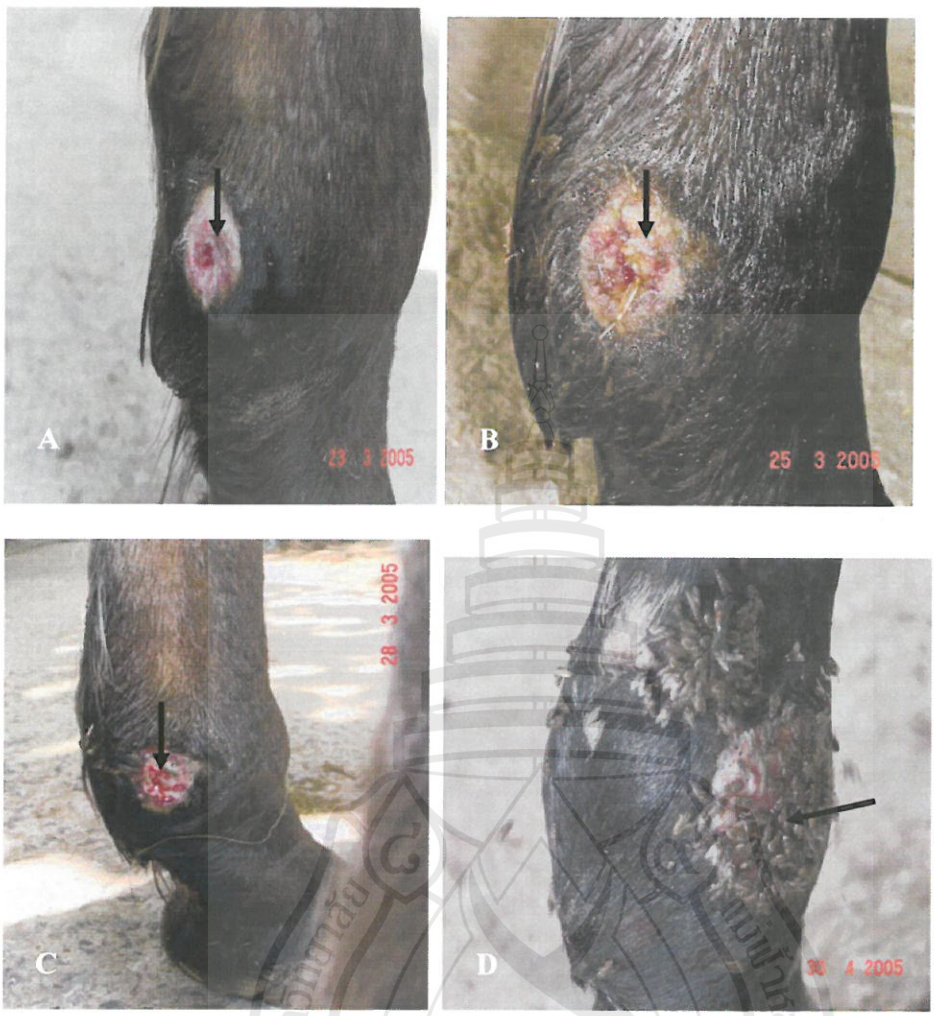


**FIGURE 13.** The photographic prints recorded changes in the surface appearance of wound on the inner part of a fetlock (A) deep chronic wound light yellow tissue (B) wounds size is increased, (C) wound is bleeding (D) bacterial burden in the wound bed resulted in no healing within the expected time.

**Wound contraction rate**

After topical application of 300 µg/ml of EEP, wound contracted from a mean initial size of 20.29±0.96 $mm^2$ to a mean final wound size of 48.07±4.06 $mm^2$. The final wound size was 48.07±4.06 $mm^2$ (Figure 14).

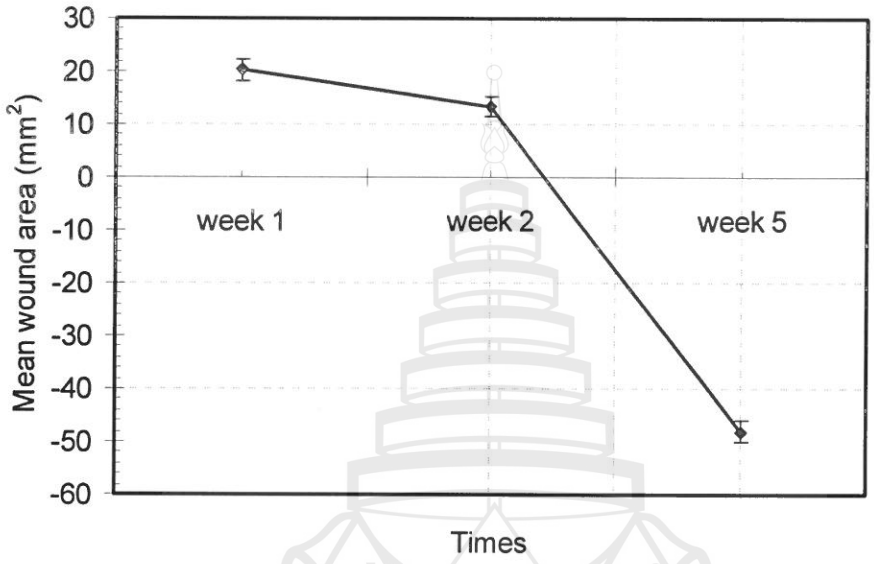


**FIGURE 14.** Mean area of wound contraction after application of 300 µg/ml of EEP. The right leg fetlock wound [♦]. Each value is expressed as a mean±SD. Wound size is expressed as $mm^2$.

**Wound recovery rate**

On week 1.7, 23.5% of all wounds recovered. After 6.5 weeks, wound increased by 138% (Figure 15).

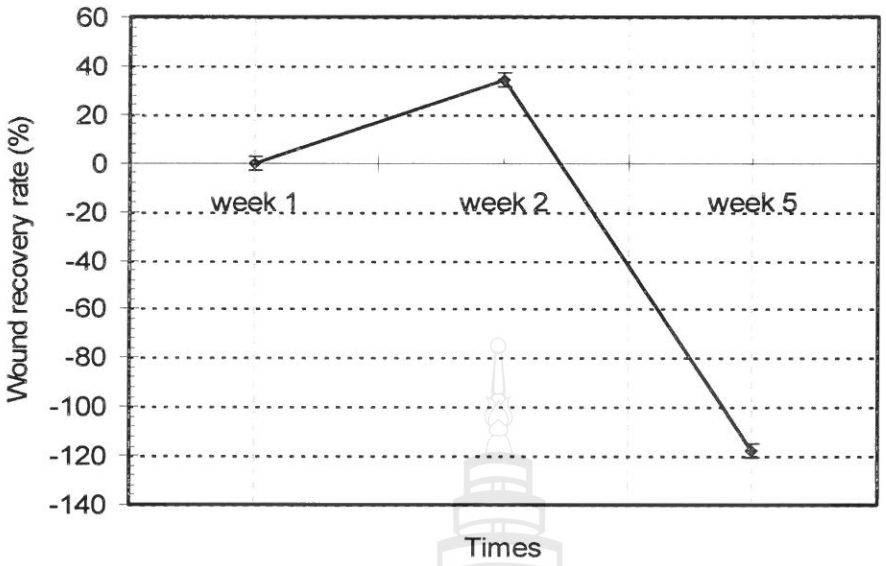


**FIGURE 15.** Wound recovery rate is expressed as a percentage. The right leg fetlock wound [♦].

**EEP against fetlock wounds**

First day, wound was bleeding, had pus, and flies were feeding. On day 3, after spraying 300 µg/ml of EEP, granulation tissue observed, and completely dry. By week 2, the wound was completely dry (Figure 16).

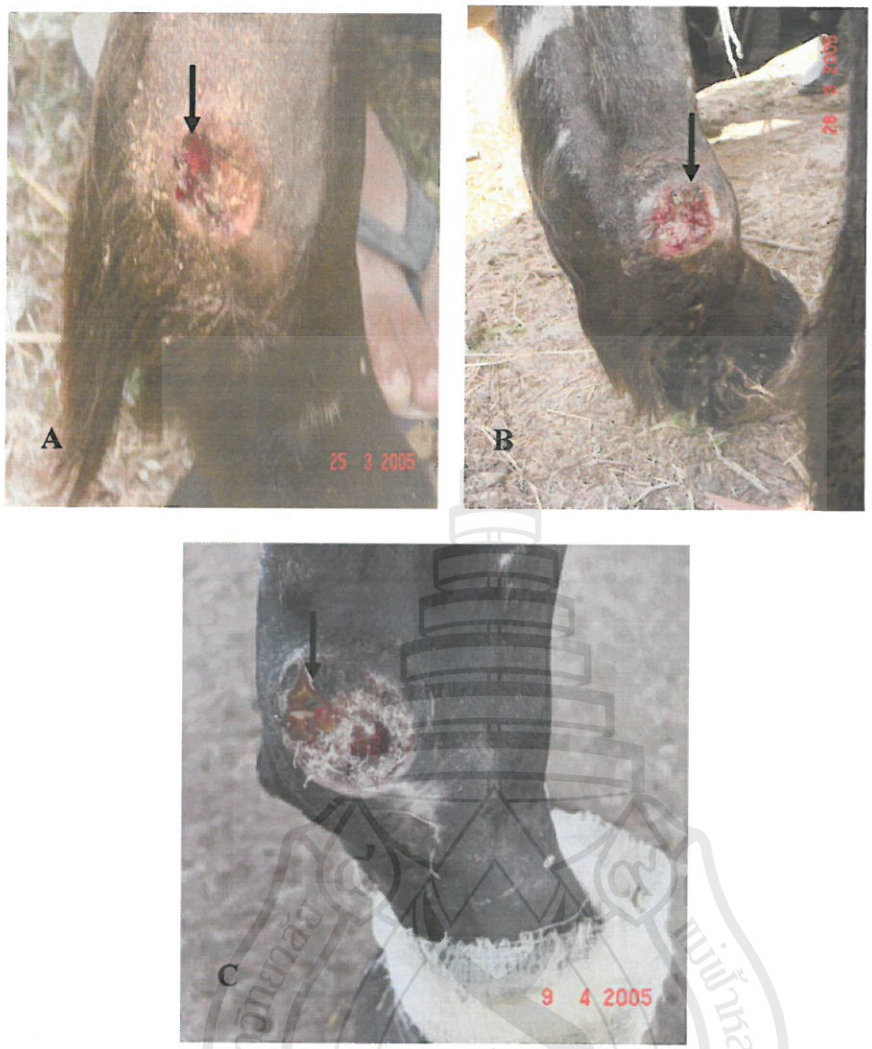


**FIGURE 16.** The photographic prints recorded changes in the surface appearance of wound on the inner part of the fetlock. (A) day 3, wound is dried, granulation tissue are observed, (B) wound is dried and epithelial tissue are observed and (C) wound is dried and partially healed.

**Wound contraction rate**

After topical application of 300 µg/ml of EEP, wound contracted from a mean initial size of 7.07±1.76 $mm^2$ to a mean final wound size of 4.44±0.45 $mm^2$. The final wound size was 4.44±0.45 $mm^2$ (Figure 17).

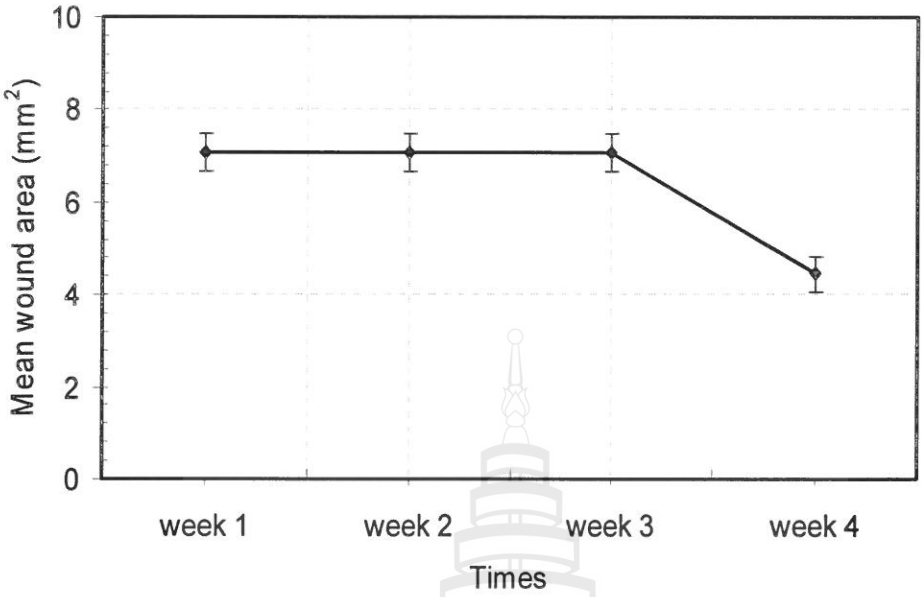


**FIGURE 17.** Mean area of wound contraction after application of 300 µg/ml of EEP. The right leg fetlock wound [♦]. Each value is expressed as a mean±SD. Wound size is expressed as $mm^2$.

**Wound healing rate**

Up to 2 weeks, wound size did not recover. After 4 weeks, wound decreased by 57% (Figure 18).

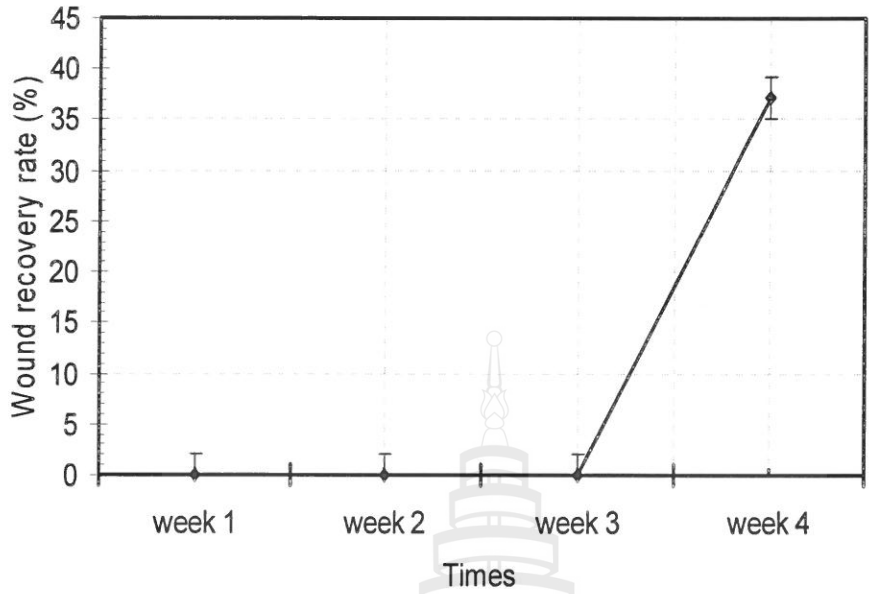


**FIGURE 18.** Wound recovery rate is expressed as a percentage. The right fetlock wound [♦].

**EEP against ulcer legs**

First day, wound was bleeding. Wound after cleaning with 0.01 NaCl were bandaged with EEP to protect from contamination, and to keep flies off (Figure 19).

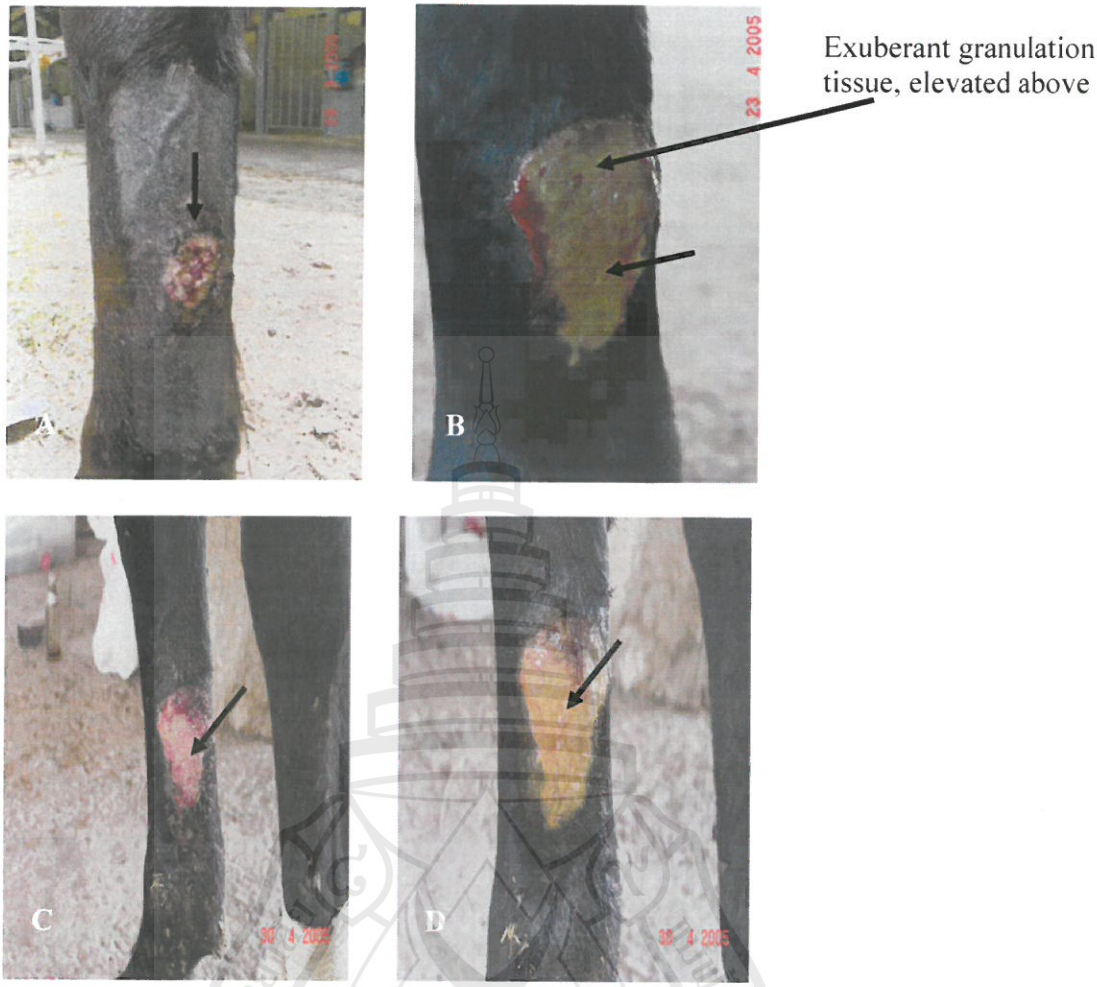


**FIGURE 19.** The photographic prints recorded changes in the surface appearance of wound on inner surface of front legs. Wound on the metatarsus of the front legs. Exuberant granulation tissue reoccurred and difficulty to cure. It seems that treatment of such a wound is more complicated. (A) first day, wound with slough, (B and C) day 26, wound is expanded, and (D) day 33, wound with granulation tissue.

**Wound contraction rate**

After topical application of 300 µg/ml of EEP, wound increased from a mean initial size of 5.90±0.28 $mm^2$ to a mean final wound size of 79.02±1.19 $mm^2$. The final wound size was 79.02±1.19 $mm^2$ (Figure 20).

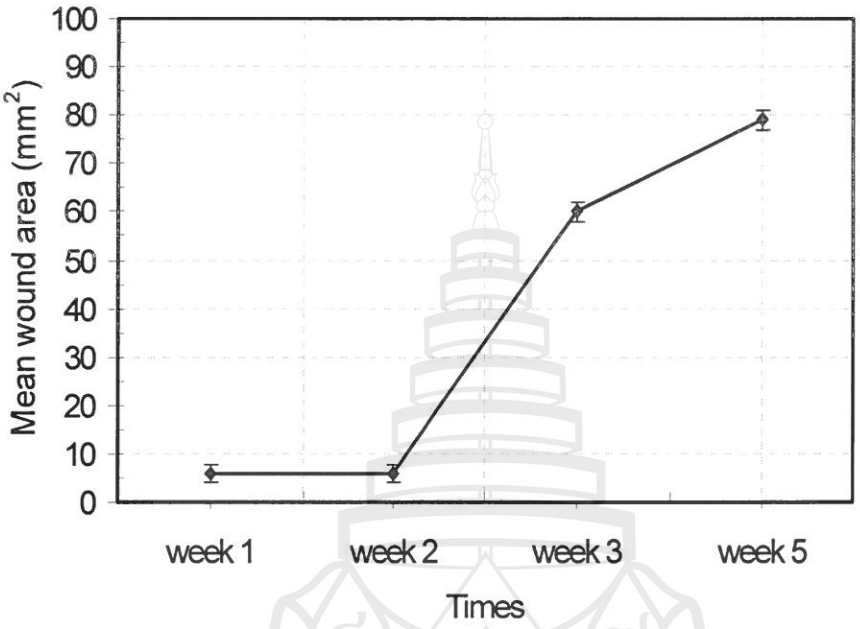


**FIGURE 20.** Mean area of wound contraction after application of 300 µg/ml of EEP. Ulcer legs [♦]. Each value is expressed as a mean±SD. Wound size is expressed as $mm^2$.

**Wound recovery rate**

First week, wound size was normal. After 5 weeks, wound was increased by 134% (Figure 21).

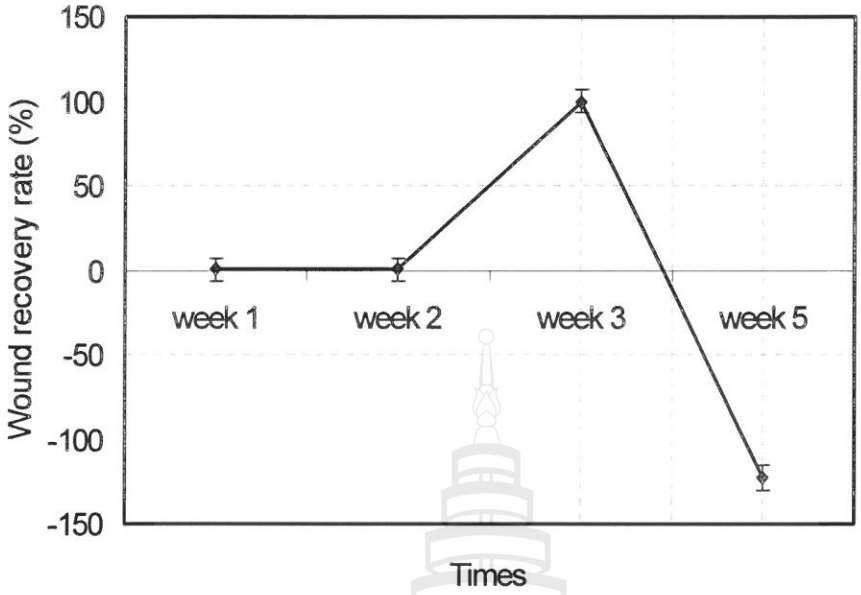


**FIGURE 21.** Wound recovery rate is expressed as a percentage. Ulcer legs [♦].

**EEP against IBH**

**Case–I.** First week, wounds were swollen, formed follicular papules, ruptured, bled, and no hairs (Figure 22A). On week 3, after the topical application of 300 µg/ml of EEP, wounds dried, and hairs started to regrowth. Hives did not rupture, and dried under the epidermis (Figure 22B). By week 28, horse was fully healed (Figure 22C).

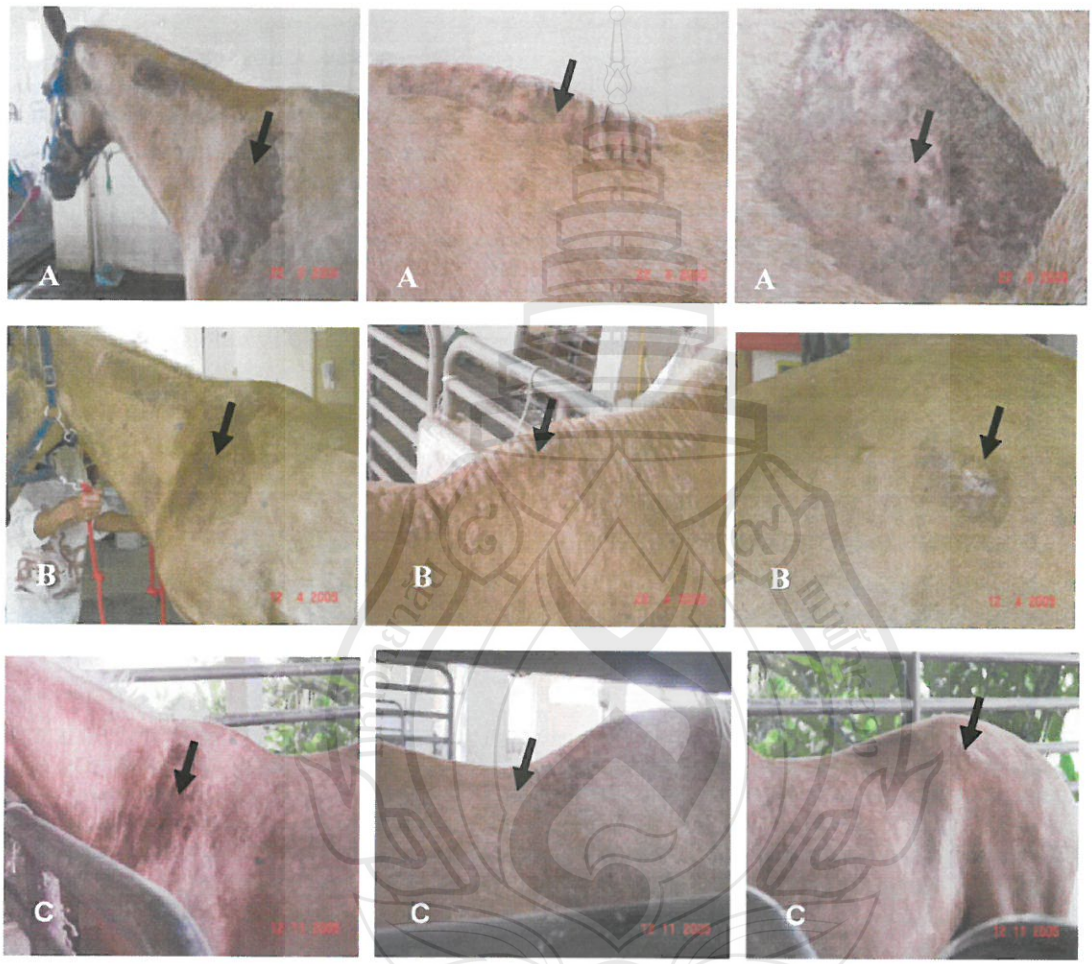


**FIGURE 22.** The photographic prints recorded changes in the surface appearance of IBH. (A) first week, allergic dermatitis on both side of shoulder, (B) week 3, wounds are dry (C) week 28, no wound trace, and hair is regrowth.

After topical application of 300 µg/ml of EEP, IBH wounds on shoulder, withers, and hip healed within 4 weeks, 12 weeks, and 9 weeks respectively (Figure 23).

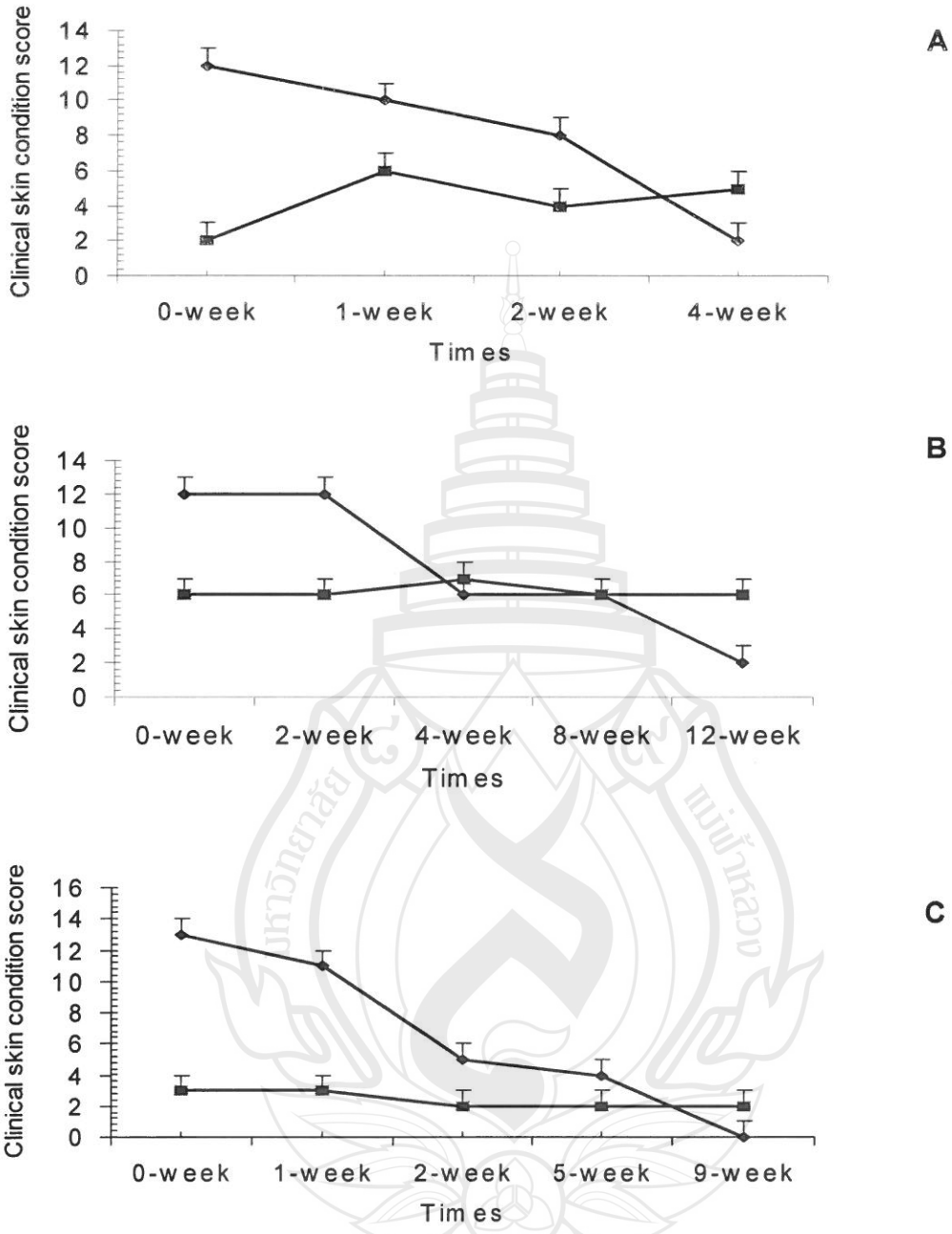


**FIGURE 23.** Recovery of IBH wounds from the different parts of the body (A) shoulder (B) withers and (C) hip. Treatment (♦) and control wounds (■).

**Case– II.** First week, IBH wound on withers, and tail bled. On week 3, after the topical application of 300 µg/ml of EEP, wounds dried, and hair started to regrowth (Figure 24).

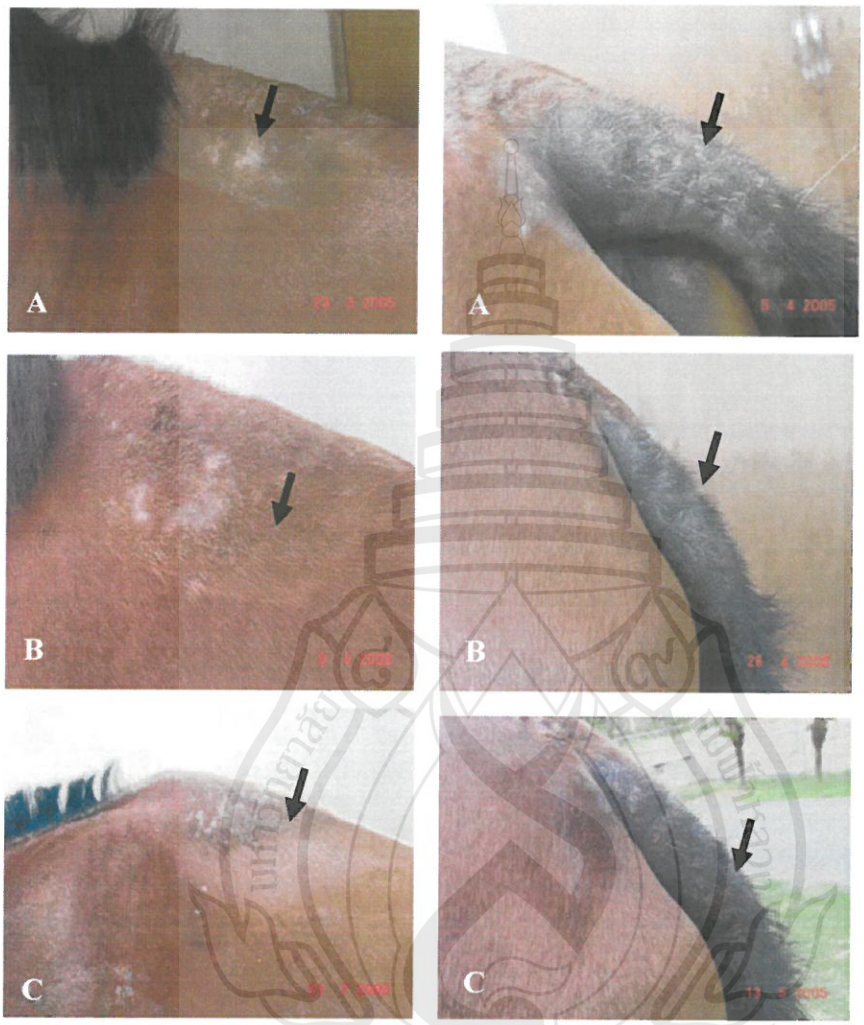


**FIGURE 24.** The photographic prints recorded changes in the surface appearance of IBH from withers, and tail (A) first week, allergic dermatitis on withers and tail (B) week 2, wounds are dried, and hair regrowth and (c) wounds are dried, and hair regrowth.

After topical application of 300 µg/ml of EEP, IBH wounds on withers, back, and tail healed within 16 weeks, 10 weeks, and 14 week, respectively (Figure 25).

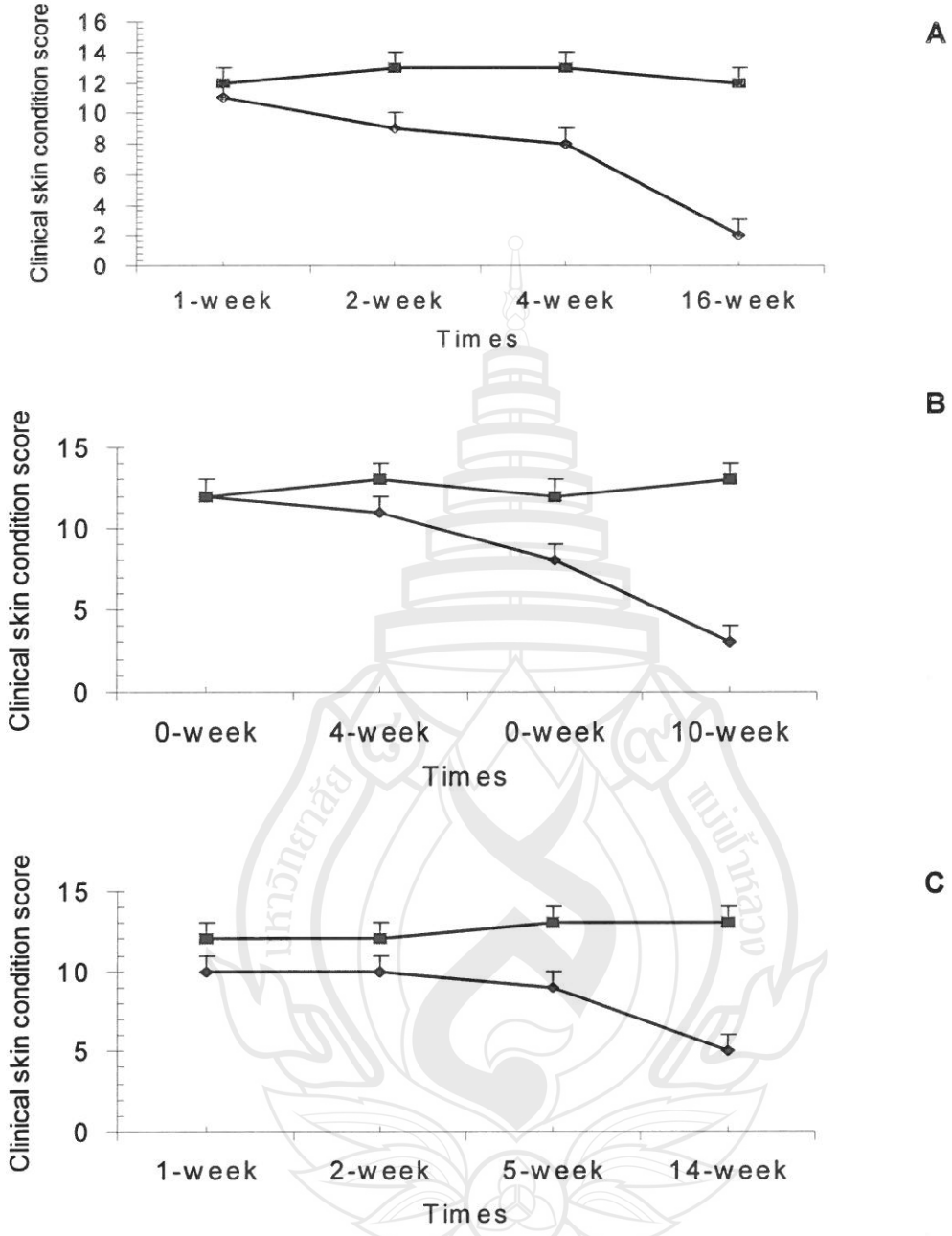


**FIGURE 25.** Recovery of IBH wounds from the different parts of body. (A) wither, (B) back and (C) tail. Treatment (♦) and control wounds (■).

**Case-III.** First week, IBH wounds swollen, and ruptured. By week 3, after the topical application of 300 µg/ml of EEP, wounds dried, and hair started to regrowth. Hives did not rupture, but dried under the epidermis. By week 28, the horse recovered (Figure 26).

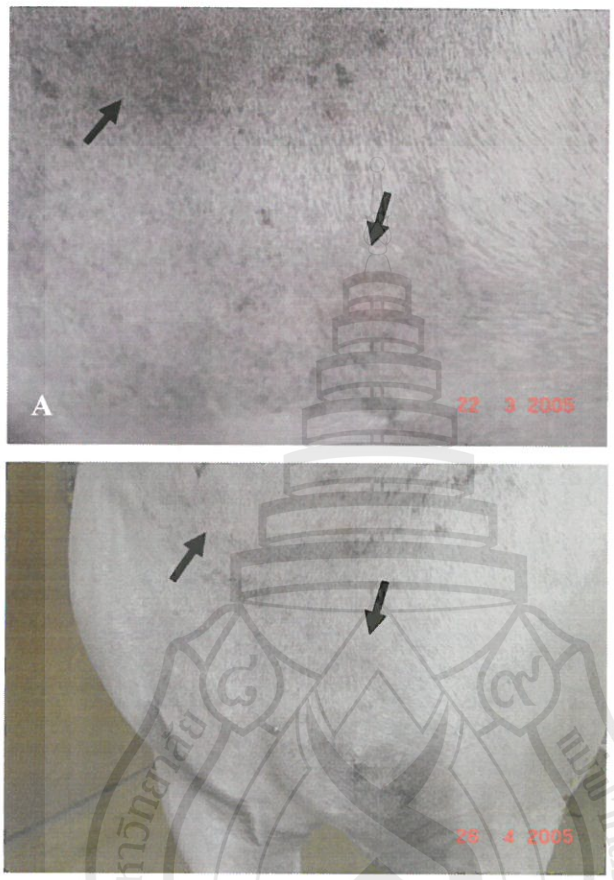


**FIGURE 26.** The photographic prints recorded changes in the surface appearance of IBH from breast (A) wound on breast (B) wound is cured and (C) IBH wound on head.

After topical application of 300 µg/ml of EEP, IBH wounds on the breast healed within 11 weeks (Figure 27).

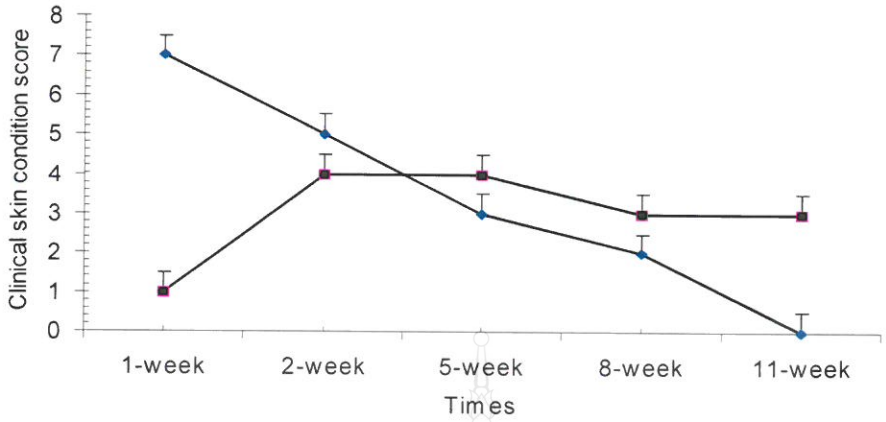


**FIGURE 27.** Recovery of IBH wounds from the breast. Treatment (♦) and control (■).

**Case-IV.** First week, IBH wounds ruptured, and bled with hair lost. On week 3, after the topical application of 300 µg/ml of EEP, wound dried (Figure 28).

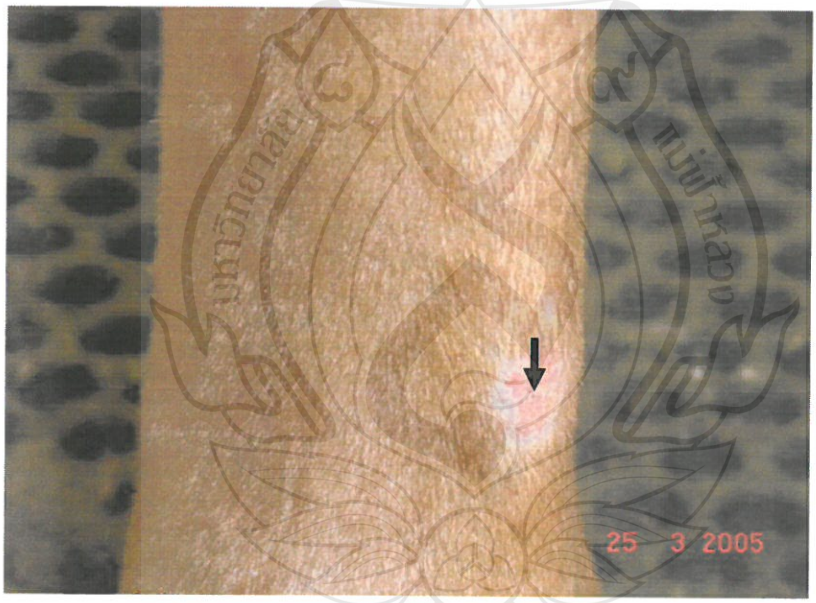


**FIGURE 28.** IBH wound on the right fore leg

After topical application of 300 µg/ml of EEP, IBH wounds on the right fore leg healed within 13 weeks (Figure 29).

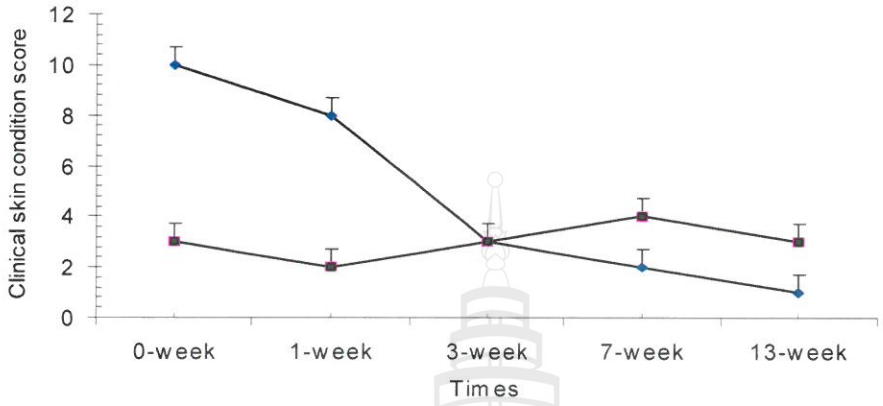


**FIGURE 29** Recovery of IBH wounds. Treatment (♦) and control (■).

**Case-V.** First week, IBH wound ruptured, and bled. On week 1, after the topical application of 300 µg/ml of EEP, wounds dried. Hives did not rupture, but wound dried under the epidermis (Figure 30).

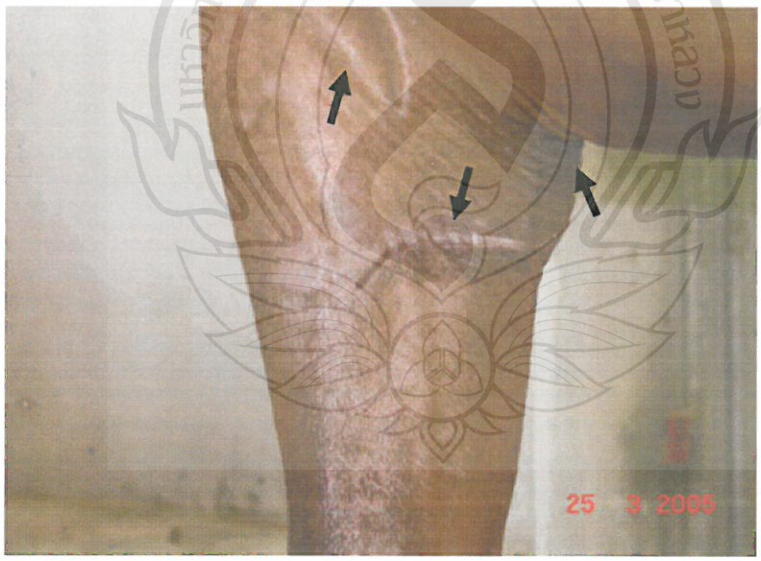


**FIGURE 30.** IBH wound trace on the inner part of the right fore leg

After topical application of 300 µg/ml of EEP, IBH wounds on the inner part of the right fore leg healed within 13 weeks (Figure 31).

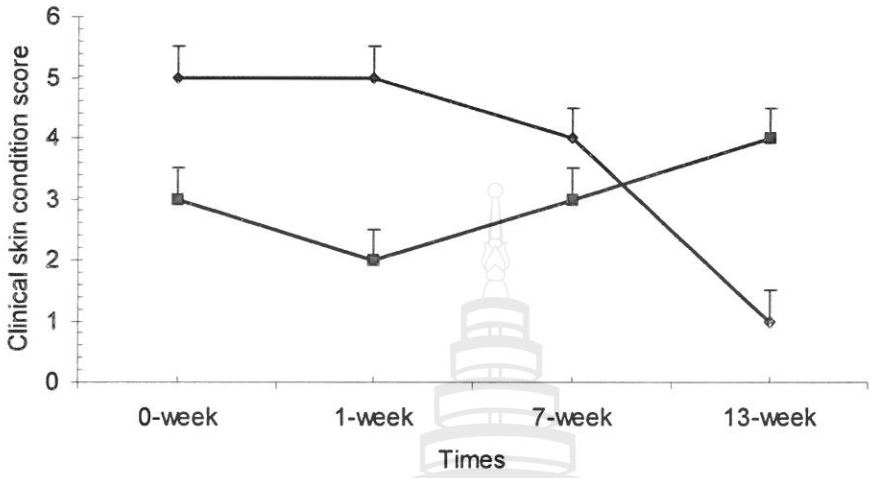


**FIGURE 31.** Recovered of IBH wound from the inner part of the for leg. Treatment (♦) and control (■).

***In-vitro* antibacterial susceptibility tests**

Three types of bacteria were identified from IBH wound. The zone of inhibition of bacterial growth on Mueller-Hinton blood agar plates is presented in (Figure 32). *S. saprophyicus*, and *S. haemolytius* were highly sensitive to EEP than *S. intermedius*. Control (distilled water) did not show inhibitory zone.

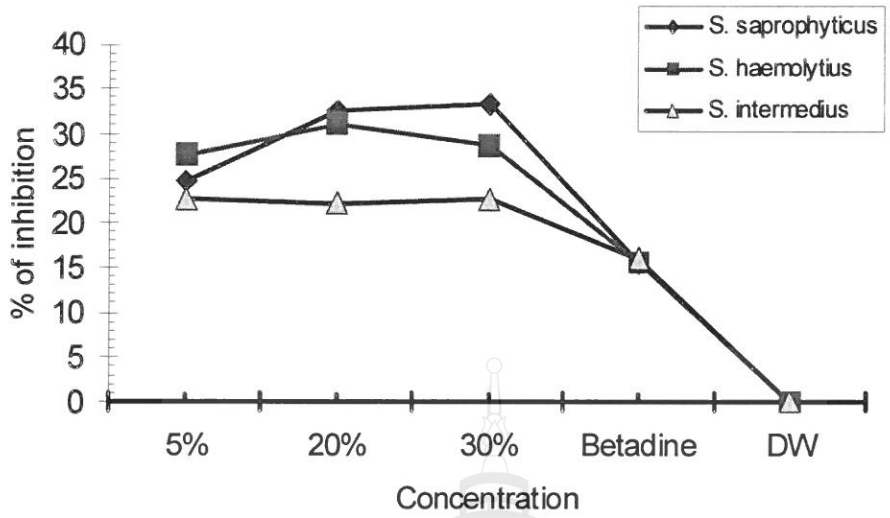


**FIGURE 32.** Inhibition of bacteria growth by different concentration of EEP, betadine and distilled water.

## DISCUSSION

### EEP against wounds

The results of this study demonstrated that EEP can heal chronic second-intention wounds of horses. The most plausible explanation is that propolis contains flavonoids including phenolic compounds and caffeic acid (CC), which are widely occurred in the plant kingdom (Havsteen, 1998; Middleton and Chithan, 1993). The main bioactive compound that assists to minimize the wound infection, wound healing, and wound closure is flavonoids (Hegazi *et al.*, 2000; Pepeljnjak *et al.*, 1982; Cheng and Woung, 1996). The flavonoids are versatile antioxidant against reactive oxygen species that cause cell damage (Cushnie, and Lamb 2005) known to reduce lipid peroxidation not only by preventing, or slowing the onset of cell necrosis tissue, but also by improving vascularity. Hence, any chemical that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, and preventing the cell damage (Getie *et al.*, 2002). Flavonoids and triterpenoids are also known to promote the wound-healing process mainly due to antimicrobial property, which seems to be responsible for wound contraction, and increased rate of

epithelialisation (Scortichini *et al.,* 1991; Tsuchiya *et al.,* 1996). Flavonoids, phenols, and CCF can break the biofilms of bacteria, and kill them. The most common pathogenic bacteria that cause wound infections are *Streptococcus* species (Mertz and Ovington, 1993), including methicillin-resistant *S. aureus*, considered to be a transient flora of horse's skin (Bikowsky, 1999). *S. aureus* presents in acute, and chronic wounds encased in a self-secreted matrix of extracellular polysaccharide (EPS), so termed *biofilms*, and can not be killed by antibiotics is the leading causes of wound infections all over the world. (Allen *et al.,*1973). The result of this research supported previous reports (Burdock, 1998; Castaldo and Capasso, 2002; Gebara *et. al.,* 2002; Nagai *et al.,* 2003; Kumazawa *et al.,* 2004). They also found the same results when they externally applied propolis to relieve various types of dermatitis caused by bacteria. Bankova *et al.,* (1995) examined the activity of Brazilian propolis against *S. aureus*, and observed the antibacterial activity, which is mainly due to polar phenolic compounds. Serra and Escola (1995) also studied antimicrobial activity of phenolic constituents from 12 samples of propolis, and established a relationship between the flavonoid components, and the bacteriostatic activity of propolis.

Propolis control bacterial proliferation, does not induce inflammation, promotes contraction, and epithelialization. The lack of bacteria infection in wounds may have contributed to a low level of inflammatory cell influx (Jorge *et al.,* 2004). Several previous reports have already demonstrated that when bacteria such as *S. aureus* at a level of $7.5x10^6$ produced little, or no overt infection at all. At this level, bacteria-host equilibrium is in balance, and the process of wound healing occurs. If bacteria-host equilibrium is not in balance, infected wounds heal very slowly. The process of wound healing is slowed when bacteria proliferate in wounds. Infected wounds can often respond to an increase microbial load with a sudden rise in exudate production. However, bacterial imbalance increases the likelihood of wound infection. Most bacteria enter wound bed through external contamination from the environment. If bacteria continuously multiply, a local infection can develop. I have demonstrated that EEP can inhibit the growth of pathogenic bacteria. The result of research is consistent with previous reports. Previous reports indicated that propolis has antibacterial activities against pathogenic bacteria (Pepeljnjak *et al.,* 1982; Toth and Papay, 1987; Petri *et al.,*

1988; Dobrowolski *et al.,* 1991; Kujumgiev *et al.,* 1993; Woisky *et al.,*1994; Meresta and Mererstа 1985). Around 57% of surface bacteria live on the skin of horses (Christopher *et al.,* 2005). Once the horse's skin is injured, or wounded, then bacteria start to colonize. Acute and chronic wounds are colonized by different populations of pathogenic bacteria (Bowler and Davis, 1999; Robson, 1997; Bikowsky, 1999). Gram-positive bacteria may be the first to invade a wound with decreased host resistance, followed by gram-negative bacteria, and then anaerobic bacterial species. To heal wounds, wounds must have bacteria (Cutting, 2003). The process of wound healing always occurs in their presence of bacteria, whether the wound is in bacterial balance (Figure 33a), or in bacterial imbalance (Figure 33b). Bacteria are of primary importance to heal wound. Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state, whereas wound contraction is the process of shrinkage of area of wound. It mainly depends on the repairing ability of tissue, types of damage, and general state of tissue. The granulation tissue of wound is primarily composed of fibroblast, collagen, and small new blood vessels. Therefore, it is very important to control microbial infection for wound healing (Levine, 1970; Muhammad, 2005). Kujumgiev *et al.,* (1999) and Castaldo and Capasso (2002) showed that propolis in *in vitro* has antimicrobial activity against gram-positive bacteria *viz Staphylococcus* spp. and *Streptococcus* spp., and gram-negative bacteria; *E. coli, Klebsiella pneumoniae, Proteus vulgaris* and *P. aeruginosa.* Takasi and Schilcher (1994) stated that propolis inhibits bacterial growth by preventing cell division. In addition, propolis has disorganized cytoplasm, which causes a partial bacteriolysis, and inhibited protein synthesis. Once bacteria are balanced (Figure 33a), or inhibited growth by propolis (Figure 33b), wound starts to heal by a complex process of inflammation, regeneration of parenchymal cells, and migration. After killing wound bacteria by EEP, myofibroblasts start to repair inflammation, proliferation, and tissue remodeling. Propolis stimulates tissue regeneration, as it caused strong activation of mitosis of cells in *in vitro*, and it enhances protein biosynthesis (Scheller *et al.,* 1977; Popeskovic *et al.,* 1977; Stojko *et al.* 1978).

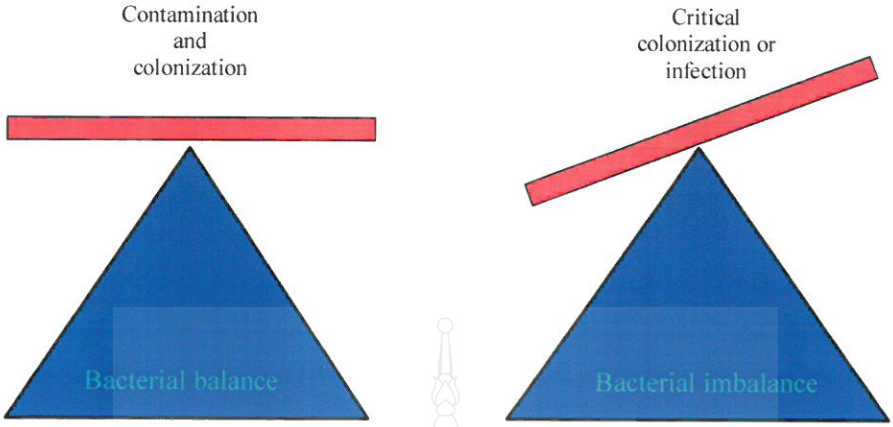


**Figure 33**. Balancing of bacterial burden in chronic wounds (a) uninfected wound (balancing of bacteria), and (b) infection of wound (burden of bacteria in wounds) (Source; Sibbald *et al.*, 2006).

**EEP against lower limb wounds**

The results show that the distal wound of horse can not be cured with ethanolic extract of propolis. The most plausible explanation is that the horse wounds are usually contaminated, sustain of trauma during wound, relative poor blood supply to distal extremities, and hypergranulation tissue cause delay in lower limb wound heal (Bertone, 1989; Stashak, 1991). Similar reports have been reported by several authors (Booth and Feeney, 1982; Clem *et al.*, 1988; Moens *et al.*, 1980). They have also mentioned that delayed wound healing of the distal limbs of horses is due to reduction of ambient oxygen tension, and reduction of local pH which may increase formation of granulation tissue (Howard *et al.*, 1993). Granulation tissue is matured, and fibrous tissue, which has less of a blood supply than newly formed granulation tissue (Teh, 1997). The slower rates of epithelialization have been attributed to inhibit cell migration, and inhibit of mitotic activity (Jacobs *et al.*, 1984; Wilmink *et al.*, 1999). Body wounds tend to repair faster than the limb, because exuberant granulation tissue usually develops in wounds on the limb (Jacobs *et al.*, 1984; Wilmink *et al.*, 1999; Theoret, *et al.*, 2001). However, horse

lower limb wounds heal by second-intention (Bertone, 1989). The plausible explanation is that granulation tissue is less abundant in the thoracic wounds. Microvessels are occluded significantly more often in limb wounds than in thoracic wounds (Lepault *et al.*, 2005). The foot wounds are frequently contaminated with the ground soil. The presence of soil, and other foreign materials massively increase the chance of wound infection. In this experiment, wound on the lower limb developed exuberant granulation tissue; as a consequence, it is very difficult to cure. This observation is quite similar to previous reports (Madison *et al.*, 1991; Bertone, 1989). They have also found that wound of lower limb usually develop exuberant granulation tissue. Chronic wounds of the lower horse limb have similar case to human leg ulcer (Cochrane, 1997). Bertone, (1989) also reported that wound healing below the hock or knee in horse is a problematic issue even in the healthiest horses. Another reason might be due to the large distance between the body and lower limbs. The tissue of the lower limbs is provided with poor blood supply, as a consequence, lower oxygen, a lower temperature and an imbalance of growth factors (Dyson, 1997; Theoret *et al.*, 2001). As healthy granulation tissue develops, this is rapidly declined in total wound area (Chvapil *et al.*, 1979; Fretz *et al.*, 1983; Ford *et al.*, 1992; Wilmink *et al.*, 1999).

**EEP against IBH**

The results revealed that the propolis extraction can control IBH allergic wounds of horse but can not totally cure. The plausible explanation is that EEP can heal the cutaneous wound by killed the pathogenic bacteria; *S. saprophyicus*, *S. haemolytius* and *S. intermedius* and maggots but can not change IgE. Because, a number of studies indicate that IgE-antibodies are involved in IBH disease (Wilson *et al.*, 2001). Therefore, EEP can not cure IBH. My results indicated that EEP has antibiotics, antiprotozoan, and antiparasite properties. The results of this study support previous reports (Higashi and Castro, 1994). They showed that ethanolic and dimethyl-sulphoxide extracts of propolis is active against the different forms of the protozoan-parasite studied.

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**EEP against pathogenic bacteria of wounds**

The results of this research demonstrated three types of wound bacteria isolated from chronic wounds of horses were susceptible to EEP. *In vitro* tests results demonstrated that all three bacteria were sensitive to EEP. Previous reports have also found several types of pathogenic bacteria *viz S. aureus*, *S. pyogenes* and *P. aeruginosa* in wounds (Lowbury and Hurst, 1959; Trengove *et al.,* 1996; Bjorland, *et al.,* 2003; Tachi *et al.,* 2004). They have also shown that all these pathogenic bacteria produce bacterial toxins, i. e. endotoxins like interleukin-1β and tumor necrosis factor-β (Stephens *et al.,* 2003). Exotoxins produced by common wound bacteria tend to have quite broad substrate specificity, attacking many types of cells with subsequent tissue necrosis. This necrosis may be exacerbated by local hypoxemia from vessel occlusion. Wound infection results in many changes in the inflammatory process, including increased consumption of complement proteins resulting in decreased chemotaxis. Tissue damage is increased by the production of cytotoxic enzymes and free-oxygen radicals. My results support previous results reported by several authors (Pepeljnjak *et al.,* 1982; Toth and Papay, 1987; Petri *et al.,* 1988; Dobrowolski *et al.,* 1991; Kujumgiev *et al.,* 1993; Woisky *et al.,* 1994). Meresta and Mererstа (1985) demonstrated that 69 species of *Staphylococcus* spp. and *Streptococcus* spp. have shown highly sensitivity to EEP. Subsequently, Grange and Devey (1990) reported that 3 mg/ml of EEP can also completely inhibit the growth of *P. aeruginosa* and *Escherichia coli*. Lavie (1957) reported that EEP has shown significant antibacterial activity against *B. subtitis*, *B. alvei*, and *Proteus vulgaris*.

## CONCLUSIONS

EEP can heal chronic wounds of horses, and also wounds caused by IBH. EEP can not cure the distal wounds of horse probably, wounds were contaminated, relative poor blood supply to distal extremities, and hypergranulation tissue cause delay in lower limb wound heal. The results revealed that EEP can control IBH allergic wounds of horse, but can not totally cure, because it involves IgE-antibodies. The results of this research demonstrated three types of pathogenic bacteria isolated from chronic wounds of horses were

susceptible to EEP. *In vitro* tests results demonstrated that all three bacteria were sensitive to EEP. EEP can control *S. aureus* and *S. intermedius* cause IBH.

## RECOMMENDATIONS AND SUGGESTIONS

### Recommendations

1 More research should on conduct on Thai propolis on different domesticated animals,

2 Research should continuous to formulate propolis as a natural medicine for domesticated animals,

3 Future research should determine potential propolis products areas in Thailand

4 Teach and train beekeepers for proper ways of propolis production and collection

5 Document the potential areas of propolis production and their pharmacological properties of Thai propolis, and

6 Botanical survey of propolis resources in Thailand.

### Suggestions for further research

Propolis is one of the most valuable and highly demanded bee products in the world market. It should receive high priorities on research and production in terms of more pharmacological documentation and to enhance the income generation of local Thai beekeepers. MFLU should look this issues which are a paramount importance for the production of Thai propolis. The main research issues which deserve future research attention are:- chemical analysis of propolis according to region as well as the season of the year, further need to do *in vitro* and *in vivo* experiment of propolis, formulations of propolis products, high yield of Thai propolis for commercial demands and refine the flavonoids.

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