EVALUATION OF ANTIFUNGAL ACTIVITY AND WOUND HEALING POTENTIAL OF HERBAL CREAM FROM AMBU LEAVE EXTRACTS

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF A MASTER DEGREE OF SCIENCE IN NATURAL PRODUCTS TECHNOLOGY AND VALUE ADDITION OF SOKOINE UNVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

This study was conducted to evaluate the antifungal activity and wound healing potential of a cream from AMBU leave extracts. In this study AMBU leaf ethanol and aqueous extract were used in the formulation of cream. The antifungal activity was determined by broth microdilution method. Wound healing potential was assessed using mice which were divided randomly into seven groups (n=7) and inflicted with wound. Group 1 received no treatment while groups 2, 3, 4, 5, 6 and 7 were given cream base, Silver sulfadiazine cream (1% SS), 15% AMBU, 7.5% AMBU, 1% AMBU and 10 mg AMBU extracts respectively in order to evaluate dose effect relationship of AMBU cream. The percentage of wound healing on day 2, 4, 6, 8 and 10 were assessed. The physicochemical properties of the cream were within acceptable limit. The pH was in the range of 6.1- 6.8 which is comparable to skin pH. The MIC for antifungal cream was 9.38 mg/ml and 12.5 mg/ml for ethanol and aqueous cream respectively. There was a significant improvement in wound healing after application of AMBU cream with 15%, 7.5%, 1% and 10 mg of pure AMBU extracts compared to the negative control on day 4 ($p<0.05$), 6 ($p<0.01$) and 8 ($p<0.01$). On day 10 wound closure was comparable in all groups ($p>0.05$). The rate of wound healing was comparable with that of reference standard of 1% SS. AMBU cream revealed antifungal activity and wound healing potential. This activity may be ascribed to the presence of phytochemical contents an observation that require further studies. It is concluded that AMBU cream has the potential for treatment of fungal infection and wound healing.

DECLARATION

I, Musa Julius Mtuga do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

_____ _____

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(MSc. Candidate)

The above declaration is confirmed by;

_____ _____

Dr. Faith P. Mabiki Date

(Supervisor)

_____ _____

Prof. Robinson H. Mdegela Date

(Co-supervisor)

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DEDICATION

This work is dedicated to my lovely father Julius Mtuga and mother Domitila who supported and encouraged my dream to undertake postgraduate studies and now my dream come true.

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LIST OF ABBREVIATION

1%SS	Silver sulfadiazine cream
AIDS	Acquired Immune Deficiency Syndrome
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
HE	Hematoxylin and eosin stain
HIV	Human Immune Virus
INT	Idonitro tetrazolium salt
ITM	Institute of Traditional Medicine
LC_{50}	Lethal concentration fifty
MIC	Minimum inhibitory concentration
MUHAS	Muhimbili University Health and Allied
O/W	Oil in water emulsion
TFDA	Tanzania Food Drug Authority
W/O	Water in oil emulsion
WHO	World health organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Skin care preparations are designed to exert activity when applied on the skin. The preparations includes creams, lotions and ointments and are used in the treatment of burns, wound, bacterial and superficial fungal infections. Considering the concentration of drug and the degree of solubility, topical formulations are said to be beneficial for treating localized skin infection (Woodruff, 1995).

Plants with medicinal properties have been used as traditional medicine from time immemorial. The extract from the leaves, stem and roots of various medicinal plants have been used for cure various ailments (Kandasamy *et al.,* 2014). Plants have always served as essential sources of therapeutic agents for human and animal diseases. Traditionally the herbal remedies may be offered in a holistic approach for maintenance and well-being of health and other body processes (Trakranrungsie *et al.,* 2008**)**. The modern herbal formulation based on scientific proven efficacy may provide a treatment of choice for specific disease conditions with a stronger clinical outcome (Trakranrungsie *et al.,* 2008).
The active constituents responsible for such medicinal values are extracted and formulated as creams, soaps and ointments for treating skin related ailments like wounds, ring-worms, as an anti-microbial agent and for cosmetic purposes (Kareru *et al.,* 2010). There have been growing interests in the use of plants and plant products to treat diseases or improve health in humans and animals (Bussmann and Sharon, 2006). Since plants are among the potential source of novel compounds, scientists and pharmaceutical industries worldwide have shown deep interest in the field of phytochemistry (Adnan *et al*., 2010). To date

traditional herbal formulations are regarded as an alternative to modern medicines. Baseline research gives phytochemical information that calls for further research on pharmaceutical formulations, pharmaceutical tests for drug validation and standardization. Traditional medicine is globally used as the primary means of health care for more than 95% of which is contributed by medicinal plants (Akerele, 1992). The world plants population is estimated to comprise about 250 000 medicinal plant species. Approximately 50 percent of the drugs commonly used clinically are derived from tropical plants (Akerele, 1992; Padulosi *et al*., 2002; Mamedov, 2012). The contemporary struggle for drug discovery from medicinal plants involves an interdisciplinary approach combining botany, pharmacology, ethnology, and anthropology (Kayombo *et al*., 2013).

In East Africa plants of the family Euphorbiaceae have extensively been used as medicinal plants. AMBU is a medicinal plant which has been used by some Tanzanian tribes to treat various diseases in humans and animals (Mabiki *et al.,* 2013). Various crude extracts including aqueous, ethanol and dichloromethane extracts have shown efficacy against viral, bacterial and fungal strains (Mabiki *et al.,* 2013).

1.2 Problem Statement and Justification

The rate of skin infections due to bacterial and fungal organisms is increasingly high. This has become a significant health problem in developed and developing countries and particularly in areas with high humidity and poor hygienic conditions. The issue of resistance of dermatological infection to some drugs available on the market has sparked up the interest in the research of the antimicrobial properties of drugs from natural sources which are active against major causative agents. Drug resistant strains cause severe problems in many infections including skin infection such as carbuncles, impetigo and

burn wound sepsis. One of the ways to prevent antibiotic resistance is by using new compounds that are not based on existing synthetic antimicrobial agents.

Plants possess a wide range of bioactive compounds which makes them potential source of antimicrobial agents and different types of medicines. AMBU as one of the studied species has been reported to possess antimicrobial activities (Mabiki *et al.,* 2013). Although the plant extract showed strong *in vitro* cytotoxic and antifungal properties (Mabiki, 2013) none of them have been used as an active ingredient in cream products for skin treatment. Despite having some formulation of medicinal products from AMBU extracts as active ingredient none of the extracts have been used for formulation of antifungal and wound healing cream. Thus this study was designed to evaluate the antifungal activity and wound healing potential of AMBU cream formulation from ethanol leave extracts. The results of this study will contribute to availability of natural bioactive cream formulations on the market for wound healing and treating fungal infections.

1.3 Objectives

1.3.1 Overall objective

To determine the physicochemical properties, antifungal activity and wound healing potential of herbal cream formulation from leaf extracts of AMBU as active ingredient

1.3.2 Specific objectives

1. To evaluate the shelf life and physicochemical properties of antifungal AMBU cream formulation.
2. To assess the antifungal activity of the formulated AMBU cream against *Candida albicans* and *C. tropicalis.*
3. To determine the wound healing potential of cream formulation from AMBU leaf extracts.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background Information of AMBU

AMBU is a medicinal plant from a family Euphorbiaceae it is used by traditional healers in treating different diseases. The members of the Euphorbiaceae family are widely utilized for different purposes in each ethnic group (Mwine and Damme, 2011). The plant grows in several regions in Tanzania such as Morogoro, Lushoto and Njombe. Indigenous people have been using them for treatment of both animal and human illnesses. The plant is used in Tanzania to treat various diseases including skin conditions, sores and wounds (Chhabra *et al.*, 1984).

Studies show that the plant is poisonous, traditional healers used a leaf decoction, mixed with lemon juice, baking soda and honey, in the treatment of asthma with no adverse effects. In Tanzania women suffering from excessive menstruation, drink the juice of fresh crushed leaves (Chhabra *et al*., 1984; Mwale *et al.,* 2005). The plants latex is highly irritating to the skin causing blisters and pain. In addition to medicinal uses, the latex is an ingredient in arrow and fishing poison in Kenya (Neuwinger, 2004).

2.2 Medicinal Value of AMBU

The diverse phytochemistry of the *Euphorbia* species revealed potent medicinal value. A number of studies have addressed medicinal significance of various extracts of AMBU. The value of AMBU in medicine is based on its antimicrobial activities against various microbes (Mabiki *et al.*, 2013). AMBU is a well-studied plant, it is reported by many sources to be a potent medicinal plant. Extracts from the plant are reported to have antiviral activities against New castle disease in chickens experimentally infected with the

virus and the extracts exhibited a significant strength upon both prophylactic and therapeutic administration of doses (Wickama *et al.*, 2006; Mabiki *et al*., 2011). The antiviral activity was tested *in vivo* in mice and the extracts suppressed the growth of the New castle Disease virus.

AMBU is well explored and from the existing data the plant exhibits a broad spectrum of medicinal utility for both, infectious and non-infectious diseases. In a study using agar well diffusion method against some species of fungi and bacteria (Mabiki *et al.,* 2013), it was revealed that different morphological parts particularly roots, root bark, stem wood and leaves have efficacy against growth of several infectious microorganisms. Among species tested were *Candida albicans*, *Aspergillus Niger*, *Pseudomonas aeruginosa* and *Escherichia coli* and all the plant parts exhibited significant antimicrobial activity against the entire microorganism tested.

Studies reveal that AMBU extracts have shown strong cytotoxic effect. The extracts from dichloromethane and ethanol display the highest level of cytotoxic. The results from brine shrimp lethality test indicates that the extracts from AMBU could be both bioactive and anticancer as they were all demonstrating the LC_{50} below 30 µg/ml lower to 0.65 µg/ml, the dichloromethane extracts were more toxic followed by pet ether and ethanol (Mabiki *et al.,* 2013). This validates that experience from traditional user the plant is very corrosive and toxic to human being and sometimes used for fishing.

2.3 Phytochemistry of AMBU

Members of Euphorbiaceae family contain a number of organic compounds in form of cyclic, aliphatic and aromatic compounds. Phytochemicals are chemicals naturally occurring in plants and many of them now are recognized to have health promoting

activity (Apostolidis *et al.,* 2006). The Phytochemical screening test of AMBU show that the plant extracts from roots, barks and leaves has many compound which are thought to have pharmacological activities. The aqueous extracts of the leaves and stem of AMBU is reported to have positive reaction for tannins, triterpenoids, and coumarins while the methanol extracts have steroids, tritepenoids, and anthocyanin and the petroleum ether extract contains carotenoids, tritepenoids, volatile oils and glucosides (Rukunga *et al*., 1990; Neuwinger, 1994). According to phytochemical screening by Mabiki *et al.* (2013) reported that dichloromethane extract indicated the presence of two main triterpenoids that best matched with lanosterol and cycloartenol. The ethanolic extract indicated the presence of polyphenolic compounds.

2.4 Classification of Fungal Infection

2.4.1 Superficial mycoses

Superficial mycoses infection is localized to the skin, hair, and nails, an example is ringworm or *Tinea*, an infection of the skin by a *dermatophyte* (Hector, 2005). Ringworm refers to the characteristic central clearing that often occurs in *dermatophyte* infections of the skin. Dermatophyte members of the genera *trycophyton*, *microsporum* and *epidermophyton* are responsible for the disease (Graser *et al.,* 2008). *Tinea* can infect various sites of the body, including the scalp (*Tinea capitis*), the beard (*Tinea barbae*) the foot (*Tinea pedis*) athlete s foot and the groin (*Tinea cruris*) (Judith, 2005; Chah *et al.,* 2012).

Candida albicans is yeast causing candidiasis or thrush in humans as a superficial mycoses, candidiasis typically infects the mouth or vagina (Oyewole *et al.,* 2013). *C. albicans* is part of the normal flora of the vagina and gastrointestinal tract and is termed a commensal. However during times of ill health or impaired immunity the balance can alter

and the organism multiplies to cause disease. Antibiotic treatment can also alter the normal bacterial flora allowing *C. albicans* to flourish (Pfaller *et al.,* 2007).

2.4.2 Subcutaneous mycoses

These are infections confined to the dermis, subcutaneous tissue or adjacent structures. Infection may arise following the wounding of the skin and the introduction of vegetable matter (Theodore *et al.,* 2008). These mycoses are rare and confined mainly to tropical regions. They tend to be slow in onset and chronic in duration example is sporotrichosis caused by sporothrix schenckii. The fungus is dimorphic, being a mould that can convert to a yeast form at 37°C on rich laboratory media or in infection (Moyne *et al.*, 2011).

2.4.3 Systemic mycoses

These are invasive infections of the internal organs with the organism gaining entry by the lungs, gastrointestinal tract or through intravenous lines. They may be caused by (i) primary pathogenic fungi (ii) by opportunistic fungi that are of marginal pathogenicity but can infect the immunocompromised host.

2.4.4 *Candida* species

Candida species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina (Shao *et al.,* 2007), and are responsible for various clinical manifestations from mucocutaneous overgrowth to bloodstream infections (Eggimann *et al.,* 2003). These yeasts are commensal in healthy humans and may cause systemic infection in immunocompromised situations due to their great adaptability to different host niches. The genus is composed of a heterogeneous group of organisms, and more than 17 different *Candida* species are known to be etiological agents of human infection; however, more than 90% of invasive infections are caused by *Candida albicans*,

Candida glabrata, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* (Pfaller *et al.*, 2007).

The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to evade host defenses, adherence, biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin (Silva *et al.*, 2011). Currently, an increase in the number of yeasts that are resistant to antifungal drugs is recognized worldwide; therefore the use of *in vitro* laboratory tests may aid the doctor in choosing an appropriate therapy (Ingham *et al.*, 2012).

The ability of *Candida* species to form drug-resistant biofilms is an important factor in their contribution to human disease. As in the vast majority of microbial biofilms (Rajendran *et al.*, 2010), sessile cells within *C. albicans* biofilms are less susceptible to antimicrobial agents than are planktonic cells (Kuhn and Ghannoum, 2004).

The progression of drug resistance within *Candida* biofilms has been associated with a parallel increase in the maturation process (Sardi *et al.*, 2011). Furthermore, some studies have also shown that biofilms of *Candida* develop statically in the presence of a minimal matrix and exhibit the same level of resistance to drugs (fluconazole and amphotericin B) as cells grown in a shaker and exhibiting large amounts of matrix (Seneviratne *et al.*, 2008; Sardi *et al.*, 2011). The increase in resistant strains necessitates a search for new targets for new antifungal agents (White *et al.*, 1998; Sardi *et al.*, 2011).

2.5 Wound Healing Process

Wounds are physical injuries that results in an opening or breaking of the skin. Proper healing of wounds is very essential for the restoration of disrupted anatomical continuity

and disturbed functional status of the skin. Wound healing is a complex but generally orderly process. Sequential waves of specialized cell types first clear the inciting injury and then progressively build the scaffolding to fill in any resulting defect. Healing process is not complete until the disrupted surfaces are firmly knit by collagen (Profyris *et al*., 2012; Kawasumi *et al.*, 2013).

Wound healing is a complicated process occurring in injured tissue to restore its construction and return the damaged tissue to its normal situation as soon as possible (Ghosh *et al*., 2012). There are three stages for wound healing: inflammation, proliferation and remodelling of the extra cellular matrix. The proliferative phase is defined by angiogenesis, collagen deposition, epithelialization and wound contraction (Atiyeh *et al*., 2002). The aim of the healing process is to prevent pathogens invasion, confirm the integrity of damaged tissue, and reconstruct the skin physiological function (Nayak and Pinto, 2006).

Oxidants are inhibitory factors to wound healing due to their cell damage ability. Studies of the topical application of compounds with free radical scavenging properties on patients or animals have been used in wound healing and protecting tissues from oxidative damage. Antioxidants could also have an important role in survival of ischemic skin flaps or promotion of wound healing (Thang *et al*., 2001).

Inflammation is a protective process conducted by the organism with the purpose of removing the harmful stimuli and initiating the operation of healing. However the excessive and unbalanced inflammation could delay the healing period and enhance scarring which suggests a promising target for future therapeutic interventions or even predisposes tissue to cancer development (Oberyszyn, 2007). Therefore, anti-

inflammatory compounds are considered as effective agents in wound healing (Chandran and Kuttan, 2008).

Despite the natural progression of the wound healing process, an infection can delay the process by several mechanisms such as decreasing blood supply, promoting disordered leukocyte function, prolonging inflammatory and debridement phases and producing proteolytic enzymes. So, infection is the major complication of injuries and antibacterial agents play an important role in the wound healing process (Chandran and Kuttan, 2008).

Plants have an extensive potential for the management and treatment of wounds and burns with their antioxidant, anti-inflammatory and antimicrobial activities (Ghosh *et al*., 2012). Anti-inflammatory and antioxidant compounds are considered as effective agents in wound healing with ability of scavenging free radicals and reducing cell damage.

2.5.1 The use of herbal cream in wound healing

Medicinal herbs have been used to help heal wounds, burns and skin ulcers for thousands years. Ancient wound healing recipes offer numerous herbs, herbal oils, creams and ointments with wound healing properties.

Scientific research conducted during the last century has expanded our knowledge about wound healing properties of many herbs (Raina *et al*., 2008). Preclinical studies have shown that certain herbs, including Sea Buckthorn, Rose hips, and others contain ingredients with anti-inflammatory, antibacterial, skin regenerative properties which are beneficial for wound healing (Hinz, 2007). It was confirmed that herbal extracts, oils and ointments traditionally are used to heal skin wounds and thus accelerating regenerative processes of the epithelial cells and promote the restoration of the skin and mucous. A

number of herbal ingredients with antibacterial and anti-inflammatory properties used in folks wound healing remedies were identified and employed in modern skin treatments (Amponsah *et al*., 2013; Kumar *et al*., 2013).

Animal studies have shown that certain herbal extracts are able to accelerate wound healing process by reducing the inflammation of the surrounding skin and accelerating the proliferation rate of epithelial cells in the wound area (Kanafani and perfect, 2008). Daily applications of the herbal medicines were shown to promote the wound closure and scar (Jackson and Shelton, 1999).

2.5.2 Medicinal plants commonly used in traditional wound healing

There are a number of plants which are used traditionally by different ethnic groups in wound healing. Generally a pharmacologist should study traditional systems of medicine in scientific way and validate by screening plant extracts for pharmacological activity.

Aloe vera the oldest medicinal plant that was used in wound healing, it has anti-inflammatory effect therefore it can be used in treating skin wound and gingivitis (Ajmera *et al.,* 2013; Khan *et al.,* 2013) Today *Aloe vera* gel is an active ingredient in hundreds of skin lotions, sun blocks and cosmetics (Ahmed *et al.,* 2015). *Aloe vera* is an excellent remedy for minor burns, cuts and sunburns. Both juice and aqueous extract from the leaves are reported to have significant healing properties. It is not only speeds up healing but also prevents injured surface from getting infected (Chitra *et al.,* 1998; Kareru *et al.,* 2008).

Gingko biloba has been found to have significant activity against both dead space and excision wound models in male rats. A dose of 50 mg/kg has significantly promoted the breaking strength and hydroxyproline content of granulation tissue in dead space wounds

and in case of excision wound model, it is found to shorten the epithelization period (Bairy *et al.*, 2001). It is also reported that the activity of *Gingko biloba* is due to its high amino acid content which is absorbed rapidly in blood stream and in combination with vitamins; they provide essential nutrients to the wound area to promote healing. Apart from wound healing, it is used as an anti-inflammatory and antiallergic agent in ancient Chinese medicine.

Alcoholic extract of neem is useful in eczema, ringworm and scabies. Neem leaf extracts and oil from seeds has proven antimicrobial effect. This keeps any wound or lesion free from secondary infections by microorganisms. Clinical studies have also revealed that neem inhibits inflammation as effectively as cortisone acetate; this effect further accelerates wound healing (Raina *et al*., 2008). Other plants with wound healing activity are *Curcuma longa, Eucalyptus***,** *Zanthoxylum leprieurii, Croton sparsiflorus morong, Camphora officinarum, Malva sylvestris, Solanum nigrum* leaves \and oil extracts of *Rosa damascena.*

2.5.3 Herbal cream formulated at the institute of tradition medicine

There are different herbal formulations which are available on the market prepared by local people. These herbal drugs are used by people in managing different health problems for example creams in managing skin related issues like fungal infection, wounds and allergic reactions. Most of these drug formulations in use are not formally registered by Tanzania food and drug authority (TFDA). Examples of formulation by institute of traditional medicine (ITM) in Muhimbili University of health and allied sciences (MUHAS) include *Aloe vera* cream, *Mangifera* cream and *Ravo* cream these cream are not registered but are in market.

Figure 1: Herbal cream formulated at ITM *Aloe vera* cream, *Mangifera* cream and *Ravo* cream

2.6 A Cream Formulation

Cream is defined as semisolid emulsions which are oil in-water (O/W) or water- in- oil (W/O) type and these semisolid emulsions are intended for external applications. Creams are often composed of two phases. Oil-in-water (O/W) emulsions are most useful as water-washable bases, whereas water-in-oil (W/O) emulsions are emollient and cleansing agents. An emulsifying agent is used to disperse the aqueous phase in the oily phase or vice versa (Singh *et al.,* 2011, Khalid *et al.,* 2015). The important components in cream formulation are emulsifying agent, water, oil, thickener and preservatives.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The study adopted an *in vitro* and *in vivo* experimental design. This study was done at Sokoine University of agriculture (SUA) in the College of Science specifically using Chemistry laboratory, and College of Veterinary and Medical Sciences in microbiology and natural products laboratories.

3.2 Sample Collection

The fresh plant materials were collected in Morogoro Municipal. The sample was identified and confirmed by a botanist from faculty of forestry and nature conservation. The leaves of AMBU were cleaned and then air dried for three weeks under a shade. The dried plant materials were grinded using a blender to get smaller particles of about 0.5 mm so as to increase the surface area for extraction.

3.3 Extraction Methods

The plant materials were extracted using organic solvents and aqueous solvent. The organic solvent used was ethanol. Maceration extraction method was used to extract the active ingredients from the dried leaves of AMBU; 100 g of dried leaves were soaked in 500 mls of ethanol and left over night with continuous stirring using magnetic stirrer for 24 hours. After extraction the sample was filtered using filter paper to obtain the filtrate. The filtrate was dried to remove solvent using rotary evaporator at temperature of 40 ºC to 70 ºC. The dried sample was stored in the refrigerator at 4 ºC.

For aqueous extraction 150 g of AMBU leaves were soaked in 2000 mls of water and heated with continuous stirring using magnetic stirrer. The temperature used for heating was around 50 ºC to 80 ºC for extracting the active ingredients from the plants. After extraction the plant materials were filtered using filter paper. The filtrate was dried using freeze dryer. The dried sample was stored in the refrigerator.

3.4 Preparation of the Antifungal AMBU Cream

Herbal cream is made up of two parts the supporting phase (base) and an active ingredient. The base was prepared separately by mixing oil portion and the aqueous portion. The oil portion was prepared from the mixture of 30% emulsifying wax, 20% liquid paraffin and 50% petroleum jelly. The materials were mixed together and melted in a container with continuous stirring until an oily mixture is achieved. After all ingredients were melted they were left to cool. The preparation above is called an emulsifying ointment which is used to provide emollient properties, moistening and non-irritating qualities of the cream to keep the skin in a moist condition.

To prepare aqueous cream a 30% of emulsifying ointment was mixed with 70% of hot water. Both were melted to obtain the uniform mixture which was left to cool. The preservative used was chloroscresol which was used to prevent the growth of bacteria and moulds. From the prepared cream base the active ingredients of the plants extracts with different percentages were added until we obtained an antifungal cream with a good activity against test organisms. The prepared cream was stored in the plastic container.

3.5 Evaluation of Physicochemical Properties of AMBU Cream

Evaluation of the herbal cream was carried out to evaluate the physicochemical properties of the prepared cream. The physical properties evaluated were,

3.5.1Appearance

The appearance of the cream was judged by its colour and roughness by visual observation and touching of cream by three volunteers. 2 g of cream was placed in the watch glass for colour and roughness examination.

3.5.2 pH determination

pH was measured by using pH meter. Measured cream of 1 g was dissolved in 50 mls of distilled water after dissolution the pH was measured and recorded.

3.5.3 Spreadability

Spreadability is a term expressed to denote the extent of area to which the ointments or cream readily spreads on application to skin or affected part. Spreadability was assessed by applying a certain amount of cream on the shaved skin area of the albino mice and the extent to which it spread was observed and graded as bad, good or very good.

3.5.4 Short term stability studies

Short term stability was done for a period of one month at different intervals of 10 days, 20 days and 30 day. A portion of the antifungal cream was kept at room temperature and another was refrigerated at $4^{\circ}C$ and changes in physical properties and activity were evaluated (Bhatia; 1998).

3.5.6 Homogeneity

Homogeneity of the creams was graded by observing the uniform distribution of extracts in the cream base. After the application of the cream, the type of film or smear formed on the skin was checked. The ease of removal of the cream applied was examined by washing the applied part with tap water.

3.6 Assessment of Antifungal Activity of AMBU Cream

Antifungal activity was carried out to determine the minimum inhibitory concentration (MIC) of the prepared herbal cream which was performed with modification by micro-plate dilution method, according to standard guideline of Clinical and Laboratory Standards Institute (CLSI) and was evaluated in the presence of standard drug ketoconazole from parchem global supplier. *p*-Iodonitrotetrazolium chloride (INT) was used as fungal growth indicator (Eloff, 1998; Mativandlela *et al.,* 2006).

3.6.1 Fungal strains

Reference strains, *C. albicans ATCC 10231* and *C. tropicalis ATCC 13803* were obtained from the Microbiology Department of Muhimbili National hospital. Sabouraud dextrose agar was used for the activation of the fungal growth while sabouraud dextrose broth was used for antifungal assays.

3.6.2 Antifungal susceptibility testing

Micro dilution method was used as described by Kuete (2010). 1 g of cream was dissolved in 2 mls of dimethyl sulphoxide (DMSO) in which Sample of 60 µl was diluted in 60 µl of sabouraud dextrose agar broth, followed by serial dilution to the tenth well. The standard 0.5 McFarland known to form 1.5 x 10^8 CFU/ml was prepared by taking two to four colonies in normal saline solution following standard procedure (Bekele *et al.,* 2015). Then 0.01 ml of 1.5x10^8 CFU/ml of the test fungal suspension was added to serial diluted drug. The standard drug ketoconazole was serial diluted the same way as the test drug. The MIC of samples was detected after 24 hrs incubation at 37 °C, following addition of 40 µL of 0.2 mg/ml INT. The MIC was defined as the sample concentration at which no colour developed following addition of the INT.

3.7 Determination of Wound Healing Activity of AMBU Cream Using Mice Model

3.7.1 Experimental animals

Forty nine mice aged 3 month male and female with an average weight of 14–27 g were kept in wood cages and served with normal commercial mice diet and water and were maintained under laboratory conditions (temperature of 28 to 30°C and normal light-dark cycle). The mice were accustomed in the laboratory for one week before the experiment. This was done to reduce the stress of experimental handling and experimental conditions. There was no difference of normal life of mice before the laboratory life and after laboratory life. Techniques and methods used in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals. (Leticia, 2010; Nelson, 2010).

3.7.2 Experimental design

Forty nine mice with average weight of 14-27 g were anaesthetized with 50 mg/kg ketamine by intramuscular injection. The dorsal furs of the animals were shaved to a circular diameter of about 20 mm by using scalpel blades and scissors, and the anticipated area of the wound was marked on the shaved skin. The area was cleaned with 70% v/v ethanol before wounds excision, wound diameter of 10 mm was created on the back of the animal which involves removal of part of skin excision at the back of mice. The wounds were wiped with saline and the entire wound left opened to the atmosphere (Diwan *et al.*, 1982). The animals were divided randomly into seven (7) groups of 7 animals each (Table1).

The animals were topically treated with herbal cream once every day for a period of ten days and the wound contraction was measured after every two days. The treatment was

monitored by assessing any effect which was seen in mice example ulcers formation and swelling.

Table 1: Treatment groups

Groups	Treatment allocation
G1	Control untreated group
G2	Treated with cream base
G3	Silver sulfadiazine (1%SS) (positive control)
G4	15% AMBU cream
G5	7.5% AMBU cream
G6	1% AMBU cream
G7	10 mg AMBU extracts alone

3.7.3 Histological examination

Wound tissues were taken on day 3 and day 10 from group 1, 3 and 4 after treatment of wounds, to assess the influence of the extracts and reference drugs on skin cells. The cross-sectional full-thickness wound scar of about 5 mm thick sections from group1, 3 and 4 were collected for the histological evaluation. Samples were fixed in 10% buffered formalin for 24 h, dehydrated with a sequence of ethanol-xylene series, processed and then blocked with paraffin at 40 to 60°C. Then trimmed with a microtome at 25 µ and sectioned into 4 µ sections. The sections were stained with hematoxylin and eosin stain (HE staining) after that they were examined by electronic microscope (Kiran and Asad, 2008; Murthy *et al.,* 2013).

3.7.4 Statistical analysis

Multiple comparison tests for different dose groups were conducted. Data were recorded and stored in excel program and analyzed to get standard deviation and standard error of the mean, then further analysed by one way ANOVA test, using SPSS version 20 and a p-value of less than 0.05 was considered to be statistically significant difference.

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Properties of AMBU Cream

The formulations were oil water (O/W) type emulsion cream. All formulation showed good stability in O/W type emulsion, the results of physical properties are summarized in Table 2. The pH of the cream was found to be in the range of 6.1- 6.8 this cream pH is neutral to the skin. These properties were stable for 30 days.

Table 2: Recorded physicochemical properties of AMBU cream

Parameter	**(15% AMBU)**	**(7.5%)**	**(1%)**
Appearance	brown	Light brown	Grey
Consistency	Easy spreadable	Easy spreadable	Easy spreadable
pH	6.1±0.047	6.4± 0.047	6.8± 0.028
Spreadability	Good	Good	Good
Easy of removal	Easy	Easy	Easy
Type of smear	Non greasy	Non greasy	Non greasy
After feel	Emollient	Emollient	Emollient

4.2 Assessment of Antifungal Activity of AMBU Cream

Minimum inhibitory concentration (MIC) values of AMBU cream formulation and positive controls against *C. albicans* and *C. tropicalis* are as shown in Table 3. The cream formulation exhibited antifungal activity against *C. albicans* and *C. tropicalis* with the organisms being most susceptible at 15% cream formulation.

Table 3: Minimum inhibitory concentration of cream extracts

Cream (% concentrations)	MIC (mg/ml)	
	C. albicans	***C. tropicalis***
Aqueous extract 5	12.5	12.5
Ethanol extract 15	9.38	9.38
Ketoconazole 1	0.5	0.5

4.3 Determination of Topical Wound Healing Activity of AMBU Cream in Mice

In this study wound healing potential of AMBU cream was determined by measuring the wound closure in all groups treated and control groups. The results show that all treated groups with 15%, 7.5%, 1% and 10 mg of pure AMBU extracts significantly increased the rate of wound closure compared to the negative control on day 4 ($p<0.05$), 6 ($p<0.01$) and 8 ($p<0.01$) as shown in (Table 4, figure 1, 2, 3 and 4). On day 10 onwards the mean diameter wound closure was comparable in all groups ($p>0.05$).

The mean diameter wound closure was calculated on day 2 to day 10 post wound treatment. The wound healing was found to be comparable with that of the reference standard cream of 1% silver sulfadiazine (1%SS).

Table 4: Mean wound diameter and percentage wound closure for ten days of treatment (expressed as Mean ± Standard error of the mean (mm))

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
G1	10	9.07 ±0.13 (9.3%)	7.5 ± 0.21 (25%)	5.42 ±0.17 (45.8%)	3.42 ±0.22 (65.8%)	1.21 ±0.26 (88%)
G2	10	9.00 ±0.15 (10%)	7.42±0.17 (25%)	5.42 ±0.17 (45.8%)	3.57 ±0.22 (64.3%)	1.28 ±0.42 (87.3)
G3	10	8.21 ±0.10 (17.9%)	5.58±0.17 (44.2%)	3.00 ±0.10 (70%)	1.64 ±0.17 (83.6%)	0.42 ±0.20 (95.8%)
G4	10	8.35 ±0.14 (16.5%)	5.78±0.18 (42.2%)	3.00 ±0.10 (70%)	1.64 ±0.17 (83.6%)	0.42 ±0.20 (95.8%)
G5	10	8.92 ±0.13 (10.8%)	6.28±0.14 (37.2%)	3.71 ±0.28 (63%)	1.85 ±0.26 (81.5%)	0.85 ±0.26 (91.5%)
G6	10	8.92 ±0.13 (10.8%)	6.21±0.18 (37.9%)	3.42 ±0.20 (65.8%)	1.71 ±0.28 (82.9%)	0.71 ±0.28 (92.9%)
G7	10	9.00 ±0.15 (10%)	6.07±0.13 (39.3%)	3.21 ±0.14 (67.9%)	1.71 ±0.28 (82.9%)	0.57 ±0.29 (94.3%)

Key: G1: untreated, G2; cream base, G3; 1%SS Silver sulfadiazine, G4; 15%AMBU, G5; 7.5% AMBU, G6; 1% AMBU, G7; AMBU extract only.

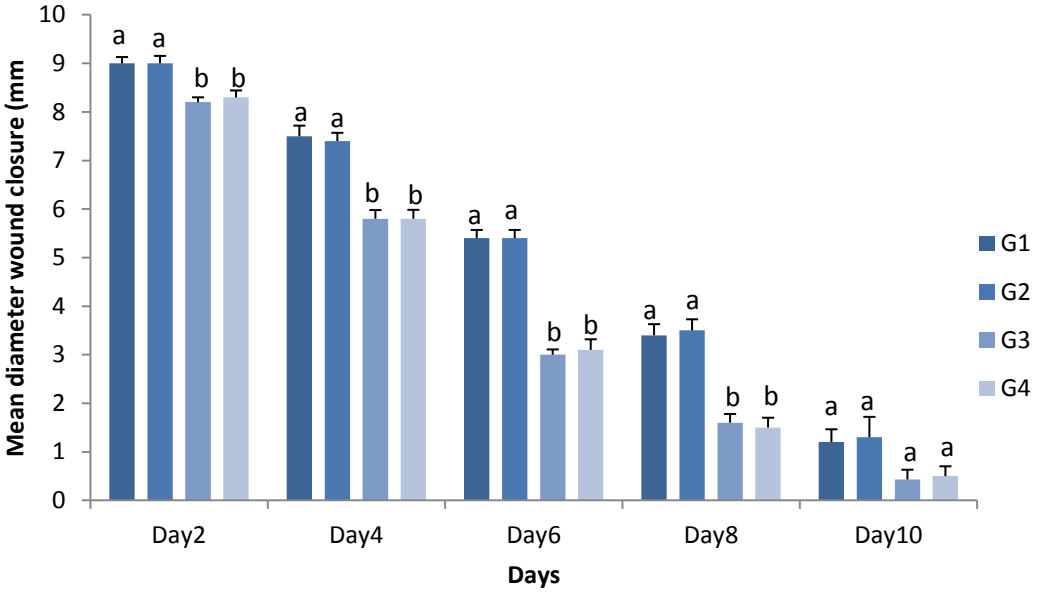


Figure 2: Effect of 15% AMBU cream on wound closure ($p<0.05$), where, G1=untreated, G2= cream base, G3= standard drug, G4= 15% AMBU

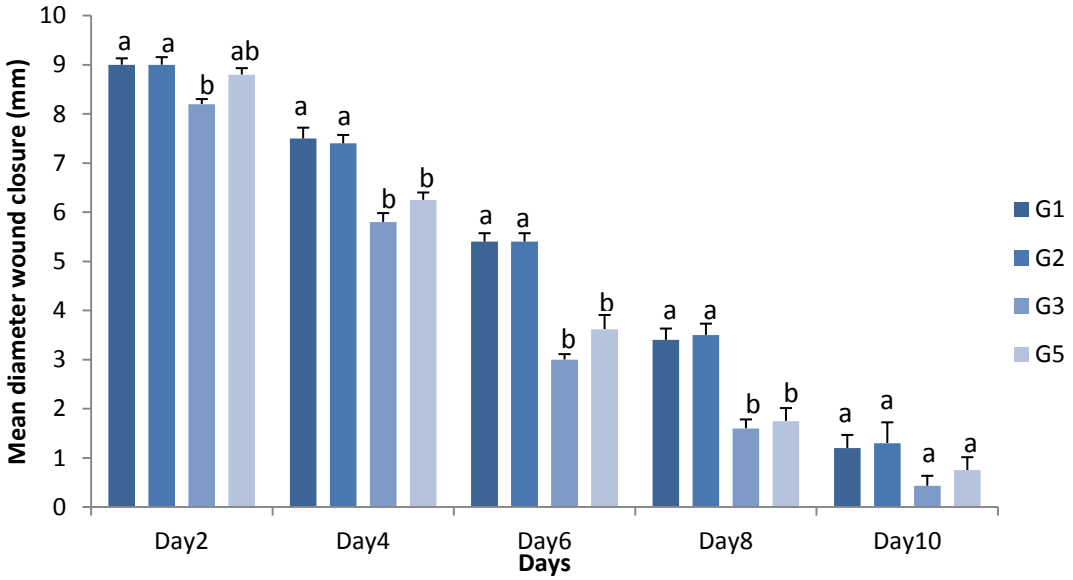


Figure 3: Effect of 7.5% AMBU cream on wound closure ($p<0.05$), where G1=untreated, G2= cream base, G3= standard drug, G5= 7.5%AMBU

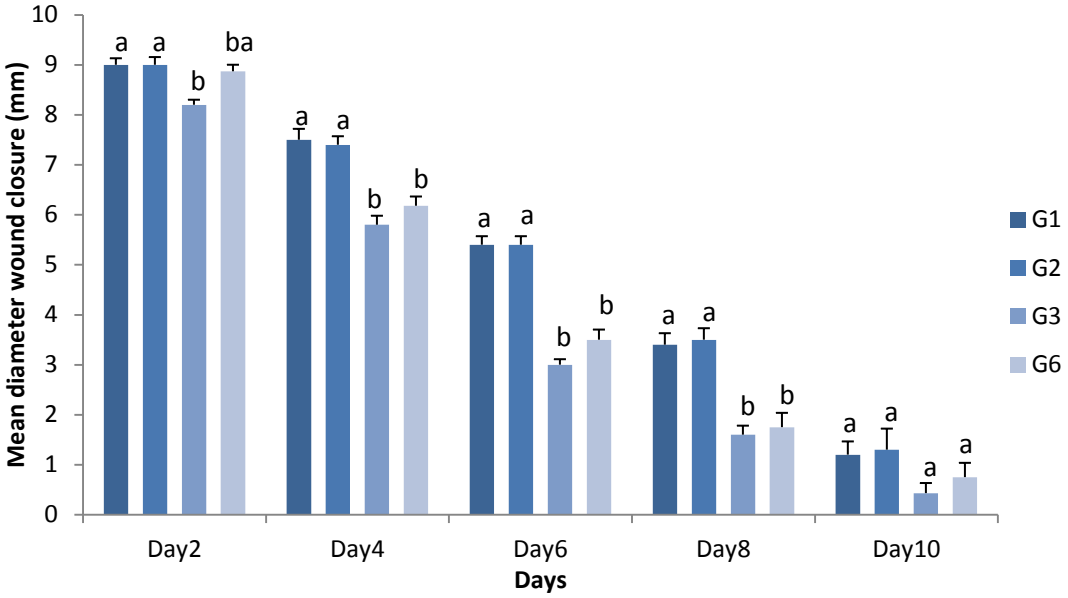


Figure 4: Effect of 1% AMBU cream on wound closure ($p<0.05$), G1=untreated, G2= cream base, G3= standard drug, G6= 1%AMBU.

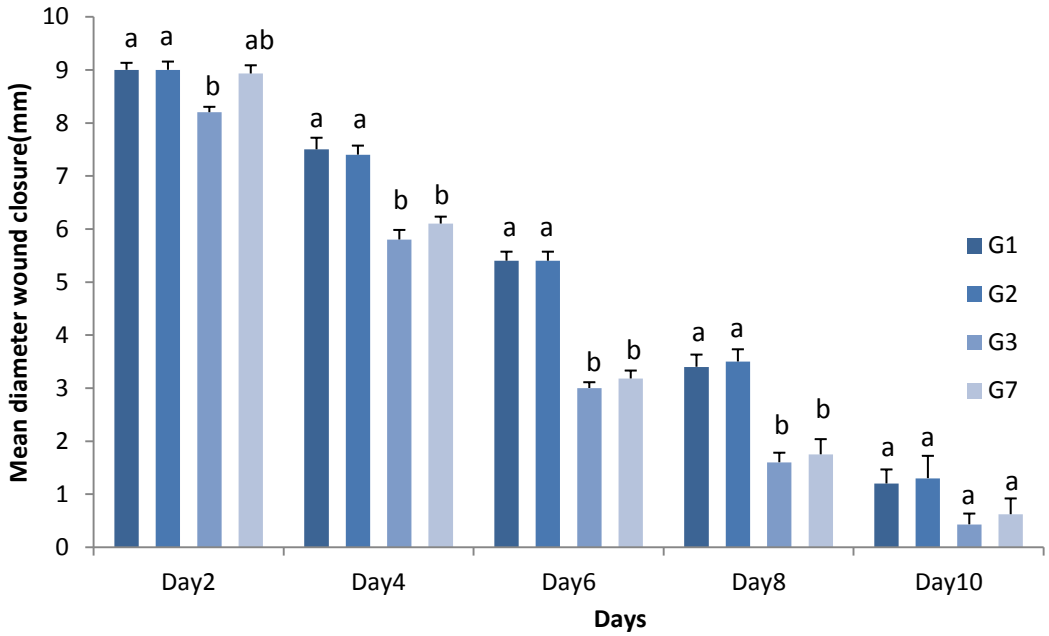
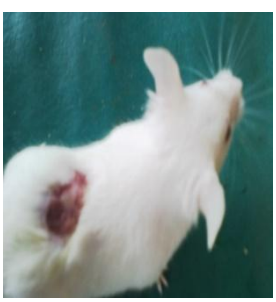
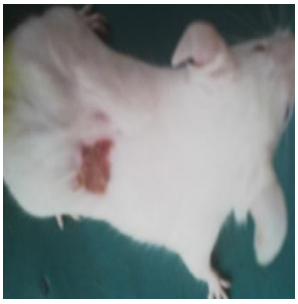


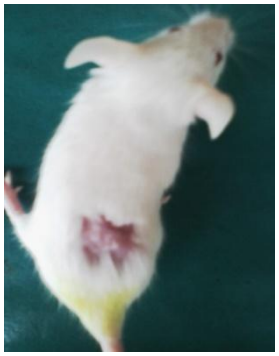
Figure 5: Effect of 10mg AMBU extracts only on wound closure ($p<0.05$) G1=untreated, G2= cream base, G3= standard drug, G7= AMBU extract only.





G1untreated day 2 G3 1% SS G4 15%AMBU

Figure 6: Wound healing after day 2 of treatment for treated and control groups


G1untreated mice G3 1%SS G4 15% AMBU

Figure 7: Wound healing after day 10 of treatment for treated and control groups

4.4 Histopathological Assessment of Skin Sections

On day three all groups had severe acute inflammatory reaction marked by neutrophils infiltration and aggregations in the subcutaneous tissue. There were no differences among the tissues from the three groups. On day 10 the skin sections from the three groups had fibrosis especially marked in the subcutaneous tissue and dermis. However there were minor differences among the three groups as outlined below: G1 the epidermal layers was not fully restored (incomplete epithelial reconstitution) healing process was characterized by fibrosis and minimal mononuclear inflammatory cells. G3 and G4 there was complete epithelial reconstitution, fibrosis and marked inflammatory reaction in the subcutaneous tissue and dermis. There were no obvious differences between the histopathological changes in skin sections from G3 and G4.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Evaluation of Physicochemical Properties of Formulated AMBU Cream

Cream are semi solid dosage form intended mainly for external use and commonly consist of two immiscible phases, an oily internal phase and aqueous external phase. Due to emulsified nature of skin surface drugs formulated as cream more effectively interact with the skin and readily penetrate through biological membrane. Some plants extracts with antifungal activity have been previously formulated as topical cream. From the results of this study the physicochemical parameters of the cream formulation from leaf ethanol extracts were within the acceptable limit.

The prepared cream was O/W type emulsion hence can be easily washed with plain water which gives better customer compliance; this was comparable with the study by Bhide and Sachin (2005) on formulation of herbal cosmetics with O/W emulsion. In another report methanolic extract of *Eucalyptus camadulensis* has been formulated as an antidermatopytic cream preparation (Moghimipour *et al*., 2009). The base formula contained reasonable amount of fat which produced no greasy sense on usage, turbidity and good consistency.

The appearance of the cream was good and the distribution of the extracts in the cream was uniformly distributed. Homogeneity test showed no turbidity and instability. Consistency showed no sticking and adhesion to skin. The pH of AMBU cream formulations was in a range of 6.1 to 6.8 for all formulation which is within the acceptable range of skin pH. According to Lambers *et al*. (2006) the range of pH of skin is acidic but with broad range from 4-7. The cream also shows good Spreadability when applied on the

skin of mice. After application of the cream the type of smear formed on the skin was found to be non-greasy and easily removed on washing with tap water.

From the stability studies, cream with 15%, 7.5% and 1% AMBU showed no changes in pH, Spreadability, drug content, consistency and phase separation after keeping at different temperature for 30 days. It has been previously reported in other studies that topical formulation in the form of cream or ointment may lead to enhancement of stability and acceptability of the active ingredient because it contains self-surfactants while the antifungal activity remains considerable (Alisa and Kadim, 2011).

5.2 Assessment of Antifungal Activities of AMBU Cream

Plants have been a rich source of medicine because it is believed that plant based drugs have less or no side effect when administered at optimal dose and affect a wide range of antibiotic resistant microorganisms (Nair *et al.*, 2015).

The results of this study (Table 3) showed that ethanol AMBU cream and aqueous AMBU cream inhibited the growth of *C. albicans* and *C. tropicalis*, the antifungal activity was enhanced with increase of the extract concentration. The MIC of 9.38 mg/ml for ethanol extracts and 12.5 mg/ml for aqueous extracts was recorded for all the test organisms including *C. albicans* and *C. tropicalis.*

The antifungal activity of these extracts was likely due to the presence of phytochemicals that have activity against the test microorganism (Mabiki *et al.*, 2013). According to Neuwinger (1994), the aqueous extracts of the leaves and stem of AMBU is reported to have positive reaction for tannins, triterpenoids, and coumarins while the methanol extracts has steroids, tritepenoids, and anthocyanin and the petroleum ether extract

contains carotenoids, tritepenoids, volatile oils and glucosides. The presence of these phytochemicals contributes to the antimicrobial activity of AMBU cream. The antifungal activities observed in this study may be due to the activity of one or combination of some of the identified constituents in plants extracts.

5.3 Determination of Topical Wound Healing Activity of AMBU Cream in Mice

Wound healing activity exhibited by most plants is due to the synergistic or additive actions of their constituents. The leaf extracts of AMBU was found to contain flavonoids, tannins, saponins and glycosides (Neuwinger, 1994). The presence of tannins and flavonoids present in the plant is very significant as these secondary metabolites have been found to act as free radical scavengers (Marja *et al.,* 1999). They exert their antioxidant property by increasing the activity of catalase and glutathione peroxidase, which detoxify hydrogen peroxide by converting it to oxygen and water (Rahman, 2007). Apart from their antioxidant properties they have also been known to promote wound healing.

Flavonoids present in the plant may increase the viability of collagen fibrils by causing an increase in the strength of collagen fibers. This reduces cell damage by promoting DNA synthesis (Panda and Tripathy, 2009). Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis (Getie *et al.,* 2002). Flavonoids have been found to exhibit antimicrobial activity and therefore may significantly help to prevent or reduce wound infections (Owoyele *et al.,* 2008). Presence of tannins in the plant may cause an increase in wound contraction and increase the rate of epithelialization due to their astringent and antimicrobial property (Panda and Tripathy, 2009). Alkaloids also if present in the plant

may increase cell proliferation and thus increase the rate of wound healing (Barbakadze *et al.,* 2009).

The results indicate that all formulations exhibited wound healing activity. The healing activity observed was not dose dependent as it was comparable in all groups ($p>0.05$) for all treatment. There were minor differences observed for 15% cream formulation which was found to have better healing activity compared to other cream formulations. The increased wound contraction for mice treated with 15% extract cream was associated with complete epithelial reconstitution and fibrosis in the subcutaneous tissue and dermis as was determined by the histological results.

The cream formulation containing 15% ethanol extracts showed significant wound healing activity that is comparable to commercial product of silver sulfadiazine (1% SS). On another hand on day 10 onwards the rate of wound healing was comparable in all groups ($p>0.05$) this may be due to their immune response which facilitate the normal wound healing of mice as days progressed. The immune response of mice may be was due to the food they consume may contain ingredients which increases the natural immunity of mice to repair the damaged cell example presence of proteins, zinc, iron, copper and vitamin C increases the rate of wound healing. The formulation did not produce any adverse effect and because of this it is possible in further studies to be recommended in treatment of wound.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The prepared cream was easily spreadable, pleasant and washable with water hence there is a chance of increased patient compliance. Formulated creams significantly enhance wound healing compared to the standard commercial cream. Also the cream has antifungal activity against the test organisms. The activity may be due to free radical scavenging activity, antioxidant activity and anti-inflammatory effect of the phytochemicals present in the extract.

6.2 Recommendations

Since the current data revealed the health benefits of cream from AMBU leaves ethanol extracts, the study put forward recommendations as follows; further studies are required to find out the bioactive ingredients in AMBU ethanol extracts which are responsible for wound healing and antifungal activity. Furthermore, a depth and structured study would be beneficial to assess the usefulness and mechanisms of the bioactive ingredients in AMBU ethanol extracts. This study can be helpful for upcoming researchers to select this plant extract for further formulation and evaluation of other cosmetic applications which can be claimed for their efficacy with scientific evidence.

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