Faculty of Resource Science and Technology

THE POTENTIAL OF SELECTED BORNEAN BUFONIDS (AMPHIBIAN: ANURAN: BUFONIDAE) AS BIOLOGICAL DRESSING IN WOUND HEALING MANAGEMENT

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DECLARATION

I hereby declare that this Final Year Project Report 2013 is based on my original work except for quotations and citations, which have been acknowledged as well, I declare that no portion of this dissertation has been submitted in support of an application for any other degree at UNIMAS or other institution of higher learning.

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LIST OF ABBREVIATIONS

dH_2O	Distilled water
DPX	Distrene, Plasticiser, Xylene
HCl	Hydrochloric acid
H&E	Haematoxylin and Eosin
IP	Intra-Peritoneal Inoculation
KTX	ketamine/xylazine solution
mm^2	millimeter square
R	Radius
°C	Degree Celsius

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ABSTRACT

This study was conducted to assess the wound healing potential of selected species in family Bufonidae. The assessment of the healing process was compared with the normal dressing wound by using dH_2O and the untreated wound. The study was conducted on day 3, day 7 and day 14. Three laboratory rats were utilised for each day of the experiment, for the purpose of the study. Ruler measurement data were collected and histological study were done. Histological sections of the skin specimens were stained by hematoxylin and eosin. Ruler measurement data suggested that the wound treated with skin *Ansonia spinulifer* healed faster than *Phrynoidis juxtasper*. However, histological evaluation suggested that both species *A. spinulifer* and *P. juxtasper* could not be justified their healing potential due to some limitations, such as no replication of the experiment, difficulty in handling the rats and error during analysing the histological slides.

Key words: wound healing, potential, Bufonidae, histological, limitations

ABSTRAK

Kajian ini dijalankan untuk menilai potensi penyembuhan luka bagi spesies yang terpilih dalam famili Bufonidae. Penilaian terhadap proses pemulihan telah dibandingkan dengan luka yang dirawat secara normal iaitu menggunakan dH_2O dan luka yang tidak dirawat. Kajian ini telah dijalankan pada hari ke-3, hari ke-7 dan hari ke-14. Tiga tikus makmal telah digunakan untuk setiap hari eksperimen, untuk tujuan kajian. Data ukuran pembaris direkod dan kajian histologi telah dilakukan. Spesimen kulit untuk histologi diwarnakan dengan menggunakan hematoxylin dan eosin. Data ukuran pembaris menunjukkan bahawa luka yang dirawat dengan kulit Ansonia spinulifer *telah sembuh lebih cepat daripada* Phrynoidis juxtasper. *Walau bagaimanapun, penilaian histologi menunjukkan bahawa kedua-dua spesies* A. spinulifer *dan* P. juxtasper *tidak dapat ditentukan potensinya dalam penyembuhan luka disebabkan beberapa batasan, seperti tiada replikasi eksperimen, kesukaran dalam mengendalikan tikus dan kesilapan semasa menganalisis slaid histologi.*

Kata kunci*: penyembuhan luka, potensi, Bufonidae, histology, batasan*

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Frog skin is said to be useful in wound healing management. Frog skin can accelerate wound healing faster than the commercial wound dressing available at the market due to its microbicidal peptides content (Raghavan *et al*., 2010). According to Mashreghi *et al.* (2013), from the latest century, traditional medicine which used the frog skin as a biological dressing in Vietnam and South America had showed good effects in wound healing. Frog skin secretions also have wound healing properties and can reduce inflammation (Mashreghi *et al*., 2013). But most of the research study of the anuran skin in wound healing is on the frog species while there is still no research study on the toad skin in wound healing management. Therefore, the selected species in family Bufonidae were chosen to screen their skin potential in wound healing management via histological method.

Wound radius reduction rate is determined by estimating the wound area (multiplying the two longest diameters) and then calculating the radius of the wound and plotting changes over time (Zimny *et al*., 2003). According to Zimny *et al*. (2003), the wounded area (A, in mm^2) was measured its maximum length and width and calculated its wound radius (R) by the equation $R = \sqrt{A/\pi}$

During the research study, the incision of the wound area was done by using punch pliers. This gives an accurate and similar surface area measurement ($25mm^2$) of each of the wound area. The finding suggested that the wound treated with skin of *A. spinulifer* healed faster as compared to the normal dressing and the untreated wound. However, the wound treated with skin of *P. juxtasper* healed slower than the normal dressing wound and the untreated wound. The untreated wound healed the fastest. Differ from the histological analysis which based on the four parameters, the overall finding suggested that both species of Bufonidae was not effective for the wound healing assessment.

1.2 Objectives

The objectives of the research are:

(i) to screen the potential of Bornean Bufonids skin of selected species among family Bufonidae in wound healing; and

(ii) to assess the healing process by determining the healing time and comparing structure between untreated, normal and biological wound dressing via histological study.

CHAPTER 2

LITERATURE REVIEW

2.1 Family Bufonidae

Bufonidae is known as true toads, from the order Anura (frogs and toads). This family has a world-wide distribution, but Borneo and Southeast Asia have a distinctive set of genera and species (Inger & Stuebing, 2005). There are a few toads that have adapted to drier environments such as deserts by burrowing (Skeel, 2011). Toads of the family Bufonidae have a thick, warty and a glandular skin (Frost, 2009). They possess large parotoid glands on the side of their heads and have rather short hind limbs than the frogs. According to Frost (2009), the parotoid gland releases an alkaloid poison when the toads are in stress condition. The poison in the glands contains a number of toxins which cause different effects to the particular predator (Frost, 2009). They also have pectoral or shoulder girdles that are not fused together and have no maxillary teeth (Skeel, 2011). They usually feed on insects and other invertebrates. According to Skeel (2011), some species in family Bufonidae are not harmful, but important members of their ecosystems and food chain which serves as both predators and preys.

Genus *Phrynoidis* was recently removed from the synonymy of *Bufo* by Frost *et al*. (2006). There are two species in this genus which are *Phrynoidis aspera* and *Phrynoidis juxtasper* (Frost *et al*., 2006). *P. aspera* is one of the two large river toad species and also known as River Toad (Haas & Das, 2012). The other species in Genus *Phrynoidis* is *P. juxtasper*. It is a very large toad and also known as Giant River Toad. The males can reach add up to 120 mm, while the females 215 mm snout-vent length and it can get as big as a small rabbit (Haas & Das, 2012). Haas and Das (2012) stated that the toads secrete large amounts of highly toxic, milky poison from their warts when molested. The skin, eggs and the tadpoles of *P. juxtasper* are poisonous (Haas & Das, 2012). *P. juxtasper* is a good swimmer and jumper as well, as stated in Haas and Das (2012). *P. juxtasper* is an adaptable species and tolerance to a degree of habitat modification (Inger *et al*., 2004a).

Genus *Ansonia* is relatively small, slender toads with dry, rough skin but lack of parotoid gland (Inger & Stuebing, 2005). Most species have slender, elongated bodies and relatively long legs. According to Inger and Stuebing (2005), the species in Genus *Ansonia* have snout which usually projects beyond the mouth. The adults are occasionally seen on the leaves of herbaceous plants and usually will move to clear, rocky streams to breed (Inger & Stuebing, 2005). Species *Ansonia spinulifer* is common lowland stream toad. The adult males are usually 30-40 mm snout-vent length, while the females are up to 45 mm (Haas & Das, 2012). Genus *Ansonia* is much smaller than genus *Phrynoidis*. *A. spinulifer* is unable to adapt to modified habitats (Inger *et al*., 2004b).

2.2 Anatomy of the Skin

Bufonidae have a rough, drier skin with warts, live on land and use water for breeding purposes. They have large parotoid gland behind their eyes. Amphibian skin is morphologically, biochemically and physiologically complex organ which fulfils a wide range of functions necessary for the organism's survival (Govender *et al*., 2012). The skin is highly vascular which facilitates dermal respiration.

Amphibians (frogs and toads) rely upon their skin to breath for the exchange of oxygen and carbon dioxide with their environment (Chiasson & Underhill, 1951). Toads spend most of their time on land and therefore are in danger of desiccation (Chiasson & Underhill, 1951). The skin provides a defence against bacteria, fungi and other invaders. The glands in the skin of amphibian produce substances that are toxic to other animals. The chemical compounds secreted by the glands play various roles, either in the regulation of physiological functions of the skin or in the defence against predators and/or pathogens (Govender *et al*., 2012). Bettin and Greven (1986) also stated that the granular glands are the site of synthesis of a wide range of chemical compounds which provide protection against bacterial and fungal infection as well as predators. In *Bufo*, the granular gland cells are covered over the skin surface or arranged in enlarged clusters which forming discrete, compact glands such as the parotoid glands (Clarke, 1997). The skin glands produce a range of biochemical active compounds that may cause death to mammalian. Daly *et al*. (1987) stated that the main categories of secretions include biogenic amines, bufogenines and bufotoxins (steroids), alkaloids and peptides. Thus, the extraordinary range of biochemical found in amphibian granular gland secretions and the high probability of their containing compounds with a novel molecular structure as well as their clinically useful function, makes the amphibians an additional target group for medicinal purpose (Clarke, 1997).

Cei *et al*. (1972) found that the biogenic amines in the skin of *Bufo* are varied between species and subspecies. Meanwhile, bufogenines and bufotoxins have cardioacceloratory properties which could increase the strength of heart beat and decrease the heart rate (Clarke, 1997). Clarke (1997) stated that dried toad skin secretions have been used in Oriental medicine for the past 3000 years and were introduced into Europe in the 1600s, while Habermehl (1981) stated that many of the bufogenines and bufotoxins were used as a marked effect for local anaesthetic. According to Daly *et al*. (1987), alkaloids are found mainly in poison dart frogs (family Dendrobatidae), however they also can be found in toads such as short-headed toad (*Brachycephalus ephippium*) and cane toad (*Bufo marinus*). Clarke (1997) stated that the literature suggested that peptides may be either absent (Cei *et al*., 1968) or present in small amounts (Daly & Witkop, 1971) in *Bufo* species, because the peptides found mainly in frog species.

In fact, toad and frog skin extracts have been used in medicinal purpose. Gomes *et al*. (2007) stated that frog and toad skin has probably been the most exploited for their antimicrobial components. It is said that the granular glands produce secretions that might be effective against microbial and fungal infections.

2.3 Histological Technique

Histology is the scientific study of biological tissues via histological techniques. Histological technique is a branch of biology which concerned with the demonstration of minute tissue structures in living organism. It is carried out by examining a thin slice of tissue under a light microscope or an electron microscope (Kiernan, 2008).

Histological stains are often used in order to examine structural details (Kiernan, 2008). During the preparation for histology slides, hematoxylin and eosin (H&E) staining protocol will be used. According to Kiernan (2008), hematoxylin solution is used for nuclear staining while eosin solution for cytoplasmic staining. Hematoxylin usually gives staining blue or blue-purple in colour while eosin gives staining pink or pink-red in colour.

2.4 Frog Skin in Wound Healing

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers (Mercandetti, 2011). Wound healing involves a complex series of interactions between different cell types (Mackay & Miller, 2003). Each phase of wound is different even though the process is continuous. The main purpose in wound management is to heal the wound in the shortest time possible without making or causing the patient suffering from the pain.

Traditional healers in India use the dorsal skin of frogs from the genus *Hoplobatrachus* to cover the wounds of their patients (Govender *et al*., 2012). According to Govender *et al*. (2012), the lack of adequate medical supplies to treat napalm burns during the Vietnam War in the 1960s lead surgeons to investigate the traditional Vietnamese remedies for burns. They found that the use of amphibian skins from the genus *Hoplobatrachus* as temporary grafts for patients with severe skin loss was a successful means of treatment. The testing of the grafts to the rats showed that experimental wounds dressed with frog skin healed much faster than the wounds dressed with cotton gauze (Govender *et al*., 2012).

Amphibian skin is a hidden treasure with exciting biomedical potential (Sai & Babu, 1998). Using frog skin as wound healer had been practically used as traditional medicine in India (Sai *et al.*, 1995). Another proof had been shown when a faster healing was observed in the experimental study which was conducted on skin wounds from female albino rats that was dressed with the dorsal skin of freshly sacrificed frogs as compared to cotton gauze. Raghavan *et al.* (2010) also found out that the frog skin possess lipid components with pharmaceutical and therapeutic potential. The current study had expected the role of frog skin lipids in the inflammatory phase of wound healing.

2.5 Rat for Animal Experimentation

Rodents are the most common type of mammal used in experimental studies. Many researchers have been using rats, mice, guinea pigs and hamsters as their study organisms. Rat is the most used as an animal experimentation due to its similarity to human in terms of genetics, anatomy and physiology (Simmons, 2008). Simmons (2008) stated that rats are being used abundantly in research studies due to their genomes which are similar to that of humans, easy to handle, highly reproductive rates and relatively low cost of use.

Rats give an excellent model for skin wound healing because it provides a standard size, type, shape and the depth of the wound injury and thus make it possible to compare the data between studies healing in all mammalian species (Dorsett-Martin & Wysocki, 2008). Dorsett-Martin & Wysocki (2008) also stated that the rat is often selected for skin wound healing models due to its availability, low cost and small size, which result in a more economical and efficient use of limited laboratory space and housing facilities.

2.6 Wound Measurement Assessment

Wound assessment is very important in wound healing management. It is a complex process which included wound appearance, wound aetiology, prediction and monitoring of healing rates, identifications of factors delaying healing and wound documentation (Shaw & Bell, 2011). Wound measurement is needed in order to investigate the healing time of that particular wounded area. According to Fette (2006), the assessment of wound measurement is important in order to monitor the progress of healing process through the changes occurring in the length, width, area or volume of a wound. Wound measurement is an important component to provide a baseline measurement and accurately determine the percentage of reduction or increase in wound area over time (Shaw & Bell, 2011).

Wound measurement should be done routinely as the size of a wound is considered as one of the main indicators in order to look for the progress in healing time. According to Shaw and Bell (2011), wound measurement techniques can be categorised as contact or non-contact in their application. Tracing of the wound, the used of depth gauges and volume measurement by using casts or saline are the examples of contact techniques (Shaw & Bell, 2011). Non-contact techniques involving the use of the structured light and lasers, photography, video image analysis, magnetic resonance imaging and stereophotogrammetry (Williams, 1997).

Simple wound measurement method is one of the popular methods. It is the simplest and cheapest method to calculate the wound surface area which include the technique that measure the length and width measurement by using a ruler (Fette, 2006; Shaw & Bell, 2011). This method assumes that the wound has a geometric surface shape, as for example, surface area of a circle is measured by (diameter x diameter), while for an oval, (maximum diameter x maximum diameter) perpendicular to the first measurement (Fette, 2006). According to Shaw and Bell (2011), in order to determine the wound surface area, two important issues are taken: the identification of the wound margin (typically using a wound tracing or alternatively a digital image) and the calculation of wound area. Another popular method in determining the wound area is by tracing method. Wound area also can be determined by tracing the outline of the wound (wound circumference) onto a grid or graph paper with 1cm squares area and the number of squares of a known area are counted (Shaw & Bell, 2011). However, the accuracy of area measurement by using this type of method depends on correct and consistent identification of the wound margins (Chang *et al*., 2011).

Zimny *et al.* (2003) state that in order to determine the wound radius reduction rate, the wound area (multiplying the two longest diameters) is estimated and then plotting the mean wound radius against time to investigate the time course of wound healing, thus the slope of the regression curve is interpreted as the daily reduction of the mean wound radius. The mean wound radius (R) is calculated from the mean wound area (A, in mm^2) by the equation $R = \sqrt{A/\pi}$ (Zimny *et al*., 2003). Thus, by taking the measurement of wound radius, the wound healing time can be measured.

2.7 Wound Healing in Rats

Rats have been widely used in the study of skin wound healing and the efficacy of different treatment model (Dorsett-Martin, 2004). Dorsett-Martin (2004) said that rat is chosen as the model due to its availability, low cost and small size. According to Vidinsky *et al*. (2006), the rat skin structure is similar to human skin. The specific structural characteristics may vary depending on the body region (Marcelo *et al*., 2003).

Most of the studies are using rat's dorsum part as the wound location. According to Vidinsky *et al*. (2006), the skin of the dorsum of a normal rat is formed by epidermis, dermis and subcutaneous striated muscle. Thus, the healing of three different tissue layers can be studied due to the ability of proliferation. Only the epidermis has the capability to regenerate (Vidinsky *et al*., 2006).

According to Kumar *et al*. (2003), the complete regeneration of the rat epidermis is finished on the fifth day after the surgery, which is comparable to human being. The process of inflammation proliferative and remodelling phases in rat are faster than in human but comparable (Kumar *et al*., 2003). Therefore, it is also important to know the histological study of wound healing in rat skin.

2.8 Wound Healing Process

A wound is defined as damage or disruption to the normal anatomical structure and function (Velnar *et al*., 2009). Normal wound healing is a dynamic and complex process which involves the restoring of cellular structures and tissue layers

(Mercandetti, 2011; Velnar *et al*., 2009). Velnar *et al*. (2009) stated that a completely healed wound is one that has been returned to a normal anatomical structure, function and appearance of the tissue within a reasonable period of time. Mercandetti (2011) stated that the human adult wound healing process can be divided into three different phases; the inflammatory phase, the proliferative phase and the remodelling phase. The inflammatory phase is marked by platelet accumulation, coagulation and leukocyte migration, while proliferative phase is characterized by re-epithelialization, angiogenesis, fibroplasias and wound contraction, whereas the remodelling phase takes place in which the dermis responds to injury with the production of collagen and matrix proteins (Kirsner & Eaqlstein, 1993).

Braiman-Wiksman *et al*. (2007) stated that the initiation of healing is primarily dependent on the epidermal migration which derives re-epithelialization. This parameter is also known as epidermal closure. Re-epithelialization is widely accepted to be one of the major processes in wound healing that ensures a successful repair (Martin, 1997). According to Braiman-Wiksman *et al*. (2007), re-epithelialization can be observed through the hyperplastic epidermis at the wound edges, constitutes an essential component of the migrating cell pool which migrates to seal the wound gap. The migration of the keratinocytes towards the gap of the wound had provided a basis for another stage, such as granulation tissue formation (Braiman-Wiksman *et al*., 2007).

Granulation tissue formation was considered fully formed which is 100% when a continuous layer of granulation tissue formed across the entire wound gap and the layer of granulation tissue filled the entire wound depth (Braiman-Wiksman *et al*., 2007).

Another major process involved in the early stages of healing is the inflammation which is related to the inflammatory response (Jones *et al*., 2004). According to Kirsner and Eaqlstein (1993), the initial inflammatory response involves the recruitment of cells that fight potential bacterial contamination of the wound and activate cytokine secretion in order to activate dermal and epidermal processes. However, the inflammation should not go beyond a certain level; otherwise it will cause healing impairment.

In the final stages of healing, remodelling occurred in which the wounds are completely re-epithelialized and the final process is the dermal reorganization or dermal closure (Braiman-Wiksman *et al*., 2007). At this stage, the wounded skin regains its strength and elasticity and thus proceeds to the reorganization of the collagen and elastic fibers for the final reconstruction of the dermis (Braiman-Wiksman *et al*., 2007). Table 1 shows the histological skin cell parameters for assessment of wound healing according to (Braiman-Wiksman *et al*., 2007).

Table 1: The histological skin cell parameters for assessment of wound healing (Braiman-Wiksman, 2007).

Healing Parameter	**Assessment Parameter**
Epidermal closure	Basal layer of the epidermis. To asses the newly formed epidermis.
Granulation tissue formation	Fibroblast, new blood vessels
Inflammation	Inflammatory cells
Dermal closure	Abscess matrix remodelling