Formulation and Quality Assessments of Topical Herbal Cream Incorporated with *Piper sarmentosum* Aqueous Extract

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A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Product Development Technology) with Honours

Faculty of Agro Based Industry

University Malaysia Kelantan

2019

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Student

Name : Koh Bao Jia

Date :

I certify that the report of this final year project entitled "Formulation and Quality Assessments of Topical Herbal Cream Incorporated with *Piper sarmentosum* Aqueous Extract" by Koh Bao Jia, matric number F15A0060 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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Date :

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ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Ikarastika Rahayu binti Abdul Wahab who enlightened me the potential of the plant species for this research. I am extremely thankful to her for providing a guidance and encouragement throughout the research as well as the writing of thesis even with her busy schedule. A special gratitude to Miss Amalina who helped me in collecting the plant sample from Kota Bharu and assisted me during my research activity at University Sains Malaysia, Kelantan. This research would not have been possible without her kind help and support.

I would like to acknowledge Mr. Qamal for providing the apparatus and materials needed throughout research at his earliest convenience. I would also like to express my sincere thanks to Mr. Suhaimi, Mrs Hidayah and other laboratory assistant for their warm assistance and advice for the use of laboratory and facilities. Their kind help has allowed me to perform my laboratory works smoothly.

I am also very grateful to my friend, Chow Jheng Wai for his companionship and helps in overcoming hardships faced during the research. Valuable time of him spent to discuss and enrich my work was much appreciated. Last but not least, I would like to thank to my family for their unconditional love and financial support in completion of this research.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
L	Litre
g	Grams
r.p.m.	Revolution Per Minute
mm	Millimetre
mJ	Mega Joule
L*	Lightness
b*	Yellowness
±	Standard Deviation
%	Percentage
E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
SPI	Scoring system of Primary Index
PII	Primary Irritation Index
NA	Nutrient Agar
NB	Nutrient Broth
TSA	Tripticase Soy Agar
TSB	Tripticase Soy Broth
Cfu/g	Colonies Forming Units per Gram

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Formulation and Quality Assessments of Topical Herbal Cream Incorporated with *Piper sarmentosum* Aqueous Extract

ABSTRACT

Herbal plants are in widespread use for medical purpose and the development of natural products. Yet, the quality of natural products continues to possess a great challenge for the during development process. *Piper sarmentosum* aqueous extract has been scientifically demonstrated to have wound healing activity using in vivo model. Hence, this research mainly emphasized on the formulation of topical herbal cream with incorporation of *P. sarmentosum* aqueous extract and natural ingredients. An optimized formulation was further assessed for its quality. Parameters used in the assessments herbal cream formulated included organoleptic and physicochemical characteristic, antibacterial property, total microbial count, stability study and sensory evaluation and sample testing for skin irritation. The result showed that the optimized herbal cream exhibited good physical properties with strong scent of extract. However, antibacterial activity against *E. coli* and *S. aureus* was not found in the herbal cream formulated. The herbal cream formulated was also found to be highly contaminated with 1.0 x10^4 cfu/g which was undesirable. One–month stability study showed that herbal cream formulated was considered stable under cold storage (5°C) and room temperature (25°C) with no evidence of phase separation nor significant change of odour, texture and pH values. Significant variation in colour ($p<0.05$) was observed after 1 week storage at 5°C and after 4 weeks storage at 25°C. The formulation was physically unstable at temperature as high as 40°C. The results of the consumers' testing (N=50) demonstrated that the herbal cream formulated had a rather high degree of preference among the respondents and it was non-irritant for skin. Future research are necessary to evaluate efficacy of the cream in wound healing activity. Optimistically, this study could contribute to the future development of natural wound healing cream.

Keywords: *Piper sarmentosum*, Wound Healing, Topical Herbal Cream, Quality Assessments.

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Perumusan dan Penilaian Kualiti Krim Herbal Topikal yang Diperbadankan dengan *Piper sarmentosum* Aqueous Extract

ABSTRAK

Tumbuhan herba digunakan secara meluas untuk tujuan perubatan dan pembangunan produk semulajadi. Walau bagaimanapun, kualiti produk semulajadi memaparkan cabaran besar dalam proses pembangunan produk. Ekstrak berair *Piper sarmentosum* telah ditunjukkan secara saintifik mempunyai aktiviti penyembuhan luka menggunakan model vivo. Oleh itu, penyelidikan ini terutama menekankan tentang perumusan krim herba topikal dengan memperbadankan ekstrak *P. sarmentosum* dan bahan-bahan semulajadi. Perumusan yang dioptimumkan dinilai untuk kualiti selanjutnya. Parameter yang digunakan dalam penilaian krim herba yang dirumuskan termasuk ciri-ciri organoleptik dan fizikokimia, aktiviti antibjakteria, jumlah kiraan mikrob, kajian kestabilan dan penilaian deria dan iritasi kulit antara responden. Hasilnya menunjukkan bahawa krim herba yang dioptimumkan mempamerkan sifat fizikal yang baik dengan bau ekstrak yang kuat. Walau bagaimanapun, aktiviti antibakteria terhadap *E. coli dan S. aureus* tidak terdapat dalam krim herbal yang dirumuskan. Krim herbal yang dirumuskan juga didapati mengandungi bakteria yang tinggi dengan 1.0 x10^4 cfu/g dan tidak memuaskan. Kajian kestabilan dalam tempoh satu bulan menunjukkan bahawa krim herbal yang dirumuskan dianggap stabil di bawah penyimpanan sejuk (5°C) dan suhu bilik (25°C) tanpa bukti pemisahan fasa atau perubahan bau, tekstur dan nilai *pH* yang ketara. Perubahan warna yang ketara ($p < 0.05$) diperhatikan selepas penyimpanan 1 minggu pada 5°C dan selepas penyimpanan 4 minggu pada 25°C. Perumusan itu tidak stabil secara fizikal pada suhu setinggi 40°C. Hasil penilaian deria menunjukkan bahawa krim herbal yang diformulasikan mempunyai tahap kepuasaan yang agak tinggi di kalangan responden (N=50) dan tidak menyebabkan iritasi kulit. Kajian masa depan diperlukan untuk menilai keberkesanan krim dalam aktiviti penyembuhan luka. Secara optimistik, kajian ini akan menyumbang kepada perkembangan masa depan krim penyembuhan luka semulajadi.

Kata Kunci: *Piper sarmentosum*, Penyembuhan Luka, Krim Herbal Topikal, Penilaian Quality.

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Herbal plants have a long tradition for medical purpose and been perceived as important sources of natural remedy (Qazi & Molvi, 2016). As far as the researchers concern, Traditional Chinese Medicine and Ayurveda are two of the oldest believes which practice on the use of plants and herbal medicine in China and India respectively since ancient. While herbal based cosmetics preparations are intended to enhance appearance and attractiveness, natural medicinal products aimed at promoting health, preventing or treating illness in human. In recent years, the popularity and demand for natural products has increased significantly not only in the field of cosmetic but also in pharmaceutical industry over the world (Pan et al., 2013). Pathare & Wagh (2012) reported that approximately 80% of the populations from all over the world are presently making use of herbal medicine for primary health. Under certain circumstance, herbal medicine provides an attractive alternative to commercial drugs (Alo, Anyim, Igwe, Elom, & Uchenna, 2012). Apparently, the growing use of herbal medicines is due to several perceptions such that herbal remedies have better efficacy, safety, poses minimal side effects and are being more affordable (Agyare, Kisseih, Yaa, & Poku,

2014; Pathare & Wagh, 2012). To exemplified, *Piper* species have been reported to be one of the effective medicinal plants used as folk medicine or natural remedies to treat various ailment and relief discomforts (Mgbeahuruike, Yrjönen, Vuorela, & Holm, 2017).

Wounds and skin injuries are common occurrence in all living organism. Minor injuries or mechanical injuries can be caused by accidents or trauma such as cut, burn or abrasion. Delay in healing minor wound could lead to development of chronic wound especially in diabetic patients. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) have been commonly reported to cause infection in wound (Builders et al., 2013). Lately, one of the preliminary study demonstrated that aqueous extract of *Piper sarmentosum* have significant potential in wound healing activity. Hence, this research intended to transform the value of the medicinal herbs into a potential product by formulating a topical herbal cream with natural ingredients and *P. sarmentosum* aqueous extract. Successful development of the natural herbal cream with good physical properties and stability may contribute to the personal health care and pharmaceutical industry in promoting wound healing.

1.2 Problem Statement

Over the years, pharmaceutical industry is expanding in a fast pace along with expansion of economic, improved accessed to health care and advancement of technologies. In current pharmaceutical and clinical practice, it is not uncommon that products available in the market are mostly chemically synthesized. For instance, synthetic ingredients such as paraben, propylene glycol, Diethanolalamine (DEA), Triethanolamine (TEA) are often added

to a consumer product to enhance the product appearance, efficacy and achieve better marketability. Besides, povidone-iodine, acriflavine, hydrogen peroxide, bisphenol and alcohol compounds are some of the chemical agents that commonly used as antiseptic or antibiotics in topical drugs especially for the prevention of wounds infection thus promote healing.

However, the use of synthetic ingredients, skin antiseptic and antibiotics have been reported to cause increasing number of skin irritation or allergic concern. This has caused more people inclined toward natural products as the consumers believed that natural products are generally safe and less probable to rise any side effect. In order to meet the growing demand of consumers, manufacturers particularly in herbal industry are always in great interest in transforming therapeutic plants into herbal based product. Hence, the aim of present study is to formulate a herbal topical cream with natural ingredients and *P. sarmentosum* extract that exhibited good physical properties and non-irritant.

On the other hand, as the market for natural products evolved, issues relating to product quality, safety, consistency and stability of natural formulation remain to possess a great challenge for formulator or product developers. As an example, natural products could have high level of contamination which lead to early product deterioration when it is not sufficiently preserved. Besides, natural products that are physically unstable tends to have short shelf life. Meanwhile, a good sensorial properties of products are likely to fulfil the expectation of consumers. For these reasons, the quality and stability of topical herbal cream developed at the present study was determined and justified in a scientific manner. The acceptance of developed cream by consumers was also determined by conducting sample testing.

1.3 Hypothesis

An optimized topical herbal cream may be successfully developed using natural ingredients and *P. sarmentosum* aqueous extract. The optimized cream exhibits good organoleptic and physicochemical characteristics. The total number of colonies forming unit (cfu) found on the optimized cream complies with the value of National Pharmaceutical Control Bureau and British Pharmacopoeia standard. Antibacterial activity of the optimized cream shows significant inhibition zone against *E. coli* and *S. aureus*. The optimized cream exhibits good stability at 5°C, 25°C, 40°C and 45°C over one-month storage period. The cream formulated has high degree of preference and acceptance among the respondents and do not cause any skin irritation reaction.

1.4 Objectives

The objectives of this research comprises of the following:

I. To formulate a topical herbal cream using natural ingredients and *P. sarmentosum* aqueous extract.
II. To determine the organoleptic, physicochemical characteristics and antibacterial property and microbiological profile of the optimized herbal cream.
III. To evaluate the physical stability of the topical herbal cream for one-month duration.
IV. To conduct sample testing for the evaluation of sensory properties and skin irritation reaction.

1.5 Scope of Study

The leave extract of *P. sarmentosum* was obtained by aqueous extraction and freeze drying. The topical herbal cream was formulated with the incorporation of natural ingredients and *P. sarmentosum* aqueous extract. The emulsion was formed by mixing the ingredients of oil phase and aqueous phase. The formulation was constantly modified and an optimized cream with desirable texture was chosen for following quality assessments. First of all, the optimized herbal cream was subjected to organoleptic and physicochemical evaluation including parameters on appearance, colour profile, odour, homogeneity, texture profile and spreadability, pH. Next, the antimicrobial activity of the formulated cream against *E. coli* and *S. aureus* was determined by measuring the diameter of zone of inhibition. Besides, the total plate count was conducted to determine the total colonies forming units (cfu/g) of the optimized. Accelerated stability test was conducted to determine the physical stability of formulated cream under different storage conditions at 5˚C, 25˚C, 40˚C and 45˚C. The evaluation was performed on every 7^{th} day, 14^{th}, 21^{th} and 28^{th} day with respect to the physicochemical parameters. Lastly, sample testing was performed randomly on 50 respondents for the evaluation of sensorial properties and skin irritation reaction. The entire research was conducted for 5 months.

1.6 Significance of Study

In previous research, the leaf extract of the *P. sarmentosum* showed bioactivity on anti-nociceptive, anti-inflammatory and enhancement of fracture healing. Lately, one of the

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preliminary study conducted by Abdullah (2018) demonstrated that aqueous extract of *P. sarmentosum* when applied topically has significant wound healing property which evaluated using both incision and excision wound model on rat. However, the potential of this herbal plant has yet to be exploit in the development of product. Coupled with that, this research was emphasizing on formulation of a topical herbal cream using natural ingredients and aqueous extract of *P. sarmentosum*. On top of that, the present research also demonstrated scientific documentation on the preparation of topical herbal cream, assessment parameters and qualitative informative for better assurance on quality, safety and consistency of the product. Optimistically, the present research contributed to novel uses of *P. sarmentosum* herbal plant by bringing the value of a medicinal herbs into a potential product.

Successful development of topical herbal cream with natural ingredients and herbal extract could also contribute to reduce reliance on the use of synthetic active ingredients and thus avoid the case of allergic or side effects. Development of this potential wound healing cream could be use in pharmaceutical and health care industry by promoting wound healing in both children and adults or even on diabetic wound as well as post-surgical wound management thus improving quality of life among patients.

1.7 Limitation of Study

The aim of the present study was to formulate a topical herbal cream using natural ingredients and *P. sarmentosum* aqueous extract. However, the mixing process of 2 immiscible liquids during the formulation of herbal has yet to improve using appropriate tool rather than constant stirring for a consistent and stable emulsion. Conceivably, the result and

the quality of herbal cream formulated may be further enhanced by using homogenizer for a better quality of emulsion. Besides, the stability evaluation was limited to one month testing period which might be insufficient to provide a strong evidence for the overall stability of herbal cream developed. Due to limited time and financial resource, the formulated herbal cream was neither tested on laboratory animals nor evaluated for its efficacy clinically on wound healing activity. Instead, antimicrobial assay will be conducted to determine the potential of formulated cream to inhibit against *E. coli* and *S. aureus*. Future research on efficacy and toxicological profile on this formulation may be carried out to overcome the limitation.

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CHAPTER 2

LITERATURE REVIEW

2.1 *Piper* Species

The *Piper* species is widely reported as woody perennial climbers and rarely shrub or trees. This species prefers moist habitat and requires little sunlight through forest gap. Approximately 1200 of *Piper* species distributed at different regions of world and Malaysia region alone has documented with over 400 species. It is an aromatic plant which give a strong pungent smell. The stem has dilated or swollen nodes and stipule (Tawan, Ipor, Fashihuddin, & Sani, 2002). Due to the vast varieties of *Piper* species, it is often difficult to identify the species by examining the leaf morphology alone as the leaf of this species consists of few different foams or they are rather similar. Indeed, proper identification of specific species are necessary to be carried out on *Piper* with inflorescences (Chaveerach, Mokkamul, Sudmoon, & Tanee, 2006). Some of the common species that can be found from Peninsular Malaysia are *Piper nigrum* L. *(lada hitam), P. betle* L. *(sireh), P. sarmentosum Roxb. (kadok), P. umbellatum (segumbar urat), P. poryphyrophyllum (sirih harimau or akar bugu), P. longum (long pepper)* (Tawan et al., 2002). To exemplified, the plant species of *P. betle* L. and *Piper nigrum* L. were shown in Figure 2.1.

(a) (b)

Figure 2.1: Plant species of (a) *P. betle* L.; (b) *P. nigrum* L.

Source: Chaveerach et al. (2006).

2.1.1 Ethnobotanical Studies

Piper species are reported to be one of the effective herbs used as folk medicine or natural remedies to treat various ailment and relief discomforts (Mgbeahuruike et al., 2017). Traditionally, this plant species are also used as vegetables and spices, traditional ceremonies purpose and home gardening decoration due to the appealing leaf shape (Ong, Zuki and Milow, 2011; Zakaria, Patahuddin, Mohamad, Israf, & Sulaiman, 2010; Chaveerach et al. 2006). Ethnobotanical studies of *Piper* species were summarized in Table 2.1.

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Table 2.1: Certain popular species of *Piper* and their traditional uses.

Piper Species	Traditional Uses
P. nigrum L.	• Black pepper and white pepper (dried fruits) are used as flavouring and spices. • White pepper is used to treat cholera, malaria and stomachache while black pepper is used to treat abdominal fullness, adenitis, cold, cholera. • The leaves are used to treat diarrhoea, headache. • In Thailand, leaves are also used as ornamental plant for home gardening.
P. betle L.	• In Thailand, it is used as ornamental plant for home gardening. • The leaves are used in ritual and wedding ceremonies. • Leaves can be used to relief kidney inflammation and reduce thirst resulting from diabetes. • Also as expectorant and reduce respiratory illness such as cough, asthma and nose bleeding. • Leaves was chewed and serve to control bad breath.
P. longum L.	• The roots are used in treating bronchitis, stomach ache and enhance appetite. • The leaves can be used as antiseptic for wound and treatment to diarrhoea.

Sources: Ong, Zuki and Milow (2011); Zakaria, Patahuddin, Mohamad, Israf, & Sulaiman, (2010); Chaveerach et al. (2006).

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2.1.2 Phytochemical Studies

Phytochemical constituents are active bioactive compounds which responsible for wide range of potential therapeutic activities in plant. Based on the previous research studies, a numbers of phytochemical compounds including alkaloid, flavonoid, tannins, saponins, phenolic compound have been isolated from certain parts of *Piper* species (Tharakan & Madhavan, 2017; Foo, Salleh, & Mamat, 2015; Ganesh, Suresh Kumar, & Saranraj, 2014; Pin et al., 2010). It has been widely established that these compounds are accounts for certain bioactivity including antioxidant, antimicrobial, anti-inflammatory, antifungal, anthelmintic and anticancer of *piper* species. The alkaloid compounds found in *Piper* species including piperine piperidine, piperettine, peperanine, piperlogumine and piperloguminine (Ganesh et al., 2014).

Specifically, alkaloid compound of piperine is widely reported in *P. longum* and *P. nigrum*. This compound is account for the pungent smell of *Piper* plant as well as the antioxidant and anthelmintic activity. Piperine has relatively low solubility in water but readily soluble in organic solvents such as alcohol and ether (Vasavirama & Upender, 2014; Simon & Henry, 2013). The chemical structure of piperine was shown in Figure 2.2.

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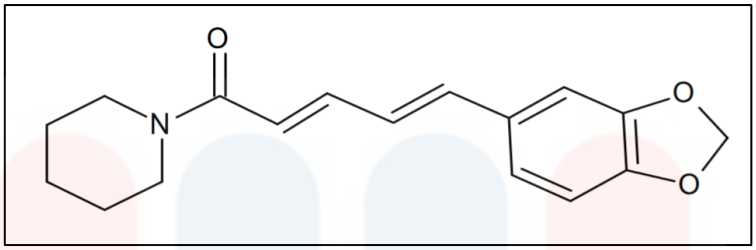

Figure 2.2: Chemical structure of piperine.

Source: Simon & Henry (2013).

Besides, hydroxylcharvicol (HC) and eugenol (EU) are 2 major phenolic compounds detected in *P. betle* leaves (Figure 2.3) (Irlan et al., 2015; Intzar et al., 2010; Pin et al., 2010). These compounds were said to be exhibit bioactivities of the plant including antioxidant and anti-inflammatory. Previous studies revealed that ethyl acetate refluxed extraction has better extraction efficacy than supercritical fluid extraction (Singtongratana, Vadhanasin, & Singkhonrat, 2013).

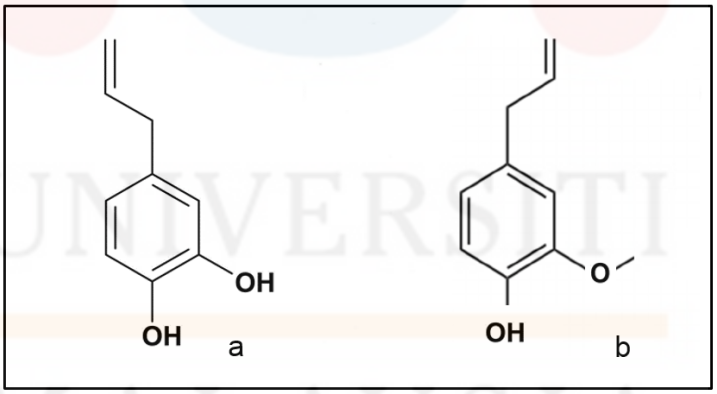

Figure 2.3: Chemical structure of (a) hydroxylcharvicol; (b) eugenol.

Source: Intzar et al. (2010); Irlan et al. (2015).

2.1.3 Pharmacological Studies

Several scientific and pharmacological studies have been investigated on *Piper* species. On the other hand, evaluation antibacterial activity of *P. nigrum* L. also showed effective inhibition effect on microorganisms especially *E. coli, S. aureus* and *S. typhi* but less effective to *Pseudomonas sp.* (Ganesh et al., 2014). Extract of *P. betle* leaves also exhibited promising antibacterial effect against *Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus and Klebsiella pneumonia.* Maximum zone of inhibition ethanol extract of *P. betle* leaves against *P. aeruginosa* was reported to be 16 ± 0.24 mm at 0% of dilution (Patwardhan, Datta, & Mitra, 2011) (Figure 2.4).

Bacterial Strain	Zone of inhibition (mm) Dilution Percentage					
	0	20	40	60	80	Ceftriaxone
Pseudomonas aeruginosa	16±0.24	14±0.15	13±41	11±0.02	9±0.75	12±0.9
Proteus vulgaris	10±0.5	9.5±0.25	8±0.5	8±0.25	7±0.5	8±0.5
Staphylococcus aureus	13±0.43	12±0.25	10±0.6	10±0.15	8±0.25	20±0.5
Klebsiella pneumonia	14±0.15	14±0.1	12±0.25	11±0.47	10±0.15	17±0.55

Figure 2.4: Zone of inhibition of *P. betle* ethanol extract and Ceftriaxone.
Source: Patwardhan et al. (2011).

In a recent study, Pin et al. (2015) have also reported antioxidant activity of *P. betle* with aqueous extract being the most effective extract. On top of that, various extracts of *P. betle* also exhibit high anti-inflammatory activity in Xanthine Oxidase (XOD) assay. Based on the literature search carried out, *Piper* species exhibit broad range of therapeutic

bioactivity including anti-inflammatory, antioxidant, antibacterial and anticancer activities which are useful in treating disease and development of antimicrobial drugs.

2.2 *Piper sarmentosum* Roxb.

Piper sarmentosum Roxb is a member of Piperaceae family and the leaves is commonly known as *"Kaduk"* in Malay. Apart from Peninsular Malaysia, *P. sarmentosum* is also widely distributed over Thailand, India, Laos, Cambodia, Vietnam, Filipina and Indonesia (Munawaroh & Yuzammi, 2017; Chaveerach et al., 2006). The shrub is about 30 to 50 cm tall with short hairy stem. The leaves are broadly ovate to elliptic and flatten and has pointed tip. It appeared in light to dark green. White flower/spike may come out from the tip of the stem and predominate during raining season (Chaveerach et al., 2006). The leaves and flowers of *P. sarmentosum* was shown in Figure 2.5.

Figure 2.5: *Piper sarmenteosum* Roxb. leaves and flowers
Source: Chaveerach et al. (2006).

2.2.1 Ethnobotanical Studies

In both Peninsular Malaysia and Thailand, the leaves of *P. sarmentosum* are usually cooked and eaten as vegetables or to impart flavour to local cuisine (Chaveerach et al., 2006). It is also traditionally used to treat pains in bones and relief discomfort such as headache, fever, toothache, cough and asthma (Mahavorasirikul, Viyanant, Chaijaroenkul, Itharat, & Na-Bangchang, 2010; Tawan et al., 2002). Decoction of the leaves used to treat malaria; crushed leaves mixed with water and used for bath to treat kidney stone and urination difficulty (Atiax, Ahmad, Sirat, & Arbain, 2011; Nordiana & Ong, 1999; Ong & Norzalina, 1999).

2.2.2 Phytochemical Studies

Several amide was extracted from the fruits of *P. sarmentosum* including pellitorine, guineensine, sarmentine and sarmentosine (Rukachaisirikul et al., 2004). On the other hand, amides and sterol including 3-(3',4',5'-trimethoxyphenylpropanoyl) pyrrolidine, N-(3-phenylpropanoyl) pyrrole and β-sitosterol found in organic solvent extraction of aerial part of *P. sarmentosum* were noted for antibacterial property (Atiax et al., 2011). A natural antioxidant superoxide scavenger, Naringenin was also found in methanolic extract of *P. sarmentosum* leaf (Subramaniam, Adenan, Ahmad, & Sahdan, 2003).

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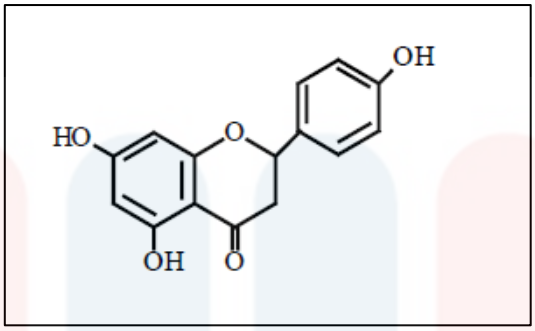

Figure 2.6: Chemical structure of Naringenin, 4’5,7-Trihydroxyflavone.

Source: Subramaniam et al. (2003).

2.2.3 Pharmacological Studies

Previous studies have investigated on pharmacological activity of *Piper sarmentosum* plant including anti-oxidant, anti-hypertensive, anti-carcinogenic, ability to reduce 11β-HSD1 enzyme activity ovariectomy-induced obese rat and enhance bone strength and structure especially against glucocorticoids-induced osteoporosis (Zainudin, Zakaria, & Nordin, 2015; Nirwana et al., 2012; Ariffin et al., 2009; Aida, Farihah, Qodriyah,& Azlina, 2009). Hexane and ethyl acetate extract of aerial part of *P. sarmentosum* showed no significant antibacterial activity against *E. coli* but positive result was obtained when tested against *S. aureus* (Atiax et al., 2011). In the research study conducted by Zakaria et al. (2010), aqueous extract of *P. sarmentosum* leaves which demonstrated potential anti-nociceptive and anti-inflammatory activities provided scientific evidence and justification on traditional use of the plant in treating pains and inflammation. *P. sarmentosum* extract was also proven to enhanced fracture healing (Estai et al., 2011).

Lately, one of the preliminary demonstrated that aqueous extract of *P. sarmentosum* leaves have significant potential in wound healing activity as shown in Figure 2.7 and Figure 2.8 (Abdullah, 2018). In the research study, the wound healing activity was investigated using excision and incision models on white rats with weight between 180 g to 200 g. The wound healing activity of rats was tested against 3 concentration of *P. sarmentosum* aqueous extract which were 50 mg/kg, 300 mg/kg and 2000 mg/kg. Vitamin E was used as positive control and the wound without applying any drugs served for control purpose. Among the concentration used, 2000 mg/kg extract applied on wound showed best healing activity. After 14 days of treatment, the wound healing rate using extract was able to achieved 100% while positive control showed was 99.89 ± 2.11%. Case of acute toxicity was not reported at each concentration used.

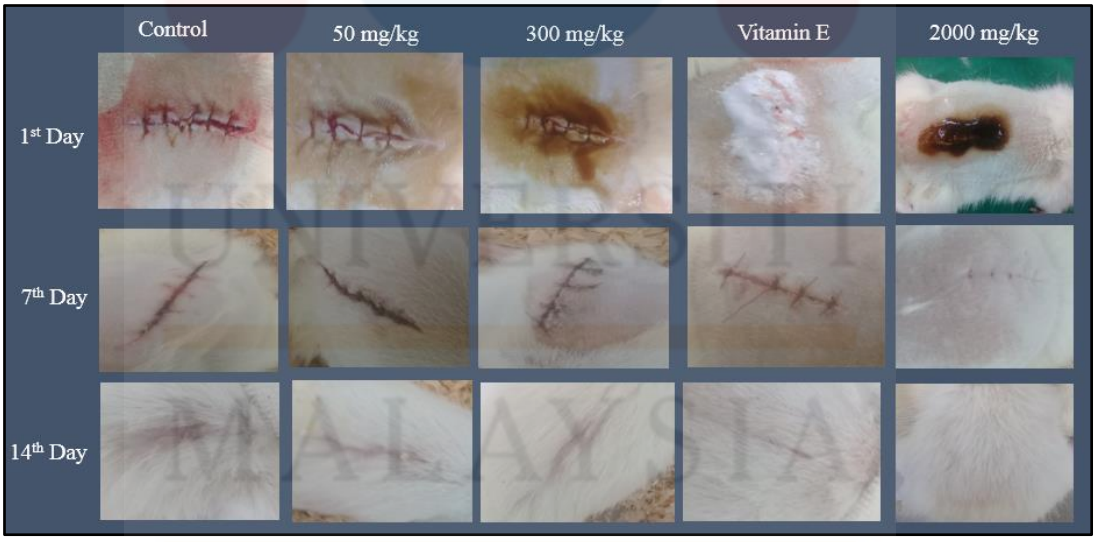

Figure 2.7: Incision wound model of *P. sarmentosum* aqueous extract.

Source: Abdullah (2018).

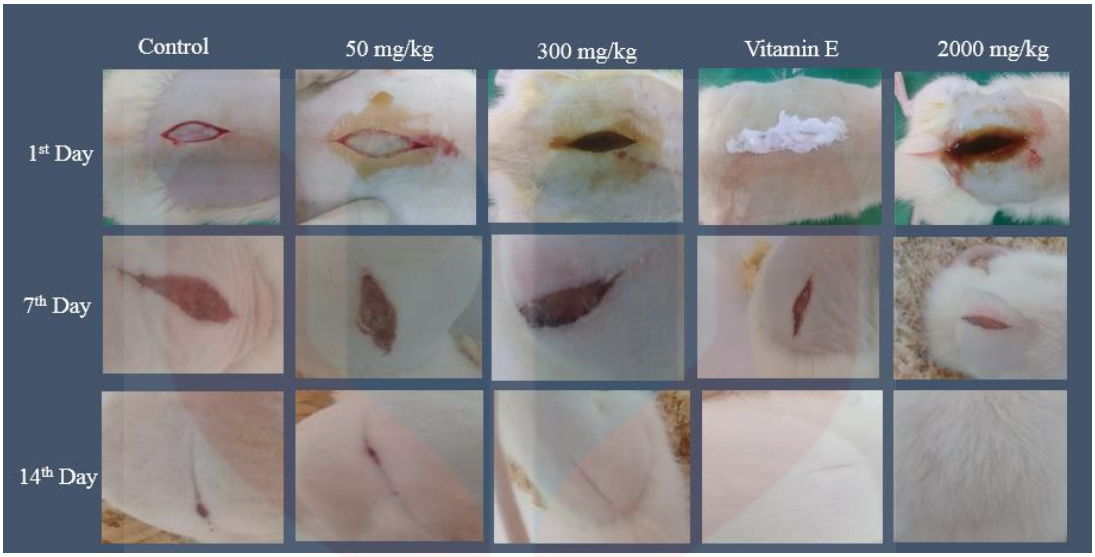

Figure 2.8: Excision wound model of *P. sarmentosum* aqueous extract.

Source: Abdullah (2018).

2.3 Pharmacological Activities of Plant Related to Wound Healing

Plants are rich in variety of active biochemical compounds have potential therapeutic value for prevention and treatment of disease (Firenzuoli & Gori, 2007). These plants promote the natural repair mechanism to maintain astatic condition which are essential for wound healing (Qureshi, Khatoon, & Ahmed, 2015). The benefits of antibacterial in wound management are widely studied. While topical antiseptic and antibiotic are commonly used as antimicrobial agent to control wound infection, plants extract which exhibits antibacterial activity is reported to have the potential to promote to the heal wound. Specifically, antimicrobials may inhibit or reduce growth of microorganism at the site of wound and prevent the bacteria to ingress the tissue though wound opening as well as prevent formation of bacteria biofilm to compete nutrient and oxygen of the body (Ikobi, Igwilo, Awodele, &

Azubuike, 2012; Templeton, 2005). Recent study conducted by Ikobi et al. (2012) demonstrated that *Gossypium barbadense* methanol extract with antimicrobial activity had the potential to heal wound on rats. Nevertheless, antioxidant and anti-inflammatory properties of botanical extracts also contribute to wound management. Antioxidant also helps to promote the repair of oxidized molecule as well as facilitates tissue regeneration at site of wound (Barku, Boye, & Ayaba, 2013). Anti-inflammatory activity is essential to control inflammation response of wound and prevent prolonged inflammation. Vitamin E has also been advocated as potential wound healing agent mainly due to the presence of antioxidant and anti-inflammatory activities (Mohanty & Sahoo, 2017).

2.4 Preparation of Plant Extract

Extraction involves the process of separating active biochemical in plant materials using particular solvents through standard procedure (Builders et al., 2013). Prior to extraction, the plant samples are usually subject to basic pre-operation process including cleaning or washing, drying, grinding and drying of samples. During the process, damage or loss of potential active constituents should be avoid through gentle handling (Sasidharan, Chen, Saravanan, Kundram, & Latha, 2011). On the other hand, different solvents are available to extract active biochemical compound such as polar and non-polar solvent. Primarily, the choice of solvents and extraction methods should consider the yield of extraction and the targeted phytochemical compounds in particular plant materials to be obtained (Sasidharan et al., 2011).

2.4.1 Aqueous Extraction of *Piper* Species

Based on literature search, aqueous extraction is commonly used in extracting *Piper* species for several bioactivities which possess the potential to heal wound including aqueous extract of *P. betle* and *P. sarmentosum* (Abdullah, 2018; Ghazali et al., 2016; Subramaniam et al., 2003). According to Pin et al. (2015), water is usually used to extract water soluble and polar compounds such as phenolic compound. As compared to ethanol, water possess greater capability to extract solute due to higher polarity and shorter chain.

A research by Zakaria et al. (2010) demonstrated that aqueous extract of *P. sarmentosum* have promising anti-nociceptive and an-inflammatory for the treatment of various pains. A study by Pin et al. (2010) also showed that aqueous extract of *P. betle* exhibit high antioxidant using DPPH and SOD assays as well as anti-inflammatory activity using XOD and LOX assays. Several advantages have been reported on the uses of water over other organic solvents for extraction such that is it safe, non-toxic, high availability and cheap in capital (Foo et al., 2015). Nevertheless, combination of *Panax ginseng* (5%), *Celendula officinalis* (5.0%), *Arnica Montana* (2%) and *Clerodendrum indicum* (1%) aqueous extract was also used by Das, Debnath, Nath, & Dash (2014) in formulating a wound healing cream. The formulation has demonstrated significant wound healing activity by excision wound model on albino white rats.

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2.4.2 Decoction

Decoction is also commonly known as hot aqueous extract (Daswani, Ghadge, Brijesh, & Birdi, 2011). According to Azwanida (2015), the principle of decoction is similar to maceration. In decoction, the plant sample is grinded and soak in a closed container with solvent. The soaked plant material is heated, and the time of extraction is often shorter. In a research study conducted by Tharakan & Madhavan (2017), decoction method was adopted to extract *P. longum*. The preliminary phytochemical analysis revealed the presence of alkaloids, phenols and tannins which are associated with antioxidant and anthelmintic effect of *P. longum*. The ethnobotanical studies have shown that decoction has been the main form of traditional medicine preparation for various ailments (Simbo, 2010; Grønhaug et al., 2008;). As discussed in section 2.3, presence of anti-oxidant and anti-inflammatory properties play a vital role in promoting wound healing.

2.5 Development of Topical Product

Generally, topical medications are drugs that are intended to be apply directly onto the skin (Amanda, 2016). As compared to oral administration, the ability to deliver drug substance to specific site of infected area and less toxicity concern to other organs have led to the widespread use of topical medication for treating skin disease and wound (Mohanty & Sahoo, 2017; "Tergus Pharma", 2015). Topical products available in the market may be broadly classified into ointments, cream, lotion, paste, gels, solution, suspension, powder, solid, aerosol spray or foam depending on the formulation of products. In spite of that,

consumers preference inclines toward semisolid form which include ointment, cream, lotions and gel (Chang, Raw, Lionberger, & Yu, 2013).

However, many issues have been arisen and reported due to associated side effects of certain synthetic ingredient in commercial products (Leelavathi, Le, Tohid, & Hasliza, 2011; Shweta & Swarnlata, 2010). The functions and associated side effect of common synthetic ingredients were summarized in Table 2.2.

Table 2.2: Common synthetic ingredients found in topical product and associated side effects.

Ingredients	**Side effects**	**Functions**
Propylene Glycol	Allergic reaction, hives, eczema	Humectant
Petrolatum	Dryness	Emollient and exclusive agent
Paraben	Allergic reaction, skin rashes	Antimicrobial agent (preservative)
Diethanolalamine (DEA), Triethanolamine (TEA)	Allergic reaction, eye irritation, dryness of skin	Emulsifier
Mupirocin, Acriflavin, Providone-iodine	Irritation, Inflammation, blister	Topical antiseptic and antibiotic (disinfectant)

Source: Leelavathi et al. (2011); Shweta & Swarnlata (2010).

2.6 Formulation of Topical Herbal Cream

The formulation of topical drug products often comprises of active ingredients or botanical ingredients, vehicle and a complex combination of excipients including humectant,

emulsifying agent, emollient, gelling agent (gel preparation), preservative and antioxidant. According to Builders et al. (2013), a good topical formulation should possess good spreadability and emollient properties without raising any skin irritation concern. Cream is a semisolid emulsion is made up of aqueous and oil phase. At the present study, the herbal cream was developed using a rather simple formulation which comprise of stearic acid, cetyl alcohol, jojoba oil, glycerine, polysorbate 80, distilled water, Optiphen and *P. sarmentosum* aqueous extract. The base ingredients are widely used in cosmetic and pharmaceutical formulation. During the development of emulsion, water is the main form of vehicle which use to dissolve and facilitate the dispersion of ingredients.

2.6.1 Stearic Acid

Stearic acid is a saturated fatty acid that are commonly derived from natural source of animal or plant. Stearic acid is hard, white or pale yellow in the form of pallet, crystal or powder. It is widely used in formulation as emulsifier and stabilizer to prevent separation of oil and water from an emulsion. It is also used as base component of oil phase of a formulation and act as a thickening agent which help to improve the viscosity of on emulsified products (Baumann, 2013). It was reported that stearic acid at concentration up to 13% was primarily non-irritating nor sensitizing ("Final Report on the Safety Assessment of Oleic Acid, Lauric Acid, Palmitic Acid, Myrinstic Acid, and Stearic Acid," 1987).

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2.6.2 Cetyl Alcohol

Cetyl alcohol is generally available in the form of white solid flakes or pallets. It is a fatty alcohol which has low solubility in water but high solubility in oil. It is renowned for its structure forming material in a semi-solid preparation which provide an emollient and softening quality to the finished product (Chang et al., 2013). Cetyl alcohol also act as an effective co-surfactant which contribute to the stability of emulsion by preventing the separation of ingredients (Watanabe, Kawai, & Nonomura, 2018).

2.6.3 Jojoba Oil

Jojoba oil is one of the most extensively used natural carrier oil in cream and lotions. It has been noted that jojoba oil has many benefits which is great for skin including moisturizing, emollient properties and excellent non-occlusive properties which leave a non-greasy feel on skin ("Provital Group Natural Efficacy", 2012). Other than that, the researchers also suggested that jojoba oil helps to enhance the absorption and skin penetration of topical drug, promote wound healing and skin repair (Ranzato, Martinotti, & Burlando, 2011; Wang, Wang, & Kuo, 2007). Jojoba oil also exhibited strong antioxidant property and high oxidative stability which are essential to enhance the shelf life of a natural formulation especially when mixed with other oil (Manoharan, Vishnupriya, & Gayathri, 2016; Sandha & Swami, 2009).

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2.6.4 Glycerine

Glycerine, also known as glycerol has been extensively used in cosmetic and topical dermatological preparations (Fluhr, Darlenski, & Surber, 2008). Glycerine is generally water soluble. It is a colourless, odourless and viscous liquid that usually derived from animal or plant oil (Fluhr et al., 2008). Glycerine play an important role as natural humectant to prevent evaporation of moisture in a formulation (Himaja, 2017; Shweta & Swarnlata, 2010). It is renowned for its moisturizing and soothing effects in dermatological preparation and help to lock water composition in skin (Chang et al., 2013; Fluhr et al., 2008). According to Cosmetic Ingredient Review (CIR) (2014), glycerine is generally safe and non-irritating when applied at concentration up to 65% to human subject.

2.6.5 Polysorbate 80

Polysorbate 80, also known as Tween 80 is commonly used in semisolid preparation as surfactant or emulsifying agent. It is renowned for its ability to reduce the inter-surface tension and improve the wetting and solubility of hydrophobic materials (Chang et al., 2013). In other words, polysorbate helps to disperse oil in water for a more stable emulsion system.

2.6.6 Optiphen

Water based formulation is susceptible to microbial growth (Budecka & Styczyńska, 2014). Optiphen is a natural preservative which is known for its preserving ability against

bacteria (Cosmetics Business, 2006). This liquid form of preservative is usually added directly to the formulation during pre or post emulsification phase below 80°C, possibly between 48°C to 60°C. While higher temperature could destroy preserving quality, temperature lower that 40°C might lead to unstable emulsion and possibly phase separation. The recommended dosage is between 0.50 % – 1.50 % ("Natural garden", n. d.).

2.7 Organoleptic and Physicochemical Property Evaluation

Topical drug products are often subjected to different assessments to ensure the strength, efficacy, consistency and safety concern. Determination of organoleptic and physicochemical properties are essential to establishing the specification and physical characteristic during formulation development. Most often, the organoleptic properties of drug products are qualitatively described in term of appearance, colour, odour and taste with certain terminologies. During the development of herbal based topical drugs, the presence of plant extracts will impact the colour and odour of finished formulation. Undesirable colour and odour are often being tackle by adding dye or fragrance to improve the consumer preference and acceptability (Desu, Vaishnavi, Divya, & Lakshmi, 2015). Nevertheless, product formulation for skin application should exhibit pH value range from 5.5 to 6.8 which are known to be average pH of human skin (Das et al., 2014).

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2.7.1 Texture Profile Analysis

Texture Profile Analysis is widely used to determine the sensory profile of cosmetics and pharmaceutical semisolid preparations apart from food samples (Tai, Bianchini, & Jachowicz, 2014). Parameters that can be determined using texture analyser including hardness, adhesiveness, cohesiveness, and resilience. Spreadability is an important attribute in semi-solid formulation which affect the delivery and penetration of topical drugs (Chang et al., 2013; Garg, Aggarwal, Garg, & Singla, 2002). Previous published application by Brookfield Ametek has demonstrated measurement of spreadability of a formulation using texture analyser. Specifically, the compression of sample using a complementary set female and male cone probe and base holder simulates the spreading application of sample on surface ("Moisturizing Cream Spreadability," n.d.; "Petroleum Jelly Spreadability," n.d.). The value of hardness and adhesiveness from the analysis has a strong positive correlation with the spreadability of the sample. Particularly, higher value of hardness and adhesiveness account for a firmness, stickier and less spreadable texture. The use of texture analyser provides a reliable, quantifiable, reproducible date on parameters assessed (Tai et al., 2014).

2.7.2 Colour Analysis

Chroma meter has been widely used as a tool for precise measure of surface colour over a wide range of application. The colour output is usually presented in the form of coordinates L*, a* and b*. As general rule of thumb, the coordinate L* represent lightness /

brightness in which brighter colour has higher L* approaching 100 and vice versa. Next, positive value of a* represent the redness whereas the negative value of a* represent greenness. Positive value of b* represent yellow intensity while negative value of b* represent blue intensity (Keskin, Karanlik, Görücü Keskin, & Soysal, 2013).

2.8 Stability Evaluation

Stability evaluation of products is critical to its efficacy, quality and safety attributes over time. Stability studies may be conducted in real time or accelerated condition. Accelerated studies are designed to intensify the rate of chemical degradation and physical changes of a product by exposing it to certain storage condition based on formal stability study. This is essential to provide information of the product stability in shortest possible time. Increasing the temperature also provide a constant degree of acceleration and more accurate attempt of predicting the product shelf life.

Accelerated temperature are usually conducted under 4°C, 25°C and 40°C to 50°C (Bhagwat, Kadam, & Hivarale, 2017; Ravindran & Ideris, 2016; Handali, Hosseini, Ameri, & Moghimipour, 2011; Akhtar et al., 2010). Finished products are said to exhibit significant change during the stability studies provided there is 5% change in the assay from its initial value; degradation of process exceeding its acceptance criterion or failure to meet acceptance criteria for appearance, physical (phase separation, pH value) and functionality test (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [ICH], 2004). It has been generally recognized that products that remained stable

for three months storage at 40°C is likely to remain stable at room temperature for 1 year (Bhagwat et al., 2017; Baertschi, Alsante, & Reed, 2011) .

2.9 Sensory Evaluation

Sensory evaluation is scientific yet subjective approach for the determination of a product's attributes particularly through human senses. Colour, aroma/odour, taste, touch and texture are some of the parameters that are usually measured. It has been used in diverse fields including food, cosmetic and personal care products as well as topical preparation (Jog, Bagal, Chogale, & Pradnya, 2012). The evaluation can be analytical and affective test. To be specific, analytical test by means of descriptive or discriminative is intended to determine the perceptible differences and detail description between samples particularly by trained panels. On the other hand, affective testing, also known as "consumer testing" involves testing for acceptance or preference predominately among untrained panels (consumers). In affective testing, the former test is mainly to obtain rating on degree of liking whereas the latter seeks to identify product that is more preferred (Choi, 2014; Jog et al., 2012). Hedonic scale is commonly employed to measure the extend or intensity of like and dislike due to its simplicity and ease of use which are well-suited to the untrained consumer panels. Consumer testing serves as a vital part for product development and marketing decision to better achieve consumers' need.

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2.9.1 Cronbach's Alpha

Reliability is one of the essential components in the evaluation of measurement instrument. Cronbach's alpha provides a useful statistic approach for the investigation of internal consistency of question. In other words, it describes the extent to which all the items in a test measures the same concept (Tavakol & Dennick, 2011). It is commonly applied to a survey or questionnaire with multiple questions Likert-scale. The reliability coefficient exhibits value range from 0 to 1. A high value of Cronbach's alpha close to "1" demonstrate good internal consistency of items in the scale and generation for trustworthy results. Alpha value above 0.8 is said to exhibit good reliability.

Table 2.3: Cronbach's alpha rule of thumb.

Cronbach's alpha	**Internal consistency**
$a \geq 0.9$	Excellent
$0.9 > a \geq 0.8$	Good
$0.8 > a \geq 0.7$	Acceptable
$0.7 > a \geq 0.6$	Questionable
$0.6 > a \geq 0.5$	Poor
$0.5 > a$	Unacceptable

Source: George and Marley (2003).

2.10 Skin Irritation Testing

Skin irritation test is an important part of the safety assessment for chemicals and product formulation (Vinardell & Mitjans, 2008). Skin testing for drug/chemical allergy is commonly to determine the possible side effect of consumer products including cosmetics and pharmaceutical products. From the journals reviewed, skin irritation is likely to happen due to the presence of synthetic ingredients, added fragrances and preservative. Possible symptoms that may arise from allergy test are mild itching, rashes, redness and swelling of skin. Generally, research involve the development and formulation of herbal cosmetic and topical products are tested against skin irritation to justify the safe use of the finished formulation (Himaja, 2017; Rao, Khaliq, Kharat, Sagare, & Patil, 2010). Primary Skin Irritation Index (PII) is one the test method that is used to categories the degree of irritation of a test sample (Kamkaen, Phuntuwate, Samee, Boonrod, & Treesak, 2007). A standard score of 0 to 4 is used to indicate different degree of with respect to erythema (redness) and edema (swelling) reaction. The Primary Irritation Index is represented by average total score of erythema (redness) and edema (swelling) which can be range from 0 to 8.

Table 2.4: Evaluation of skin irritation reaction.

Skin reaction	Degree of reaction	Score
Erythema	No erythema	0
	Very slight erythema (barely perceptible)	1
	Well-defined erythema	2
	Moderate to severe erythema	3

	Severe erythema (beet redness) to slight eschar formation	4
Edema	No edema	0
	Very slight edema (barely perceptible)	1
	Slight edema (edges of are well delineated by definite swelling)	2
	Moderate edema (raised approximately 1mm)	3
	Severe edema (raised more than 1 mm and extending beyond area of exposure)	4

Source: Ko et al. (2010) and Kamkaen et al. (2007).

Table 2.5: Primary irritation index and irritation classification.

Primary Irritation Index	Irritation Classification
0 – 0.9	Non – irritant
1 – 1.9	Mild
2 – 4.9	Moderate
5 - 8	Severe

Source: Ko et al. (2010).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

The plant sample (*Piper sarmentosum*) was collected from Kota Bharu, Kelantan, Malaysia. It was authenticated by botanist from Herbarium University Kebangsaan Malaysia, Selangor earlier on 5 April 2018 with voucher no. UKMB40387.

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3.1.2 Chemicals and Reagents

The chemicals and reagents used throughout the research were listed in Table 3.1.

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Table 3.1 Chemicals and reagents used for research activities.

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Item	Supplier / Brand
Stearic acid	Take It Global Sdn Bhd

Cetyl alcohol	Take It Global Sdn Bhd
Polysorbate 80	Take It Global Sdn Bhd
Jojoba oil	Green Herbology Enterprise
Optiphen	The Soap Cellar Sdn Bhd
Distilled water	*Unit Peralatan dan Kemudahan (UpKem),* UMK
Glycerin	*Unit Peralatan dan Kemudahan (UpKem),* UMK
75% ethanol	*Unit Peralatan dan Kemudahan (UpKem),* UMK
Dettol Antiseptic Cream	Dettol
Buffer Solution	Bendosen
Nutrient agar powder	Difco ™
Nutrient broth	Difco ™
Trypticase Soy Agar	Oxoid
Trypticase Soy Broth	Oxoid

3.1.3 Apparatus

Laboratory apparatus used in this research were beaker (50 ml, 100 ml, 250 ml, 1000 ml), measuring cylinder (5 ml, 25 ml and 100 ml), spatula, thermometer, cloth filter, inoculating loop, Bunsen burner, pipette and pipette tips, glass petri dish, sterile petri dish, gloves and masks, parafilm, glass rod, forceps, aluminium foil, Duran bottle (500 ml and 100 ml), falcon tube (50 ml). These apparatuses were borrowed from *Unit Peralatan dan Kemudahan Makmal (UpKem),* University Malaysia Kelantan, Campus Jeli.

3.1.4 Equipment

Equipment used in this research were oven, blender, lamina flow cabinet (Azteclab), electronic analytical balance (KERN EMB 200-2,) vacuum pump, autoclave machine, refrigerator, stirring Hotplate, texture analyser (Brookfield CT3 Version 2.1), chroma meter (Kinolta Minolta), incubator, incubator shaker (Lab Companion), digital pH meter. Nevertheless, activity involved concentrate machine and freeze dryer were conducted in University Sains Malaysia, Kelantan.

3.1.4 Bacteria and Culture

Staphylococcus aureus (S. aureus) and *Escherichia coli (E. coli)* pure culture was obtained from Puan Hidayah, laboratory assistant of Faculty of Agro-Based Industry.

3.2 Methods

3.2.1 Preparation of Plant Sample

The plant sample *P. sarmentosum* was collected from Kota Bharu and washed thoroughly to remove all the soil sediments and foreign particles. The plant was distributed evenly on tray and dried using oven at 40˚C for 1 week. Upon complete drying, the dried

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leaves were grinded into powder using blender and kept in zip lock bag for subsequent aqueous extraction.

3.2.2 Extraction of Plant Sample using Decoction and Freeze Drying

P. sarmentosum powder was decocted using sterile distilled water in a ration of 1:20. Particularly, 50 g of the sample was added with 1000 ml of sterile distilled water and heated to 80°C for 3 hours using stirring hotplate (Zakaria et al., 2010). The mixture was constantly stirred to at 15 minutes' interval to ensure even heating process. Next, a cloth filter was used to separate the plant residue from the liquid preparation. The liquid preparation was then further filtered using a vacuum pump and Whatman no. 1 filter paper until a clear brownish crude leave solution was obtained. The solution obtained was immediately kept at 4˚C. At the end of filtration process, approximately 35 ml of solution was filled into a 50 ml falcon tube. The falcon tube containing plant extract solution was then sent to University Sains Malaysia, Kelantan for concentration process followed by freeze drying. The freeze dried extract was kept at 4˚C until use. The yield of extraction was calculated using the Equation (3.1).

$$Yield\ (\%) = \frac{weight\ of\ dried\ extract}{weight\ of\ original\ sample}\ x\ 100\% \qquad (3.1)$$

3.2.3 Formulation of Herbal Topical Cream

At the present study, a formulation by Handali, Hosseini, Ameri, & Moghimipour (2011) was adopted with slight modification for the preparation of cream. The ingredients used in the formulation consists of both oil phase and aqueous phase. The composition of the formulae as to produce 150 g of cream were presented in Table 4.1. Prior to the formulation process, all the equipment including beakers, measuring cylinder, glass rod, spatula, distilled water, glass petri dishes were autoclaved at 121°C for 15 minutes.

Initially, the oil phased ingredients including stearic acid, jojoba oil and cetyl alcohol were weight, mixed and heated to 65°C using hotplate and stirred until homogenous. At the same time, the aqueous phase was prepared by adding glycerine, of polysorbate-80 into sterile distilled water and heated to 65°C. Next, 0.2% of freeze-dried extract of *P. sarmentosum* were added to the aqueous phase and mixed well until homogenous. Upon heating, both phases were allowed to cool down to 60°C followed by adding 0.5% of Optiphen to the aqueous phase. The oil phase was then slowly added to the aqueous phase and stirred well. The stirring process was continued for approximately 15 minutes until a consistent emulsion was formed. Modifications were done throughout the formulation process until a desirable texture and sensory properties were obtained. An optimized cream was chosen for further characterization and quality assessment. At the end of formulation process, 45 g of the cream was weighed and filled into a glass petri dish.

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3.2.4 Organoleptic and Physicochemical Analysis of Optimized Cream

The formulated cream was assessed and characterized for basic organoleptic properties which included appearance, colour, odour, homogeneity, spreadability and immediate after feeling such as stickiness and greasiness. These characteristics were mainly determined though visual inspection, smell and touch.

Texture profile analysis was performed using texture analyser (Brookfield CT3 Version 2.1) to evaluate the hardness, adhesiveness and spreadability. Under the guidance of lab assistant Puan Aisyah and Brookfoeld Amtek manual, the spreadability test was performed using Fixture Dual Extrusion Cell (TA-DEC), with a conical probe of angel 45°. Approximately 45 g of cream sample was filled into a 50 ml beaker. The probe was allowed to penetrate the sample at test speed of 2 mm/s to a depth of 10 mm with a trigger force of 5 g. The force exerted to the probe was recorded using Texture Pro CT V1.7 Build 28 Software. At present study, the texture profile of marketed cream (Dettol Antiseptic Cream) was determined and used as benchmarking. The results were collected in triplicate and the average value and standard deviation were tabulated.

The colour profile of optimized cream and base cream were determined using chroma meter. The 5 g sample was filled in a beaker (50 ml) and the colour of the cream was measured from the bottom of the beaker. The data output in the form of L*, a* and b* displayed on the screen and recorded down. The results were collected in triplicate and the average value and standard deviation were tabulated.

Lastly, pH of the optimized cream was measured using digital pH meter. Prior to the analysis, the pH meter was calibrated using buffer solution pH 7. 0.5 g of the cream was obtained and dissolved in 50.0 ml of sterile distilled water. The pH value was collected in triplicate and the average value and standard deviation were tabulated.

3.2.5 Total Plate Count

Nutrient Agar (NA) plates were prepared by mixing 14 g of agar powder with 500 of distilled water in a media bottle. The mixture was then autoclaved at 121 ˚C for 15 minutes and allowed to cool down. At the same time, 9 ml of distilled water were pipetted into a 50 ml of beakers and 18 x 9 ml of distilled water were pipetted into 10 ml test tube and autoclaved. The cooled mixture was poured into sterile petri dish under lamina flow cabinet and allowed to harden for 30 minutes. Next, 1 g of the optimized cream was added to 9 ml of sterile distilled water in beaker and mixed well until homogenized. The mixture was serially diluted for 9 times by pipetting 1 ml of the solution to the respective test tube. The test tubes were shaken well and 0.1 g of the solution from each dilution factor was pipetted onto the NA plates prepared in earlier. A sterile hockey sticks was then used to spread the solution on the NA plates evenly. The plates were then wrapped appropriately using parafilms, labelled and incubated at 37 ˚C for 2 days. Total microbial count of each dilution were observed and calculated in term of colony forming unit (cfu/g). Finally, the results for cfu/g of each dilution factor were recorded and tabulated.

$$Total\ Colony\ Forming\ Unit\ (CFU/g) = \frac{\text{No. of colonies}}{\text{Volume of culture plate}} \times Dilution\ factor \quad (3.2)$$

3.2.6 Antimicrobial Assay of Optimized Cream and Preservative

Two of the common bacteria species which caused infection on skin was used to determine the antibacterial property of optimized cream sample. The test microorganism used at the present study was *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)*. Trypticase Soy Agar (TSA) and Nutrient Agar (NA) was prepared and used for antibacterial assay. 20 g of the TSA powder and 14 g of NA powder were weighed and mixed with 500 ml of distilled water respectively in a media bottle. The media solutions were autoclaved at 121°C for 15 min and left to cool at room temperature for 30 minutes. The cooled was poured into sterile petri dish under a lamina flow cabinet and allowed to solidified. Next, pure culture of *E. coli and S. aureus* were obtained from lab assistant, Puan Hidayah. *E. coli* and *S. aureus* were sub-cultured by dipping an inoculating loop into the glycerol stock and streaked on one NA and TSA plate respectively. The sub-culture of *E. coli* and *S. aureus* were incubated in incubator 37˚C for 18 hours.

Trypticase Soy Broth (TSB) was prepared for inoculation of bacteria. 6 g of TSB was weighed and mixed with 200 ml of distilled water in a media bottle. The broth was autoclaved at 121°C for 15 min and left to cool at room temperature. Later, 40 ml of cooled TSB was poured into 2 sterile falcon tubes respectively. After 18 hours, single colony of the sub-

cultured *E. coli and S. aureus* was scoped using an inoculating loop and inoculated in the TSB respectively. The falcon tubes were wrapped with parafilm and incubated at incubator shaker at 30˚C, 120 rpm for 24 hours.

On the next day, a sterile cotton swab was used to spread the *E. coli* and *S. aureus* culture suspensions on NA and TSA plates respectively in a manner of back and forth followed by 45°C rotation to ensure the plates were thoroughly covered with bacteria. A well of 6mm diameter were then punch on the agar plates using a sterile cork borer. 0.10 g of base cream and optimized cream and cream with increased concentration of preservative (0.75%, 1.00%) were then filled into the well to three-quarters full with the aid of sterile micropipette tip. In this test, a sterile Whatman no.1 filter paper disc (6 mm diameter) was infused with distilled water and used as negative control while Dettol Antiseptic Cream was used as positive control. The plates were incubated at 37˚C for 24 hours. The results were collected in duplicate and the diameter of clear zone was measures as zone of inhibition.

Subsequently, paper disc diffusion assay was used to determine the antibacterial property of preservative alone against *E. coli* and *S. aureus*. Preservatives was prepared in various concentration by diluting it with sterile distilled water. The preservative was prepared in concentration of 0.50%, 0.75%, 1.00% and 100% without any dilution. The plates were incubated at 37˚C for 24 hours. The results were collected in duplicate and the diameter of clear zone was measures as zone of inhibition.

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3.2.7 Accelerated Stability Test of Optimized Cream

The accelerated stability test was performed under 4 different storage conditions of 5˚C ± 1°C (refrigerator), 25˚C ± 1°C (room temperature), 40˚C and 45˚C (oven). 45 g of sample which was filled in the petri dish kept under the conditions for 28 days. The tested parameters including texture, colour, pH, odour and phase separation was observed and evaluated for any changes on every 7-days interval The results were collected in triplicate and compared with the initial value of respectively parameters. This study was carried out in accordance with International Council for Hominization (ICH) guideline and World Health Organization (WHO) stability guideline.

3.2.8 Sample Testing for Sensory Evaluation

A sensory evaluation test was conducted to determine the sensory attributes of the optimized cream which included colour, aroma, texture/appearance, spreadability, greasiness, stickiness, ease of removal and overall acceptance. 5-piont hedonic scale was used which included the following score 1 = dislike very much; 2 = dislike, 3 = neither like nor dislike; 4 = like; 5 = like very much. 50 respondents were selected randomly from University Malaysia Kelantan, Campus Jeli.

In preparing the samples, approximately 4 x 5 g of sample was served in one petri dish. The samples were given to 4 respondents at one time. Prior to sample testing, a consent letter was distributed to the respondents to document their decision to participate. The

respondents were examined to ensure no chronic skin disease or damage on left hand dorsal surface and forearm. A brief introduction and instructions were given to ensure the respondents to ensure the evaluation was performed in a professional manner. Each of the respondents were required to rate the particular attribute according to their preference and degree of likeness. During the period of testing, the respondents were given the opportunity to had theirs inquires answered. However, the respondents were discouraged to discuss among each other. They were also encouraged to leave their opinions or comments on space provided.

The sample testing was followed by skin irritation test and performed on the same respondents after sensory evaluation. During the skin irritation test, the respondents were required to take a small amount of cream and applied on the left hand dorsal surface. The cream was allowed to sit on the skin for 30 minutes. The evaluation was done using the scoring system of Primary Irritation (SPI) to reflect the degree of irritation including erythema (redness) and edema (swelling). The individual score was summed up and the cream was categorised based on the average score calculated according to Primary Irritation Index (PII). As for this assessment, feedbacks collected from respondents for any discomforts appeared to be highly subjective. The evaluation form was presented in Appendix.

3.2.9 Statistical Analysis

The results obtained from each analysis was expressed in mean ± standard deviation. During the stability testing, the effect of storage time and storage temperature on

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physicochemical properties of optimized cream was determined using two-way analysis of variance (ANOVA) of IBM SPSS Software (Version 21). At 5% significant level, p-value less than 0.05 ($p<0.05$) was considered to be statistically significant. Besides, results collected from sample testing was interpreted using descriptive analysis. Cronbach's alpha was performed using SPSS to determine internal consistency or reliability for scales of the sensory evaluation form.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preparation of *Piper sarmentosum* Aqueous Extract

Plant sample *P. sarmentosum* collected from Kota Bharu and washed thoroughly to remove all the soil sediments and foreign particles. The plant was then dried using oven at 40˚C for 1 week to remove excess moisture. Relatively low drying temperature between 30˚C to 50˚C was recommended to preserve the active ingredient of herbal plant (Müller & Heindl, 2006). As stated by Mediani, Abas, Tan, & Khatib (2014) , drying process of herbal plants is beneficial in preserving the photochemical efficiency throughout storage or before further use. Upon grinding the dried leaves into powder, the powder was extracted using hot water at 80˚C for 3 hours. Previous studies revealed that extraction on *Piper* species using water as solvent has been one of the reliable way to isolate active compounds which may contribute to heal wound property (Abdullah, 2018; Ghazali et al., 2016; Subramaniam et al., 2003). In the end of extraction process, the liquid preparation was filtered, concentrated and freeze dried. The process of freeze drying was essential to preserve the extract through substantial removal of water content (Shukla, 2011). Freeze drying process using low temperature also

helped to avoid decomposition of extract at higher temperature of drying. Eventually, the total freeze-dried extract obtained was 11.33 g which adequate to 8.15% of yield.

4.2 Formulation of Topical Herbal Cream

In the course of this research, a topical herbal cream was formulated by manual mixing of oil phase and aqueous phase ingredients. The basic ingredients used in the formulation of cream bases including stearic acid, jojoba oil, cetyl alcohol, glycerine, polysorbate 80, Optiphen and distilled water. A total of fourteen different cream formulations (F) were prepared and the composition of each cream was given in Table 4.1. Particularly, F1 to F8 were prepared with the same aqueous and oil phase ingredients in varying concentration in order to obtain a desirable base cream. Out of these 8 formulations, F1 was selected as optimal formulation of cream base based on overall physical evaluation and immediate skin feel. Meanwhile, F2 to F8 were eliminated as the physical characteristic of the cream base were less desirable. Particularly, the issues encountered during the formulation were overly soft texture of cream, insufficiently stable emulsion, present of tiny white residue due to lack of homogeneity.

Using F1 as the cream base, F9 and F10 were formulated with the incorporation of *P. sarmentosum* extract at 0.2% and 2% respectively. However, a consistent emulsion did not form in F10 which was due to incompatibility of high extract concentration (2%) with base cream while F9 incorporated the active ingredient well at 0.2%. According to Abdullah (2018), *P. sarmentosum* aqueous extract exhibit good wound healing activity and does not

cause acute toxicity to the test animals. At later stage, Optiphen was also added to improve the natural preserving quality of the herbal cream at different concentration of 0.5% (F11), 0.75% (F12), 1.00% (F13) and 1.25% (F14). It was found that, addition of Optiphen at 1.25% did not form a consistent emulsion and thus eliminated. Among F11, F12 and F13, the texture and after-feel of formulated cream F11 was considered to be the most satisfactory and used for further quality assessments.

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Table 4.1: Compositions of topical herbal cream formulation.

Phase	Ingredients	Amount of ingredients (%)													
		F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**
Oil	Stearic acid	10	9	8	12	11	10	10	8	10	10	10	10	10	10
	Jojoba oil	5	5	5	4	4	5	5	5	5	5	5	5	5	5
	Cetyl alcohol	5	5	5	3	4	5	6	6	5	5	5	5	5	5
	Glycerin	5	5	7	4	4	5	5	5	5	5	5	5	5	5
	Polysorbate 80	1	1	1	1	1	2	2	1	1	1	1	1	1	1
Aqueous	Extract	-	-	-	-	-	-	-	-	0.2	2	0.2	0.2	0.2	0.2
	Preservative	-	-	-	-	-	-	-	-	-	-	0.5	0.75	1.0	1.25
	Distilled water	74	75	74	76	76	73	72	75	73.8	72	73.3	73.05	72.8	72.55

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4.3 Organoleptic and Physicochemical Characteristics of Optimized Cream

The organoleptic properties of the formulated cream were given in Table 4.2. The optimized cream (F11) exhibited good appearance which was smooth, homogenized and complete absence of any tiny residue by visual inspection and touch. The optimized cream possessed very light brown colour and rather unpleasant aroma of *P. sarmentosum* extract. The researchers reported that pungent smell of *Piper* species is attributed to the presence of alkaloid compound, piperine (Vasavirama & Upender; 2014). In the formal studies, cream formulated using *P. betle* extract (2%) shown dark green in appearance with slight pungent smell which was attributed to the *P. betle* extract while Unani extract (6.25%) also render to dark brown colour of cream formulated (Fatima, Zaman, Shamsi, & Alam, 2017; Ravindran & Ideris, 2016). Lighter colour was observed in the herbal cream formulated at the present study was presumably due to the lower concentration of extract used (0.2%). The cream also showed good physical properties such as good spreadability, non-greasy and non-sticky after application. Besides, the cream can be easily removed by water. The physiochemical properties of cream including texture, colour and pH was also determined using appropriate equipment.

Table 4.2: Organoleptic characteristics of optimized cream.

Characteristic	Description
Appearance	Smooth, opaque
Color	Nude color / Very light brown
Odor	Strong scent of *P. sarmentosum* extract

Homogeneity	Homogenous
Spreadability	Easy to be spread
Immediate After Feel	non-greasy and non-sticky on application

4.3.1 Texture Profile Analysis (TPA) and Spreadability

As the herbal cream formulated at present study was intended to treat wound and minor cut, commercialized Dettol Antiseptic Cream was used as benchmark. By visual inspection and touch, it was distinguishable that the texture of the optimized cream was thicker and firmer than the commercialized cream. In fact, the texture of commercialized cream was much softer, slippery and emollient. The objective measurement of both cream textures was performed using texture analyser. The texture was particularly evaluated on 2 attributes including hardness and adhesiveness. The texture profile of the optimized cream and a commercialize sample (Dettol Antiseptic Cream) using texture analyser was detailed in Table 4.2.

Table 4.3: Texture profile analysis of optimized and Dettol cream.

Parameters	Optimized Cream	Commercialize Cream (Dettol Antiseptic Cream)
Hardness (g)	27.00 ± 1.73	23.67 ± 2.08
Hardness Work Done (mJ)	1.27 ± 0.11	1.13 ±0.06
Adhesiveness (mJ)	0.90 ± 0.06	0.77 ± 0.06

The values were presented in mean ± standard deviation (n=3).

Hardness is measurement of firmness at a specific depth while energy need to attain a given deformation to the defined depth during the first compression cycle is known as hardness work done ("Moisturizing Cream Spreadability," n.d.). Besides, adhesiveness is work needed to overcome attractive force between sample and probe which resemble that trait of stickiness. The greater the adhesiveness, the stickier the sample. From the result obtained, the hardness of the optimized cream was slightly higher than the commercialize cream which was 27.00 g ± 1.73 and 23.67 g ± 2.08 respectively. This explained that greater force was required for the cone probe to penetrate the cream to defined depth of 10.00 mm. Correspondingly, the optimized cream has higher work done value than the commercialize cream which was 1.27 mJ ± 0.11 and 1.13 mJ ± 0.06 respectively. These values provided a rationale for the firmer texture of optimized cream as compared to commercialized cream.

The measurement of hardness, or firmness, has also been recognized to have positive correlation with sensory estimate of spreadability where higher firmness and hardness work done value accounts for a less spreadable sample Therefore, it was presumed that the optimized cream has less spreadability than the commercialized cream due to the higher value of hardness. Nevertheless, the optimized cream had greater adhesiveness than the commercialized cream which is 0.90 mJ ± 0.06 and 0.77 mJ ± 0.06 respectively. This indicated that optimized cream was relatively stickier than the commercialized cream.

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4.3.2 Colour Analysis

In term of visual appearance, the base cream without extract was white in nature and the optimized cream exhibited light brown colour. Colour profile of the cream sample was specifically determined using chroma meter with respect to the L*, a* and b* parameter as presented in Table 4.3. From the result obtained, L* value of the formulated cream was found to be 67.24 ± 0.41 and the b* value was 15.74 ± 0.04. As compared to the base cream (L*=81.47; b*= 12.54), optimized cream was less bright and more yellowish which was attributed to the colour of extract incorporated. Similar finding was found in a study conducted by Fatima et al. (2017) and Ravindran & Ideris (2016) such that extract will impart colour to an herbal preparation. However, the presence of extract had no effect on a* value as the both base cream and optimized cream has similar a* which is 2.73 and 2.70 respectively.

Table 4.4: Colour analysis of formulated base cream and optimized cream (F11).

Parameters	Base Cream	Optimized cream
L*	81.47 ± 0.20	67.24 ± 0.41
a*	2.73 ± 0.08	2.71 ± 0.02
b*	12.54 ± 0.17	15.74 ± 0.04

The values were presented in mean ± standard deviation (n=3).

4.3.3 pH Value

A product intended for the application of skin should have a close pH to the skin (pH= 6.8) or falls within range of 5.5 to 6.8 (Das et al., 2014; Himaja, 2017). At the present study, the pH value of the formulated cream was found to be 6.0 which was compatible with skin pH and considered safe to apply. It is essential to obtain pH within the required range as products with too high or too low pH will affect skin or potentially induced irritation (Campana, Scesa, Patrone, Vittoria, & Baffone, 2006; Lambers, Piessens, Bloem, Pronk & Finkel, 2006;).

Table 4.5: pH value of the optimized cream.

Parameter	Optimized cream	Standard
pH	6.0 ± 0.00	5.5 – 6.8

The values were presented in mean ± standard deviation (n=3).

4.4 Antimicrobial Assay

Agar well diffusion method was performed to determine the antibacterial activity of the optimized cream particularly towards *Escherichia coli* (*E. coli*) and *Staphylococcus aureus (S. aureus)*. The antibacterial activity was assessed based on the diameter of halozone observed. From the result obtained, it was shown that base cream and optimized cream incorporated with 0.2% of *P. sarmentosum* extract did not revealed any significant zone of inhibition (Table 4.6). Later, the antimicrobial activity was further determined on

formulation with increased concentration of preservative at 0.75% and 1.00%. In spite of that, none of the formulated cream exhibited antimicrobial activity against *E. coli* and *S. aureus* with complete absence of clear zone (Table 4.6). These result obtained was incomparable with marketed product of Dettol Antiseptic which recorded 25.00 mm ± 0.00 and 34.50 mm ± 0.70 against *E. coli and S. aureus* respectively.

Table 4.6: Zone of inhibition of herbal cream formulated and Dettol cream.

Test Microorganism	Zone of Inhibition (mm)					
	Base cream	Optimized Cream with Preservative			Negative Control (Distilled water)	Positive Control (Dettol Antiseptic Cream)
		0.5%	0.75%	1.0%		
E.coli	-	-	-	-	-	25.00 ± 0.00
S. aureus	-	-	-	-	-	34.50 ± 0.70

The value was presented in mean ± standard deviation (n=3); (-) represent no inhibition.

E. coli and *S. aereu*s are two bacteria that commonly reported to cause infection on wound (Builders et al., 2013). Hence, significant antibacterial activity was highly anticipated in the optimized cream to promote wound healing process by prevent bacterial infection by *E. coli* and *S. aureus*. However, the result obtained was clear that optimized cream containing extract did not exhibit antibacterial activity against *E. coli and S. aureus.* Presumptions was made such that the extract itself does not exhibit antibacterial property or the concentration of extract incorporated was insufficient to inhibit the test

microorganism. In fact, there has been very limited studies on antibacterial properties on *P. sarmentosum* aqueous extract. Several researches have reported that aqueous extract of medicinal plants exhibited the least significant antibacterial activity against *E. coli* and *S. aureus* when compared with extract using organic solvent (Kaur, Aggarwal, & Dhiman, 2016; Azzam, Hazaa, Mostafa, & Bayoumi, 2014; Dahiya and Purkayastha, 2012). Handali et al. (2011) also reported that increasing concentration of herbal extract will has positive influence on antibacterial activity. During the formulation of herbal cream, Optiphen was added as natural preservative to inhibit bacteria growth. However, 0.5% of Optiphen added to the optimized formulation was said to be ineffective in inhibiting the test microorganism. Nevertheless, antibacterial activity was also absence in herbal cream with increased concentration of Optiphen (0.75% and 1.00%).

Subsequently, antibacterial activity of the Optiphen alone at 0.50%, 0.75%, 1.00% and 100% without any dilution were tested respectively using paper disc diffusion assay. It was found that Optiphen at 0.50%, 0.75% and 1.00% had small clear zone of inhibition against *E. coli and S. aureus* of 6.5 mm to 7.00 mm (Table 4.7). Optiphen without any dilution marked significant zone of inhibition of 14.50 mm ± 0.70 against *E. coli* and 16.00 mm ± 0.00 against *S. aureus*. In this context, ineffectiveness of the preservative when incorporated to the herbal cream formulated could be due to compatibility issue with the formulation which might have limit or hinders the preserving quality. Further studies should be performed to determine the compatibility of Optiphen or other preservative agent with the formulation for effective antibacterial activity.

Table 4.7: Zone of inhibition of Optiphen and Dettol cream.

Test Microorganism	Zone of Inhibition (mm)				
	Optiphen (Preservative)				Positive Control (Dettol Antiseptic Cream)
	0.5%	0.75%	1.0%	100%	
E.coli	7.0 ± 0.00	7.0 ± 0.00	7.0 ± 0.00	14.50 ± 0.70	23.50 ± 2.12
S. aureus	6.5 ± 0.70	6.5 ± 0.70	7.0 ± 0.00	16.00 ± 0.00	32.50 ± 3.50

The value was presented in mean ± standard deviation (n=3).

4.5 Total Plate Count of Optimized Cream

Total plate count was performed to determine the total colony forming unit (cfu) of cream samples. According to National Pharmaceutical Control Bureau and British Pharmacopoeia (2012), the acceptance criteria of microbial count is ≤ 100 cfu/g. Initially, the total microbial count of the optimized cream was found to be 1.0×10^4 cfu/g which had far exceeded the standard value. In this case, high bacteria count could be several factors such as presence of indigenous microorganisms from the plant sample as a consequence of inadequate washing during the preparation of plant extract. Madhuri et al. (2011) also pinpointed that the naturally occurring bacteria on plant sample could be originated from soil or contaminated by animal manure or slurries. Contamination could also be associated to the impurity of raw material and possibly arose from the preparation of cream in an opened space (shared laboratory) which was crowded with students who was also working on final year project (Budecka & Styczyńska, 2014). Besides, the optimized cream containing large amount of water tend to be conducive to microbial growth (Budecka &

Styczyńska, 2014). On top of that, the effectiveness of preservative (Optiphen) in inhibiting bacteria may have had been lost when incorporated into the formulation as discussed earlier.

During the storage period, cream samples at both storage temperatures exhibited increased colony forming unit. At day 28th, greater number of colonies was found in sample stored at 25°C has than at 5°C which was 4.5 x 10^6 cfu/g and 2.0 x 10^6 cfu/g. This may be justified such that room temperature was optimum for microbial growth yet the growth was delayed at lower temperature (Budecka & Styczyńska, 2014). High microbial count was undesirable because they may cause product spoilage and negative impact on product safety.

Table 4.8: Total plate count of optimized cream.

Storage Time (day)	Total Plate Count			
	5°C		25°C	
	cfu/g	Log cfu/g	cfu/g	Log cfu/g
1	1.0 x 10^4	4	1.0 x 10^4	4
14	1.0 x 10^5	5	1.0 x 10^6	6
28	2.0 x 10^6	6.3	4.5 x 10^6	6.65

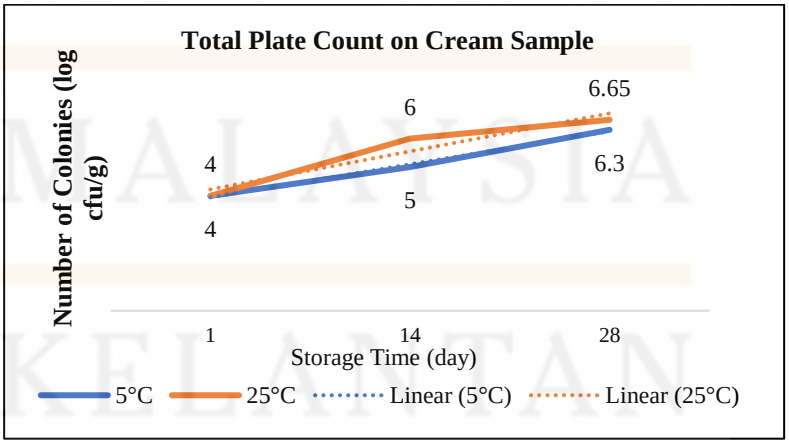

Figure 4.1: Total plate count on optimized cream.

4.6 Stability Testing of Optimized Cream

At the present study, the optimized cream sample were stored at 4°C (refrigerator), 25°C (room temperature), 40°C and 45°C (oven) for 1 month stability study. On every 7th day, the optimized cream was evaluated on various parameters involving odour, hardness, adhesiveness, L* value, b* value, pH value, phase separation. This stability test was carried out by storing the cream sample in glass petri dish. This was to minimize the influence of container on the formulation stability or avoid any uninviting changes in the product due to the interaction that may occur between the formulation and container used. Glass packaging has been noted for the fact that it is the most inert material and thus less likely to affect the performance of a product (Campbell & Vallejo, 2015; Shivsharan, Raut, & Shaikh, 2014). Particularly, a stable formulation shall not have exhibited 5% change in assays from its initial value or any degradation beyond acceptance criteria. The summary of physicochemical characteristics of optimized cream stored at 5°C ± 1°C and 25°C ± 1°C over 28 days were presented in Table 4.9 and Table 4.10 respectively. Yet, all the samples stored at 40°C and 45°C were discontinued as the liquefaction was observed on the samples after 1 day storage. which was considered unfit for further stability study. The researcher has reported on liquefaction as the most significant sign of instability which might attributed to passage of water from internal phase to external phase and decreased in viscosity (Smaoui et al., 2012). In this context, breakage of the formulation presumably due to inadequate mixing of emulsion during formulation.

Table 4.9: Physicochemical characteristics of optimized cream stored at 5°C over 28 days.

Physicochemical characteristic	Storage Temperature (5°C)					P-value
	Day1	Day7	Day14	Day21	Day28	
Hardness (g)	27.00	27.33	28.00	26.00	25.67	$p > 0.05$
Adhesiveness (mJ)	0.90	0.93	0.90	0.90	0.87	$p > 0.05$
L*	67.24^{a}	68.88^{b}	69.70^{c}	69.87^{c}	69.83^{c}	$p < 0.05$
b*	15.74^{d}	15.08^{c}	15.00^{c}	14.42^{b}	13.68^{a}	$p < 0.05$
pH	6.0	6.0	5.9	5.9	5.9	$p > 0.05$
Odour	-	NM	NM	NM	NM	-
Phase separation	-	NO	NO	NO	NO	-

The value was presented in mean (n=3). The values followed by different superscript letter (a, b, c and d) in the same row of each individual parameter were statistically significant ($p<0.05$) by Duncan's multiple range test.

Table 4.10: Physicochemical characteristics of optimized cream stored at 25°C over 28 days.

Physicochemical characteristic	Storage Temperature (25°C)					P value
	Day1	Day7	Day14	Day21	Day28	
Hardness (g)	27.00	27.00	28.00	27.00	26.00	$p > 0.05$
Adhesiveness (mJ)	0.90	0.90	0.87	0.90	0.90	$p > 0.05$
L*	67.24^{a}	67.22^{a}	67.71^{a}	67.77^{a}	68.39^{b}	$p < 0.05$
b*	15.74^{a}	15.78^{a}	15.73^{a}	15.70^{a}	15.08^{b}	$p < 0.05$
pH	6.0	6.0	5.9	5.9	5.8	$p > 0.05$
Odour	NM	NM	NM	NM	NM	-
Phase separation	-	NO	NO	NO	NO	-

The value was presented in mean (n=3). The values followed by different superscript letter (a and b) in the same row of each individual parameter were statistically significant ($p<0.05$) by Duncan's multiple range test.

Based on the result obtained, the initial value of hardness of optimized cream recorded with 27.00 g. When stored at 25°C, increased slightly of hardness was observed from day 1 to day 14^{th} (28.00 g) followed by slight decreased to day 28^{th} (26.00 g) while

adhesiveness remained almost consistent at 0.90 mJ over 28 days. On the other hand, the hardness of cream at 5°C exhibited a similar trend as samples at 25°C throughout 21 days of storage. However at day 28th, cream stored at 5°C exhibited lowest hardness value which was found 25.67 mJ. Slight decrease of hardness value of indicted that the cream was slightly losing its firmness. When stored at 5°C, unexpected increased in adhesiveness was observed on at day 14th. Despite that, adhesiveness of cream sample exhibited inconsiderable decreasing trend from 0.90 mJ to 0.87 mJ. Inconsistency of cream texture could had been influenced by several factors such as emulsifier type, concentration, thickeners/stabilizer, preservative as well as method of preparation. In this study, homogenization process is a major concern to emulsion consistency and stability. Due to the lack of appropriate equipment, all the cream samples were produced by manual mixing of oil phase and aqueous phase ingredients using a spatula with constant stirring until a consistent emulsion was formed. This had become one of the dominant factors that could have impaired the texture consistency over the testing period. In fact, homogenization process should be thoroughly performed using a homogenizer at high shear mixing rate (Atikari et al., 2014). Dhankhar (2014) has also reported homogenizer is an essential tool to create a stable finished product and consistent product quality via efficient particle size reduction. As stated by Ahtikari, Bansal, Jackson, & Solanki (2014), smaller droplets size is essential for the tighter distribution and more stable emulsion.

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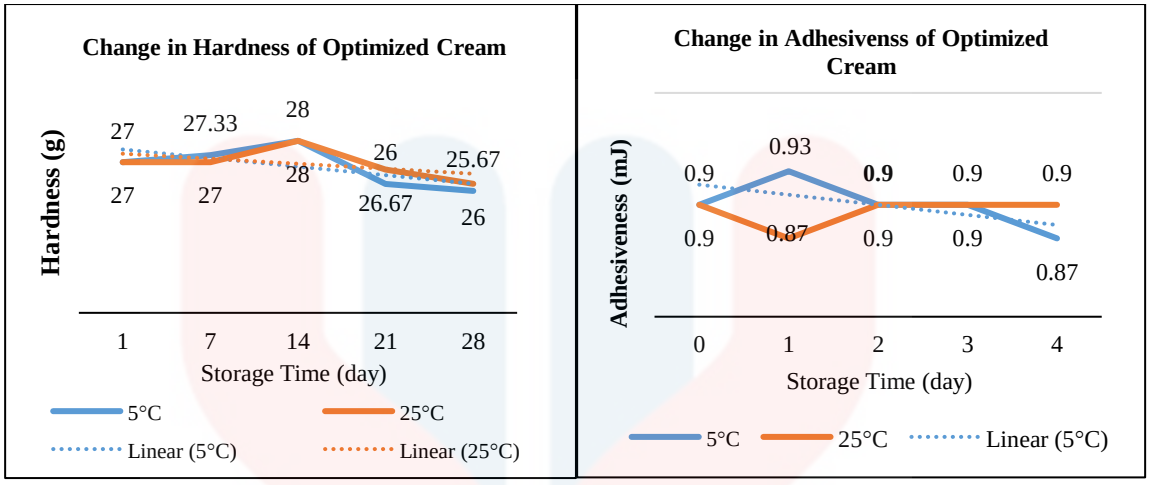

Figure 4.2: Change in the textural properties (a) hardness; (b) adhesiveness of optimized cream.

Moving on to colour profile analysis, the initial value of lightness and yellowness were found to be 67.24 and 15.74 respectively. However, a* value was not tabulated and further analyse as the colour coordinate (redness) has no concern with the presence of extract as discussed in section 4.3.2. From the result obtained, it was shown that the L* value increased while the b* value decreased during the testing period when stored at both 5°C and 25°C. This indicated that cream samples stored at both temperatures were getting brighter and less yellow intensity over one-month storage. To justify, introduction of air into the herbal cream during mixing process might led to oxidative degradation of the pigment as given by the extract over one-month storage. However, the result obtained in this study was not in accordance with any of the previous studies as most of the studies reported stable colour of cream/gel/ointment over the testing period. Greater decreased in b* value of cream was also observed at storage temperature of 5°C as compared to 25°C. Indeed, the cream sample at 5°C was visually getting light-coloured as the yellow intensity faded off as compared to samples stored at 25°C. This may be justified such that cooler

temperature might have had cause greater alteration of pigment or herbal extract in the formulation which requires further determination. As mentioned by Tucker (2017), certain ingredients of a formulation could be sensitive to extreme cold causing inconsistency or impact on stability. To overcome this issue, natural anti-oxidation compounds may be incorporated to the formulation to prevent oxidative degradation (Chang et al., 2013).

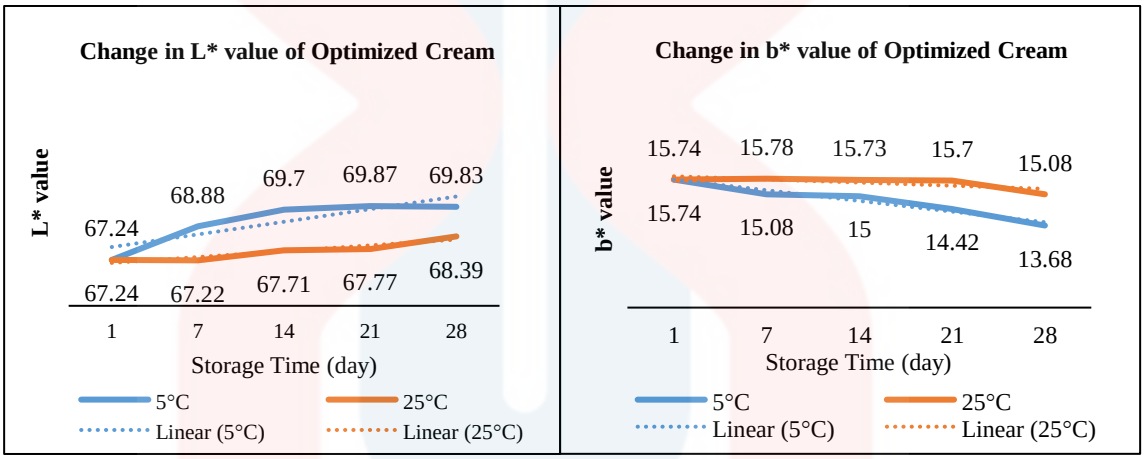

Figure 4.3: Change in the colour profile (a) L* value; (b) b* value of optimized cream.

Next, consistency of pH is also one of the essential parameter to reflect a product quality. Throughout the storage period, pH of cream samples at both temperatures were considered stable. Slight decreased in pH observed in samples stored in both cream was presumably due to the production of acidic by-product of ingredients used such as stearic acid or possibly due to bacteria growth (Fatima et al., 2017; Chang et al., 2013; Smaoui et al., 2012; Akhtar et al., 2010). Nevertheless, no sign of phase separation nor change in odour were observed in all samples during one month storage at 5°C and 25°C.

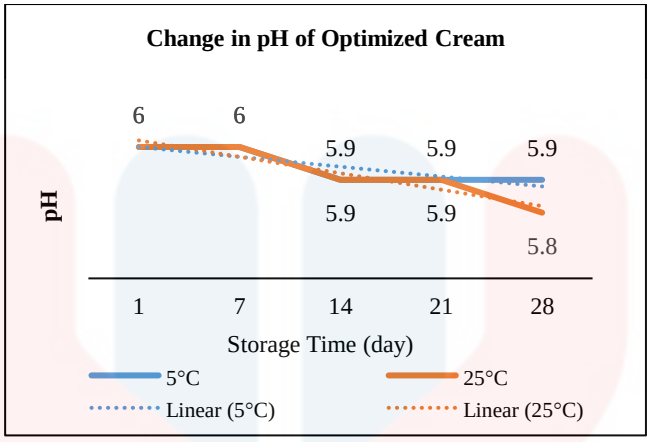

Figure 4.4: Change in the pH of optimized cream.

Two-way analysis of variance (ANOVA) were performed to determine effect of storage temperature and storage time on all parameters discussed. At 5% significant level, it was showed that the variation in hardness, adhesiveness and pH of cream samples were statistically not significant ($p>0.05$) at different level of storage time and temperature (5°C and 25°C). This indicated that hardness, adhesiveness and pH were less likely to be affected by storage temperature over one-month testing. Yet, the colour of the optimized cream in term of L* value and b* value were statistically significant ($p<0.05$) at different level of time and temperature. Duncan test was used to check the L* value and b* at different storage temperature over storage period. The result showed that variation in L* value and b* were significant ($p<0.05$) after 1 week storage at 5°C whereas significant change ($p<0.05$) was observed only after 4 weeks storage at 25°C.

Despite significant variation in colour, one–month stability study showed that herbal cream formulated was considered stable under cold storage (5°C) and room temperature (25°C) with no evidence of phase separation nor significant change of odour,

texture and pH value. Certainly, the formulation was physically unstable at extreme temperature as high as 40°C.

4.7 Sample Testing

A total number of 50 respondents had participated in this study. The respondents were randomly chosen from student and staffs in University Malaysia Kelantan Campus Jeli. Particularly, the demographic information collected involved gender and comprised of three different races with wide range of age group. From the data tabulated, female respondents dominated this study with 66% compared to male with only 34%. The age group were range between 20 to 60 years old. Out of all respondents, 12% of them aged between 31 to 40, followed by 4% of them aged between 41 to 50, and 2% aged between 51 to 60. The highest percentage of respondents (82%) was grouped between 20-30. In term of races, most of the respondents in this study were Malay (48%). 26% of the respondents were Chinese and 22% of them were Indian. Only 4% of the respondents were of other races.

Table 4.11: Gender group of respondents.

Gender	Frequency	Percentage (%)
Female	33	66.00
Male	17	34.00
Total	50	100

Table 4.12: Age group of respondents.

Age Group	Frequency	Percentage (%)
20-30	41	82.00
31-40	6	12.00
41-50	2	4.00
51-60	1	2.00
Total	50	100

Table 4.13: Races of respondents.

Gender	Frequency	Percentage (%)
Malay	24	48
Chinese	13	26
Indian	11	22
Others	2	4
Total	50	100

4.7.1 Sensory Evaluation

At the present study, consumers' acceptance testing for sensory attributes was conducted to determine the degree of liking of respondents on certain attributes of the optimized cream. Particularly, the attributes included colour, aroma, texture/appearance, spreadability, stickiness, greasiness, ease of removal and overall acceptance. While 9-point hedonic scale is widely used in performing sensory evaluation, 5-points hedonic scale was used at the present study considering that respondents might not be proficient in quantifying the intensity of attributes due to lack of training.

Visual appearance, in this study, colour was first evaluated followed by aroma and texture as these are some of the primary senses involved when the respondents first came into contact with the formulated cream sample. The colour attribute was rated as "5" when the colour of the cream sample was presentable and satisfactory to the respondents at the first sight, otherwise rated from "4" or below. Next, aroma attribute referred to the smell of the formulated cream sample. It was given "5" for pleasant smell or "1" for disagreeable smell. Next, the term "texture/appearance" was evaluated and rated by visual inspection and by touch. In this case, the score of "5" denoted a smooth and homogenized texture which meets their satisfaction while rating below "3" accounted for less desirable texture/appearance.

Subsequently, the spreadability and immediate skin feel including stickiness and greasiness were evaluated when the respondents tested the cream by applying a small amount of cream sample at the back of the hand. The attribute "spreadability" was rated "5" for good spreadability or "1" for poor spreadability trait. On top of that, the immediate skin feel was rated "5" for not being greasy nor sticky after the application of cream and greatly preferable. Rating below "3" denoted as sticky and greasy traits which was less preferable. In the following attribute, the score of "5" was given when the respondents can easily remove the cream applied at the back of hand by using tap water, or otherwise rated from "4" or below. Nonetheless, the overall acceptability was used to determine the overall satisfaction, preference and acceptability of the respondents toward the herbal cream formulated.

Table 4.14 reported the mean score with respect to each sensory attributed. The formulated cream received relatively high mean score of which above 4 out of 5 for nearly

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all the attributes including colour (4.40 ± 0.67), texture/ appearance (4.41 ± 0.57), spreadability (4.58 ± 0.61), stickiness (4.62 ± 0.60), greasiness (4.58 ± 0.64), ease of removal (4.78 ± 0.47). This resulted signified that even though texture profile analysis showed lower spreadability and greater stickiness of optimized cream than commercialized cream, the respondents were contented with the spreadability and stickiness of the optimized cream. In brief, the respondents find agreeable the sensory attributes with respect colour, texture/appearance, spreadability, immediate skin feel and ease of removal.

Of all attributes evaluated, the aroma of the cream received the lowest mean score of 2.94 ± 0.74 out of 5. This can be justified such that most of the respondents not satisfied with the aroma of cream which was given by the extract of *P. sarmentosum*. Taking account of feedbacks provided by the respondents, the aroma of the cream should be improved or added with natural fragrance to make it more presentable. In term of overall acceptance, the formulated cream received mean score of 4.22 ± 0.51. This demonstrated that the herbal cream formulated was acceptable and had a rather high degree of preference among the respondents.

Table 4.14: Consumer acceptance testing based on sensory attribute.

Sensory Attribute	Mean Value
Colour	4.40 ± 0.67
Aroma	2.94 ± 0.74
Texture/Appearance	4.41 ± 0.57
Spreadability	4.58 ± 0.61
Stickiness	4.62 ± 0.60
Greasiness	4.58 ± 0.64

Ease of Removal	4.78 ± 0.47
Overall Acceptance	4.22 ± 0.51

The value was mean ± standard deviation. N= 50. Evaluation based on 5-point hedonic scale (1= Dislike very much; 2= Moderately dislike; 3= Neither like nor dislike; 4= Moderately like; 5= Like very much).

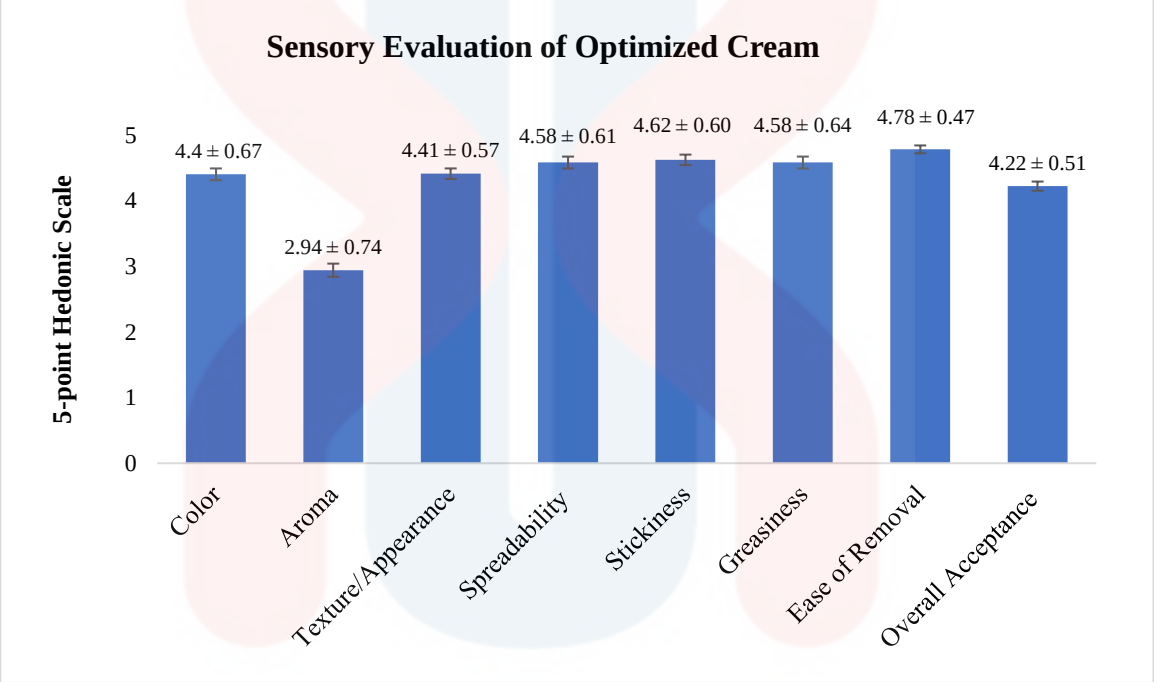

Figure 4.5: Sensory evaluation of formulated cream.

Reliability test on sensory evaluation of formulated cream were detailed in Appendix. At the present study, the Cronbach's alpha reliability coefficient surpass the minimal standard requirement ($\alpha = 0.60$) that is needed to determine whether there are internal consistencies within the studied components (George & Marley, 2003). The value obtained where $\alpha = 0.780$ suggested the Likert scale devised components (N=8) has acceptable internal consistency and fit for this study.

4.7.2 Sample testing on Skin Irritation

At the end of sensory evaluation, skin irritation test was conducted on the same respondents to evaluate the safety of the cream. It was revealed that out of 50 respondents, only 1 respondent experienced very slight erythema (redness) which is barely perceptible while another respondent experienced slight itching feeling. No sign of edema (swelling) was observed in any of the respondents. The result showed that the optimized formulation was primarily non-irritant, representative by average PII of 0.01 (Table 4.15). This may be justified such that the pH of the formulated cream (6.0) was nearly neutral and closed to the pH of the normal skin. On top of that, the cream was formulated using natural ingredients and minimal natural preservative that are less likely to cause irritation or allergic reaction.

Table 4.15: Summary of primary irritation index and classification.

Scoring for skin reaction	Frequency		PII	Average PII (N=50)	Irritation Classification
	Erythema (redness) formation	Edema (swelling) formation			
0	49	50	0.50	0.01	Non-irritant
1	1	0			
2	0	0			
3	0	0			
4	0	0			
Total	50	50			

Evaluation based on Primary Irritation Index (PII) (See also Table 2.3).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

To summarized, the optimized topical herbal cream exhibited good organoleptic and physicochemical characteristics except for the natural aroma of the extract. The optimized cream was also found to exhibited good stability in term of texture (hardness and adhesiveness), pH and odour with no sign of phase separation at both at 5°C and 25°C. On top of that the optimized cream also gained high degree of preference and acceptance among the respondents and the cream was found to be non-irritating. However, improvements are need to enhance the natural aroma of the cream with the incorporation of natural fragrance and to limit the total microbial count of the optimized cream. Natural antioxidant compound may also be added to improve the colour stability of the cream. Hence, the topical herbal cream incorporated with *P. sarmentosum* aqueous extract has the potential to be developed into natural product for wound management with the extract which has been proven to heal wound. Proper botanical identification and documentation of scientific research played a vital role for future development of herbal based product.

5.2 Recommendation

Particularly, this research was emphasizing on the formulation and quality assessments of a natural topical herbal cream with the incorporation of aqueous extract *P. sarmentosum* leaves for potential wound management. However, the pure compounds contribute to wound healing activity has not been studied. In future, determination of the active compounds of the *P. sarmentosum* extract could be carried by spectroscopy technique such as HPLC/MS or LCMS which is essential to correlated to its biological activity and activity on wound healing. The herbal cream formulated should also be tested on laboratory animal to evaluate the in-vivo efficacy of wound healing to better justify its potential for the development of natural wound healing cream.

Besides, it was evident that the herbal cream formulated did not exhibit antibacterial activity against *E. coli and S. aureus*. Further research may be performed to justify the antibacterial property of the aqueous extract alone and possibly the optimum concentration for significant antibacterial activity. Alternatively, further studies on the different combination of various preservative and essential oil with antibacterial property are necessary, particularly to characterize the interaction concerning the synergetic or antagonistic effect when incorporated to the formulation. This is important to enhance the antibacterial activity of herbal cream and overcome contamination problem which has direct impact on the safe use of product and its quality before reaching to consumers. On top of that, the stability evaluation of was limited to one month testing period which might be insufficient to provide a strong evidence for the overall stability of herbal cream developed. Hence, a longer testing period of minimum 3 months should be conducted in

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order to predict or determine the shelf life of the product in real time. Conceivably, the result and the quality of herbal cream formulated may be further enhanced by using homogenizer for a tighter distribution and better consistency of emulsion.

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APPENDICES

A Authentication Certificate of Plant Species

UNIVERSITI KEBANGSAAN MALAYSIA
The National University of Malaysia

Pusat Pengajian Sains Sekitaran dan Sumber Alam *School of Environmental And Natural Resource Sciences*

Date : 5 April 2018
Reference : UKMB 17/2018

To,

Noramalina Abdullah
Kg. Pauh Janit, Beris Kubur Besar,
16050 Bachok, Kelantan,
E-mail: Amalinaabdullah71@gmail.com
Contact no.: 0111-7029280

Mr/Mrs.

CONFIRMATION OF PLANT SPECIES

With all respect the above matter is referred.

Please be informed that the plant specimens submitted for identification as below:

Scientific Name	Family	Common name	Voucher Number
Piper sarmentosum	Piperaceae	Kaduk	UKMB40387
Cinnamomum iners	Lauraceae	Medang Teja	UKMB40388

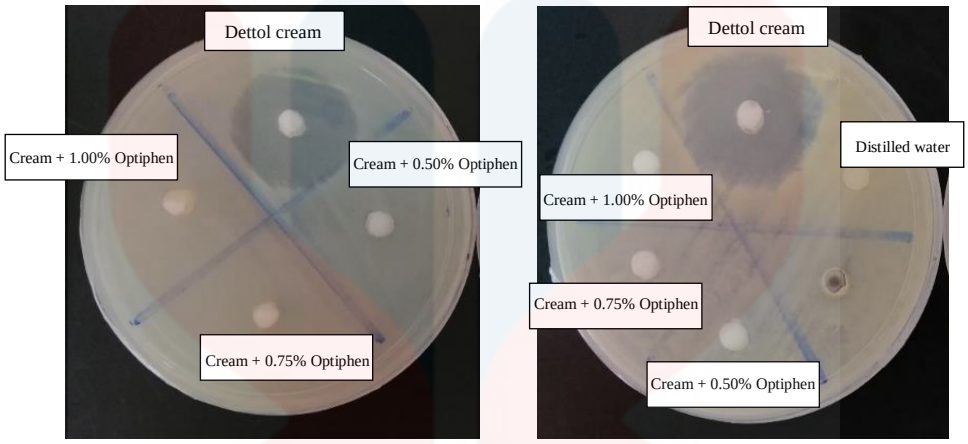
It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you.

Sincerely,

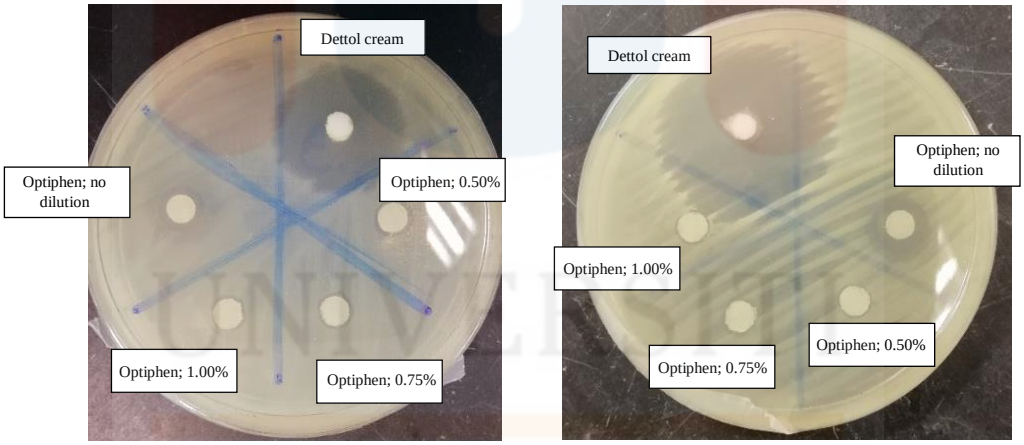
.....
Botanist
Universiti Kebangsaan Malaysia's Herbarium
School of Environmental and Natural Resource Sciences
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
Bangi, 43600, Selangor
Office : +603-89213970
Email : herbariumukm@gmail.com
Web: www.ukm.my/ukmb

B Antimicrobial Assay of cream formulated and Optiphen



(a) (b)

Antibacterial activity of formulated cream against (a) *E. coli* and (b) *S. aureus*.



(a) (b)

Paper Disc Diffusion Assay of different concentrations of Optiphen against:

(a) *E. coli* and (b) *S. aureus*.

C Evaluation Form for Sample Testing

PART 1: SENSORY EVALUATION

SENSORY EVALUATION FORM OF FORMULATED CREAM SAMPLE

Age : _____ years Date:
Race : Malay / Chinese / Indian / Others Sample Code:
Gender: Male / Female

Directions:
You are given some samples for test of attributes. Please state your degree of likeness based on the characteristics below at the mark ( ) provided. Circle which is appropriate.

1. Colour

1 2 3 4 5
Dislike very much Neither like nor dislike Like The Best

2. Aroma

1 2 3 4 5
Dislike very much Neither like nor dislike Like The Best

3. Texture/Appearance

1 2 3 4 5
Dislike very much Neither like nor dislike Like The Best

4. Spreadability

1 2 3 4 5
Dislike very much Neither like nor dislike Like The Best

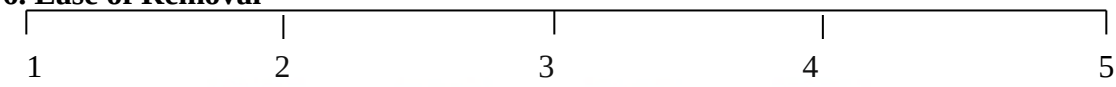
5. Immediate After Feel - Stickiness

1 2 3 4 5
Dislike very much Neither like nor dislike Like The Best

6. Immediate After Feel - Greasiness

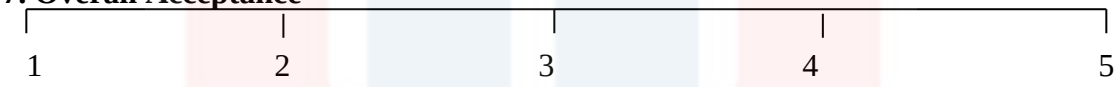
1 2 3 4 5
Dislike very much Neither like nor dislike Like The Best

6. Ease of Removal



1	2	3	4	5
Dislike very much		Neither like nor dislike		Like The Best

7. Overall Acceptance



1	2	3	4	5
Dislike very much		Neither like nor dislike		Like The Best

PART 2: SKIN IRRITATION TEST

Directions:
You are given some sample for test of skin irritation. An area (1sq.cm) on the hand dorsal surface or forearm will be mark and apply for the cream for 30 minutes. Please state the degree of irritation based on the description below. Tick which is appropriate at the column provided.

Skin reaction	Score	Degree of reaction	(/)
Erythema (redness) formation	0	No erythema.	
	1	Very slight erythema (barely perceptible).	
	2	Well-defined erythema.	
	3	Moderate to severe erythema.	
	4	Severe erythema (beet redness) to slight eschar formation (injuries in depth).	
Edema (swelling) formation	0	No edema.	
	1	Very slight edema (barely perceptible).	
	2	Slight edema (edges of area well-defined by definite raising).	
	3	Moderate edema (raised approx. 1 mm).	
	4	Severe edema (raised more than 1 mm and extending beyond area of exposure).	

Precaution: Please consult a doctor if skin irritation persists or any abnormal symptoms occur.

We greatly appreciate any feedback. Please leave your comment here.

_____

_____

THANK YOU

D Two-way ANOVA

Tests of Between-Subjects Effects

Dependent Variable: Hardness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17.367[a]	9	1.930	1.158	.371
Intercept	21708.300	1	21708.300	13024.980	.000
Day	15.533	4	3.883	2.330	.091
Temperature	.300	1	.300	.180	.676
Day * Temperature	1.533	4	.383	.230	.918
Error	33.333	20	1.667		
Total	21759.000	30			
Corrected Total	50.700	29			

a. R Squared = .343 (Adjusted R Squared = .047)

Dependent Variable: Adhesiveness

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.010[a]	9	.001	.107	.999
Intercept	24.120	1	24.120	2412.033	.000
Day	.005	4	.001	.117	.975
Temperature	.000	1	.000	.033	.857
Day * Temperature	.005	4	.001	.117	.975
Error	.200	20	.010		
Total	24.330	30			
Corrected Total	.210	29			

a. R Squared = .046 (Adjusted R Squared = -.383)

Dependent Variable: L

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	32.939[a]	9	3.660	47.008	.000
Intercept	140236.442	1	140236.442	1801213.022	.000
Day	13.794	4	3.449	44.293	.000
Temperature	14.925	1	14.925	191.697	.000
Day * Temperature	4.220	4	1.055	13.549	.000
Error	1.557	20	.078		
Total	140270.938	30			
Corrected Total	34.496	29			

a. R Squared = .955 (Adjusted R Squared = .935)

Dependent Variable: b*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	13.374[a]	9	1.486	302.026	.000
Intercept	6925.729	1	6925.729	1407668.512	.000
Day	6.423	4	1.606	326.392	.000
Temperature	5.076	1	5.076	1031.678	.000
Day * Temperature	1.874	4	.469	95.248	.000
Error	.098	20	.005		
Total	6939.201	30			
Corrected Total	13.472	29			

a. R Squared = .993 (Adjusted R Squared = .989)

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Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.068[a]	9	.008	1.139	.382
Intercept	1050.208	1	1050.208	157531.250	.000
Day	.053	4	.013	2.000	.133
Temperature	.003	1	.003	.450	.510
Day * Temperature	.012	4	.003	.450	.771
Error	.133	20	.007		
Total	1050.410	30			
Corrected Total	.202	29			

a. R Squared = .339 (Adjusted R Squared = .041)

E Post Hoc Test

Storage temperature at 25°C

L*

Duncan[a]

Day	N	Subset for alpha = 0.05		
		1	2	3
1	3	67.2367		
7	3		68.7400	
14	3			69.7033
28	3			69.8300
21	3			69.8700
Sig.		1.000	1.000	.476

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

b*

Duncan[a]

Day	N	Subset for alpha = 0.05			
		1	2	3	4
28	3	13.6767			
21	3		14.4167		

14	3			15.0000	
7	3			15.0767	
1	3				15.7433
Sig.		1.000	1.000	.105	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Storage temperature at 25°C

L

Duncan[a]

Day	N	Subset for alpha = 0.05	
		1	2
7	3	67.2200	
1	3	67.2367	
14	3	67.7100	
21	3	67.7733	
28	3		68.3867
Sig.		.057	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

b

Duncan[a]

Day	N	Subset for alpha = 0.05	
		1	2
28	3	15.0767	
21	3		15.7033
14	3		15.7267
1	3		15.7433
7	3		15.7767
Sig.		1.000	.342

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

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