# DEVELOPMENT OF ORAL WOUND HEALING FORMULATION FROM *Ocimum tenuiflorum* EXTRACT

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**UNIVERSITI SAINS MALAYSIA**

**2020**

# DEVELOPMENT OF ORAL WOUND HEALING FORMULATION FROM *Ocimum tenuiflorum* EXTRACT

**By**

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**Thesis submitted in fulfilment of the requirements**
**for the degree of**
**Doctor of Philosophy**

**May 2020**

## ACKNOWLEDGEMENT

First of all, I thank the Almighty God for giving me strength and blessings throughout the process of completing my experiment and thesis. I acknowledge my gratitude to my supervisors, Dr Rabeta Mohd Salleh and my co-supervisors Dr Uthumporn Utra @ Sapina Abdullah and Associate Professor Dr Amin Malik Shah. I have gained a lot of new experiences, knowledge and skills with their supervision and guidance.

Furthermore, I would love to convey my sincere gratitude to my mother Saroja, father Jayaraman, sisters Munissha and Dakshynee, my husband Tanes, all family members and my friends who continuously gave moral supports and prayers. They encouraged me to keep calm and patient in finishing this project.

I would like also to thank my well-wishers Muhammad Ezzudin, Mansoureh, Jeyanthy, Krissilla, Valarmathi and Kasthuri for their continuous supports and prayers in all my ups and downs throughout my PhD journey. Not forgetting all my FTD PG friends who have spent their valuable time for teaching and guiding me to complete my research.

Finally, I would like to extend my thankfulness to Research University (RU) fund and Graduate assistant (GA) scheme for providing me financial aids in fulfilling my study. Thanking everyone who contributed support and help directly and indirectly.

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# LIST OF SYMBOLS

| | |
|---|---|
| α | alpha |
| ~ | approximate |
| β | beta |
| °C | degree Celsius |
| γ | gamma |
| λ | lambda |
| < | less than |
| - | negative |
| % | percentage |
| ± | plus minus |
| : | ratio |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| API | Apigenin |
| BW | body weight |
| cm | centimeter |
| CI | Carr index |
| DSC | Differential scanning calorimetry |
| DMSO | dimethyl sulfoxide |
| FT-IR | Fourier-transform infrared |
| g | gram |
| h | hour |
| HPLC | High performance liquid chromatography |
| HR | Hausner ratio |
| kg | kilogram |
| kHz | kilohertz |
| kV | kilovolt |
| LCMS | Liquid chromatography mass spectroscopy |
| LOD | limit of detection |
| LOQ | limit of quantification |
| µg | microgram |
| µm | micrometer |
| mg | milligram |
| ml | milliliter |
| $mm^2$ | millimeter square |

| | |
|---|---|
| mV.s | millivolt per second |
| min | minute |
| nm | nanometer |
| RH | relative humidity |
| RP | reversed phase |
| rpm | rotation per minute |
| RSD | relative standard deviation |
| RT | room temperature |
| SD | Sprague dawley |
| s.d | standard deviation |
| SEM | Scanning electron microscope |
| SEM | Standard error mean |
| TEM | Transmission electron microscope |
| UV | ultraviolet |

**PEMBANGUNAN FORMULASI ORAL PENYEMBUHAN LUKA DARIPADA EKSTRAK *Ocimum tenuiflorum***

## ABSTRAK

Penyelidikan ini bertujuan untuk menghasilkan formulasi penyembuhan luka untuk meningkatkan keberkesanan ekstrak tumbuhan *Ocimum tenuiflorum*. Kajian ini terdiri daripada 4 fasa analisis yang mana pada fasa 1, ekstrak air tumbuhan dinilai untuk potensi penyembuhan luka menggunakan tiga bahagian yang berbeza (daun, batang, dan bunga) pada ujian penyembuhan luka *in vitro*. Keputusan menunjukkan bahawa ekstrak daun mempunyai potensi penyembuhan luka dengan signifikannya ($p<0.05$) meningkatkan kesan sel migrasi dan angiogenesis yang merupakan faktor penting dalam proses penyembuhan luka. Dalam fasa 2, ekstrak diformulasikan dengan menggunakan jenis surfaktan yang berbeza (betasiklodekstrin, hidroksil-betasiklodekstrin dan lesitin soya) pada nisbah 1:1 dan 1:2 dengan kaedah penyejatan larutan. Sebelum itu, ekstrak dianalisis secara kualitatif untuk mengenalpasti kandungan sebatian dan apigenin dipilih sebagai kompaun penanda untuk menganalisis kandungan kompaun. Kandungan kompaun dalam formulasi yang disediakan diukur menggunakan peralatan HPLC dan keputusannya menunjukkan kompleks lesitin pada nisbah 1:1 mempunyai kandungan kompaun penanda API yang lebih tinggi ($p<0.05$) berbanding dengan ekstrak daun dan jenis formulasi lain. Dalam fasa 3, formulasi berasaskan lesitin diuji untuk ketoksikan oral akut, keberkesanan penyembuhan luka dan kestabilan penyimpanan diuji pada suhu dan bentuk yang berbeza (kering dan cair). Hasil ujian ketoksikan oral akut menunjukkan tiada tanda-tanda ketoksikan, seperti yang ditunjukkan oleh kajian histopatologi. Sementara itu,

perbandingan kajian penyembuhan luka yang dijalankan di antara keempat-empat kumpulan mencadangkan bahawa aktiviti penyembuhan luka secara signifikannya ($p < 0.05$) lebih cepat di dalam kumpulan formulasi berbanding dengan kumpulan ekstrak daun dan kawalan negatif. Aktiviti penyembuhan luka yang ditambah baik terus disokong dengan kajian pelepasan kompaun penanda yang berterusan sehingga 12 jam yang dapat diperhatikan di dalam formulasi berbanding dengan ekstrak daun. Selain itu, formulasi yang disediakan juga menunjukkan peningkatan kestabilan kompaun penanda sehingga 8 minggu pada suhu 4 °C dalam bentuk kering dan juga dibandingkan dengan formulasi dalam bentuk cair dan kedua-duanya (kering dan cair) untuk ekstrak. Dalam fasa 4, teknik semburan kering digunakan untuk menghasilkan serbuk formulasi kering dan sifat fizikokimianya dicirikan. Hasil analisis fizikokimia menunjukkan bahawa formulasi serbuk semburan kering menghasilkan serbuk dengan kebolehterimaan kandungan lembapan, aktiviti air, ketumpatan pukal dan diketuk. Walau bagaimanapun, kekurangan serbuk formulasi ialah mempunyai ciri-ciri pertengahan kemampuan mengalir, kesepaduan dan ciri higroskopi yang lemah yang membolehkan penyerapan kelembapan serbuk-serbuk partikel. Oleh itu, menjadikan serbuk tersebut melekit dan bergumpal yang boleh dilihat di dalam mikrograf SEM. Selain itu, formulasi yang telah disediakannya turut dikaji untuk sifat DSC dan FT-IR. Dalam keputusan itu, menunjukkan bahawa formulasi yang disediakan mempamerkan berlakunya reaksi kimia apabila ekstrak tumbuhan dan lesitin dicampurkan di dalam penyediannya dengan kemunculan puncak termogram yang baru untuk dalam ujian DSC sementara peralihan dan kehilangan spektrum inframerah dalam ujian FT-IR. Akhirnya, formulasi semburan kering telah menghasilkan serbuk yang mempunyai saiz partikel <100 nm diameternya dan sebarannya yang stabil dengan nilai potensi zeta -28.3 mV dan juga dapat dilihat dalam mikrograf TEM sama ada partikel-partikel

tersebut berada di dalam bentuk yang diskret dan individu. Kesimpulannya, didapati bahawa ekstrak daun berkesan dalam penyembuhan luka dan kestabilannya bertambah apabila diformulasikan dengan lesitin pada nisbah 1:1.

**DEVELOPMENT OF POTENTIAL WOUND HEALING FORMULATION FROM *Ocimum tenuiflorum* EXTRACT**

## ABSTRACT

The research aims to develop a wound healing formulation to improve the efficacy of the plant *Ocimum tenuiflorum* extract. The study consists of 4 phases of analysis whereby in phase 1, the plant was evaluated for wound healing potential using three different parts (leaves, stems, and flowers) of aqueous extract on *in vitro* wound healing assays. The results showed that leaves extract has a potential wound healing effect by significantly ($p<0.05$) increasing cell migration and angiogenesis effect, which are important factors in the wound healing process. In phase 2, the extract was formulated with different types of surfactants (betacyclodextrin, hydroxyl-betacyclodextrin and soy lecithin) at ratio 1:1 and 1:2 by solvent evaporating method. Prior to that, the extract was qualitatively analysed for the presences of its phytocompounds and apigenin was selected as target marker compound to analyze the drug content. The drug content of the prepared formulations was quantified using HPLC equipment and the result showed that lecithin complex at ratio 1:1 has significantly higher amount of API ($p<0.05$) compared with leaves extract and other types of formulation. In phase 3, the lecithin based formulation was tested for its acute oral toxicity, wound healing efficacy and its storage stability which was evaluated at different temperatures and form (dry and liquid). The result of the acute oral toxicity test showed no sign of toxicity, as corroborated by a histopathological study. Meanwhile, the comparative wound healing study conducted between four groups suggested that wound healing activity was significantly ($p<0.05$) faster in formulation

group compared with leaves extract and negative control groups. The improved wound healing activity further supported with drug release study done where a sustained releases of marker compound up to 12 h was observed in formulation compared to leaves extract. Besides that, the formulation prepared also showed improved the stability of the maker compound studied in dry form up to 8 weeks at 4 °C when compared to liquid form formulation and both (dry and liquid) forms of extract. In phase 4, spray drying techniques were used to produce spray dried formulation and its physicochemical properties were characterized. The physicochemical analysis results suggest that spray dried powder formulation produced acceptable value for powder's moisture content, water activity, bulk and tapped densities. However, the drawbacks for the spray dried powder were the intermediate properties of flowability, cohesiveness and hygroscopicity which allow the moisture absorption of the powder particles. Hence, allowed the powder to be sticky and agglomerates which can be seen in SEM micrograph. Moreover, the prepared formulation was further analysed for it DSC and FT-IR properties. In that results, it showed that the prepared formulation exhibited some chemical reaction when mixture of extract and lecithin was used in preparation with the appearance of new thermogram peak for formulation in DSC while shifting and disappearance of infrared spectra in FT-IR. Finally, the spray dried powder formulation produced particles with size <100 nm in diameter and stable dispersion with zeta potential value of −28.3 mV and it can also be seen in TEM micrograph whereby the particles were in discrete and individual manner. In conclusion, it was found that the leaves extract wound healing efficacy and its stability were improved when formulated with lecithin at 1:1 ratio.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Wound is often termed as the break or injury of the skin and its layers. This is a condition resulted from external sources like physical, thermal or medical (Boateng et al. 2008). Annually, around 280 million wounds occur everywhere in the world which are either due to medical or accidental causes (Fletes-Vargas et al. 2016). Wound can be broadly divided into two categories which are the acute and chronic wounds (Whitney 2005).

In acute wound, the healing process is not affected and complete healing occurs while in chronic wound the process get disrupted at any stages and the wound fail to heal at appropriate time (Halim et al. 2012). Chronic wounds are usually resulted from non-healing acute wounds like surgery, trauma or insects bite which happened due to some biological factors (Han & Ceilley 2017). Chronic wounds also include pressure ulcers, diabetic foot ulcers and venous or arterial leg ulcers (Frykberg & Banks 2015).

Since the incidence of diabetes is increasing in Malaysia, the prevalence of limb amputation also increasing simultaneously (Arifin et al. 2017). Wound healing is the process which responsible to repair and restore the function of the skin and injured tissues or cells. This is an continuous overlapping process consist of four levels which are the bleeding, inflammation, proliferation and remodeling (Pazyar et al. 2014).

Herbalism defines as the usage of different types of medicinal plants which are the backbone of traditional medicines (Singh 2015). Taid et al. (2014) stated that, human beings have been dependent on herbal medicines since ancient time to treat various illness. In developing countries, about 80% of people are completely dependent of herbal medicine as their main healthcare sources (Chen et al. 2016). Recently, medicinal plants have attracted the researchers to work on it because of their broad spectrum of medicinal properties (Morales et al. 2017). Consequently, it helps the inventions and development of novel medicines due to their distinct qualities as huge sources for therapeutics applications (Azwanida 2015).

*Ocimum tenuiflorum* or called as “ruku” in Malay is one of the potential medicinal plant that have been used traditionally (Rabeta & Lai 2013). This aromatic herbal plant is widely distributed throughout tropical and Eastern regions of the world (Subramanian et al. 2014). Almost all the parts (leaves, stem, flowers, root, seeds) of the plants have been used traditionally for various illness (Pattanayak et al. 2010). Numbers of scientific studies have been conducted to evaluate the medicinal properties of the plant like antibacterial (Joshi et al. 2011), antidiabetic (Mousavi et al. 2016), antioxidants (Rabeta & Lai 2013), anticancer (Lam et al. 2018), hepatoprotectivity (Lahon & Das 2011), anti-inflammatory (Kumar et al. 2015), wound healing (Francis et al. 2017) and many more researches have been conducted to evaluate the medicinal properties of the plants.

## 1.2 Problem statement

Wound healing is a challenging task for many surgeons in the world (Vijay 2017). There is a must to have more ways to manage the chronic complex wounds comprehensively with minimal or no complications (Harikrishna 2017). Statistic in United States reported that about 6.5 million of patients are suffering with non-healing chronic wounds (Sen et al. 2009). Moreover, it has been reported that lower limb amputation happened every 30 s around the world in chronic wound cases (Järbrink et al. 2017). Although the wound care system has evolved extensively, it still shows the failure of current treatment method (Gwak & Sohn 2017).

Hence, effective wound care from plant-based could be an alternative for those who don’t prefer snakehead fish and sea cucumber, popular wound healing preparations. Besides, the religious consideration and vegetarian lifestyle choices may prohibit certain consumer groups from taking animal-based wound healing products (Boyer 2013). So, for these reasons medicinal plant can be the substitutes for the treatment. Application of crude extract will be beneficial because it gives synergistic effect due to mixture of compounds (Soonthornsit et al. 2017).

Literature study shows that *Ocimum tenuiflorum* has been widely tested for wound healing properties, but study on formulating the extract is still lacking. Researchers have proved that, formulating the herbal extract could improve its therapeutics properties than its original state (Armendáriz-Barragán et al. 2016). Besides, literature survey also suggests that, most wound healing product or studies was topical application (onto the skin) while lack of study on attempting to prepare an oral based product whereby oral route of administration was the post preferable and easy (Hu et al. 2012).

Hence, this project was initiated to develop an oral based wound healing formulation from *Ocimum tenuiflorum* plant using the most widely used surfactants which are the cyclodextrin and phospholipids agent (Semalty 2014). The overall flow of the project was presented as chart in Figure 1.1. The chapters of the thesis will be representing the phases of the project conducted.

## 1.3 Objectives

### 1.3.1 Main objective

- To develop an oral wound healing formulation from *Ocimum tenuiflorum* extract

### 1.3.2 Specific objectives

1. To carry out and select the potential wound healing parts of the plant using preliminary wound healing assays *in vitro* with of different parts (leaves, stems, flowers) of the plant.
2. To develop wound healing formulation with one part of the plant extract using different types of surfactants at different ratio.
3. To evaluate the acute oral toxicity, wound healing efficacy *in vivo*, stability and drug release efficiency of the formulation
4. To study the physicochemical properties of the prepared oral wound healing formulation.

## 1.4 Flow chart of the project

Figure 1.1 display the overall structure of the project flow.

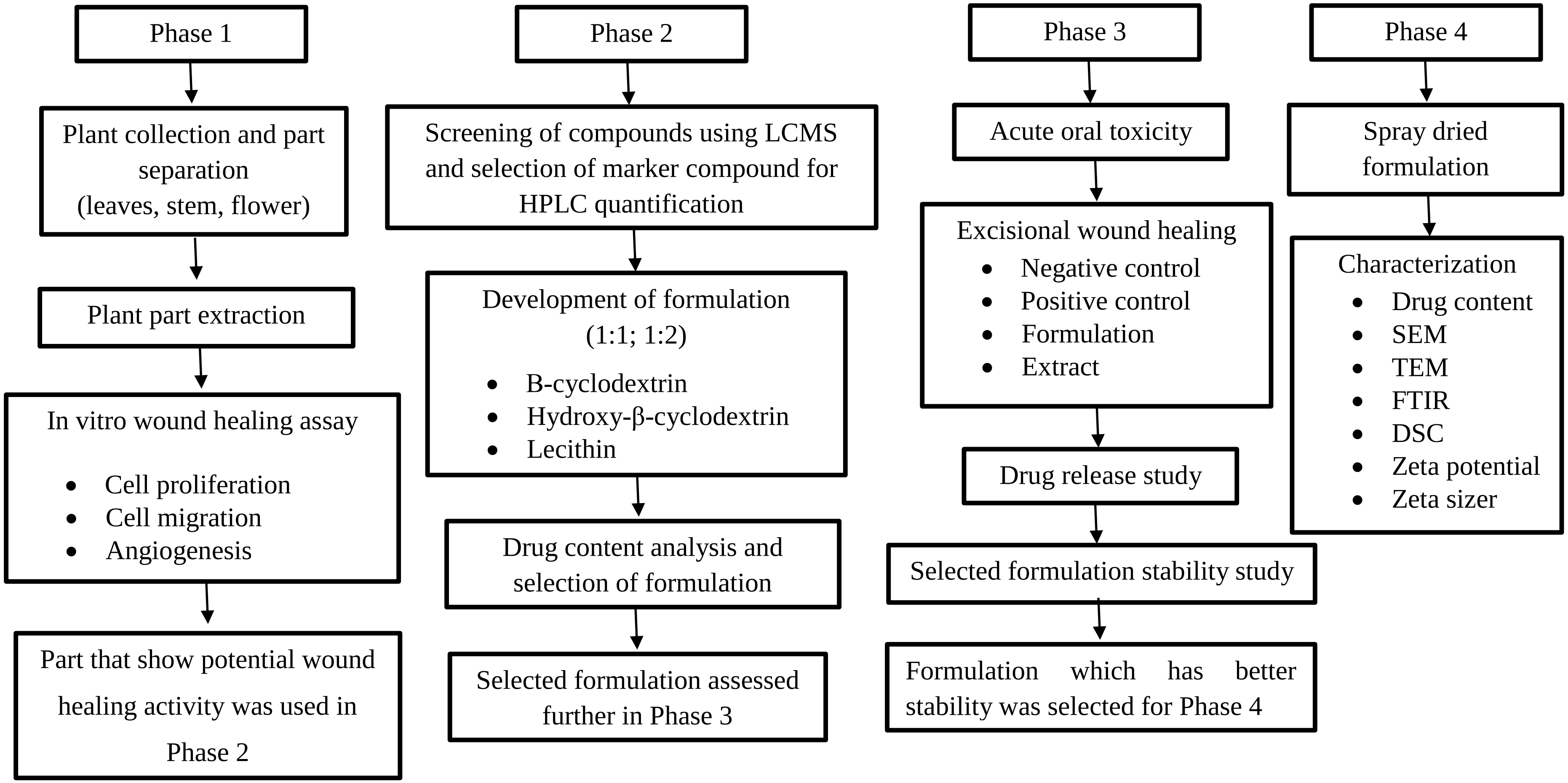


Figure 1.1 Overall project flowchart

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Ocimum tenuiflorum*

Among many medicinal plants, plant from Lamiaceae family and genus *Ocimum* are the most famous for its medicinal properties (Triveni et al. 2013). *O. tenuiflorum* (Figure 2.1) sometimes known as *Ocimum sanctum* is an herbal plant that is available in most South Asian countries (Hannan et al. 2006). This plant commonly called as Holy Basil or Tulsi in most countries (Kalabharathi et al. 2011) while in Malaysia it is called as "ruku" and usually planted as an ornamental plant (Mousavi et al. 2016).

Figure 2. 1 *Ocimum tenuiflorum* plant

### 2.1.1 Scientific classification

*O. tenuiflorum* has been scientifically classified as mentioned below (Palla et al. 2012):

| | |
|---|---|
| Kingdom | : Plantae |
| Division | : Magnoliophyta |
| Class | : Magnoliopsida |
| Order | : Lamiales |
| Family | : Lamiaceae |
| Genus | : *Ocimum* |
| Species | : *O. tenuiflorum* |
| Botanical Name | : *Ocimum tenuiflorum* or *Ocimum sanctum* |

### 2.1.2 Morphological description

*O. tenuiflorum* is an erect, much branched sub-shrub 30–60 cm tall, with simple opposite green or purple leaves that are strongly scented and hairy stems. Leaves have petiole and are ovate, up to 5 cm long, usually somewhat toothed. Flowers are purplish in elongate racemes in close whorls (Pattanayak et al. 2010).

### 2.1.3 Medicinal properties

*O. tenuiflorum* have been widely studied for it medicinal values. According Selvam et al. (2013), the medicinal properties executed by the plant is due to the bioactive phytochemical constituents that produce a potential physiological action. Table 2.1 shows the summary of literature survey on different pharmacological studies done on *O. tenuiflorum* plant.

Table 2.1 Medicinal properties of *O. tenuiflorum* plant

| Therapeutic properties | Parts | Findings |
|---|---|---|
| Anti-inflammatory | Leaves | Inhibiting the matrix metalloproteinases-9 expression on liposaccharide induced inflammatory cells (Manaharan et al. 2014). |
| | | Reduces inflammation in carrageenan-induced oedema in rats (Mirje et al. 2014) |
| Antioxidants | Leaves | Free radical scavenging that reduced cadmium induced oxidative stress in rats (Ramesh & Satakopan 2010). |
| | Leaves, stem | The leaves own better antioxidant activity by scavenging of free radicals than stem (Balaji et al. 2011). |
| Antidiabetic | Leaves | The leaves extract shows potent antidiabetic effect in streptozotocin + nicotinamide induced diabetic in rats (Parasuraman et al. 2015). |
| | | Antidiabetic effect (hypo- and hyperglycaemic) were similar to standard drug metformin when tested on diabetic rats (Mousavi et al. 2016). |

| | | |
|---|---|---|
| Hepatoprotective | Leaves | Protection against liver steatosis by increasing glutathione peroxidase and calatase (Kamyab & Eshraghian 2013). |
| | | Provide synergistic protection to liver when administered simultaneously with silymarin drug than extract alone (Lahon & Das 2011). |
| Antibacterial | Leaves, inflorescence | Essential oils extracted from the parts exhibit antibacterial effect against *S. aureus* and *E. coli* than *P. aeruginosa* species (Yamani et al. 2016). |
| | Leaves | Ethanolic extract shows better antibacterial activity on *P. aeruginosa* strain when compared to other strains like *S. aureus, E.coli, K. pneumonia and* strains (Joshi et al. 2011) |
| Anticancer | Leaves | Promoted anticancer effect against lung cancer cell A549 *in vitro* and *in vivo* (Magesh et al. 2009). |
| | | Anticancer effect was better on MCF-7 than MDA-MB-231, the breast cancer cell lines (Lam et al. 2018). |

| | | |
|---|---|---|
| Cardioprotective | Leaves | The fixed oil extracted protect the heart by inhibiting the fat deposition in blood vessels of rats fed with high fat diet (Suanarunsawat et al. 2010). |
| | | Cardioprotection was exhibited by the extract to the rats under chronic restraint stress via central effect (Sood et al. 2006). |
| Antihyperlipidemia | Leaves | Essential oil extracted able to lower the serum lipid level in rats fed with high cholesterol diet (Suanarunsawat et al. 2009). |
| | | Lipid lowering effect of fresh leaves in male rabbit induced with atherosclerosis (Vasudevan 2007). |
| Wound healing | Leaves | Topical application of the extract possess quicker wound healing effect by tumor necrosis factor (TNF)-alpha elevation (Goel et al. 2010). |
| | | Oral administration of fresh leaves enhances wound healing by reduction in lipid and sugar level in blood (Sharma & Paul 2011). |

### 2.1.4 Phytoconstituents of plant

Ocimum tenuiflorum plant owned the same chemotype although the plant was purple or green. The main constituent of the plant is the eugenol (27-83%) along with methyl eugenol (3– 24%), methyl chavicol (10–15%) and sesquiterpene hydrocarbons (Bariyah 2013). Besides, preliminary phytochemical screening conducted by Umamageswari and Kudagi (2015) on aqueous leaf extract of *O. tenuiflorum* revealed the presence of saponins, triterpenoids, flavonoids, tannins, and eugenol. Different extraction methods yield different composition of phytoconstituents content. However, listed are the major compounds found in most situations such as eugenol and its derivatives volatile fatty acids, mucilage, polysaccharides, ursolic acids, and compounds from flavonoids, alkaloids, tannins and carbohydrates (Kumar et al. 2013a). Among many constituents present in *O. tenuiflorum*, pharmacologically essential active compounds are from polyphenolic phenolic flavonoids such as apigenin, vicenin-1, vicenin-2 caffeic acid and ursolic acid (Bariyah 2013).

## 2.2 Wound care management

Over past decade, the wound management practice has been changed tremendously whereby scientific research have come up with dry healing approaches from moist treatment, multiple and novel wound care substitutes in market and greater suitability of product with patient's conditions (Lait & Smith 1998). In a review article published by Sarabahi (2012), it can be understood that most wound care products were topical based application which means mostly applied externally and typically on skin. Those products ranging from different type of approaches like antiseptics and antimicrobials, foams, debridement, alginates, hydrogels, hydrocolloids, transparent films, growth factors, collagen, negative pressures wound therapy and many others.

Ideally, wound dressings should own several properties for proper wound healing. Those properties are accelerating epithelization and improve cell migration, provide wet environment, stimulate angiogenesis for gas exchange and improve blood flow, non-sticky, compatible with patients, non-toxic, protect from microbial contamination and clear the debris at wound site (Dhivya et al. 2015). With these properties, cell proliferation and migration are important factors in wound healing process because they help in re-epithelization, an attempt of covering wound area and restoring the skin integrity (Landén et al. 2016).

In simple terms, cell proliferation can be defined as production of more number of cells while cell migration is ability of the cells to move forward to close the wound area (Landén et al. 2016). Besides that, another important phenomenon that aids wound healing process is angiogenesis. Angiogenesis is the formation of new blood vessels from the preexisting blood vessels (Honnegowda et al. 2015). Angiogenesis is essential in wound healing process as it transfer nutrients and oxygen to wound site and tissues for repairs (Yeh et al. 2017).

However, not all types of dressings fulfilled all these requirements hence, an alternative approach is essential. This can be done by allowing the formation of scab, the natural wound dressing which provide all needed functions for proper wound healing (Lionelli & Lawrence 2003). In daily life, the tendency of wound to occurs via cuts, burns or abrasion is common (Kuhlmann et al. 2019). As results of this incident, wound healing process takes place in order to maintain the skin integrity and restore the function of the skin (Wang et al. 2018). Wound healing is a complex process which involves multiple group of cells and mediators (Sorg et al. 2017).

### 2.2.1 Wound healing process

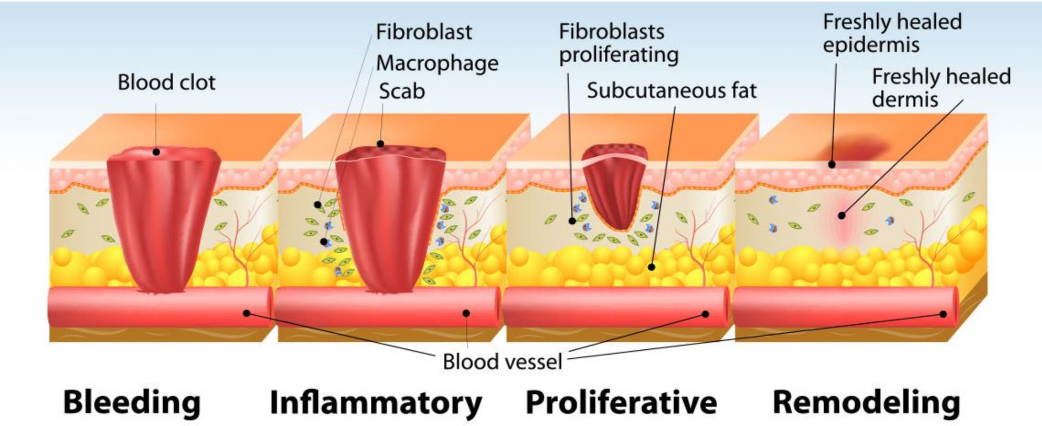


Figure 2.2 Stages of wound healing process

The skin is the largest organ of the body, which accounts about 15% of the total body weight in adult humans. The anatomy of skin consist of layers of the epidermis, dermis and hypodermis (Kanitakis 2002). Wound healing is the repairing process of skin or tissues after the injury (Shetty et al. 2008). A quick wound healing is needed to restore the integrity and prevent severe damage to the body (Goel et al. 2010). The process is divided into four stages (Figure 2.2) which are the haemostasis, inflammation, proliferation and remodeling (Velnar et al. 2009).

Haemostasis or bleeding is the first stage which occurs right after the injuries to the skin. This phase is induced by platelets via fibrin clot formation. During this stage, other mediators were released to attract fibroblasts and macrophages to the injury site (Thiruvoth et al. 2015). Activated platelets are themselves an important source of growth factors such as platelet-derived growth factor, CXCL4, basic fibroblast growth factor, transforming growth factor-β (TGFβ), vascular endothelial growth factor and known to promote various aspects of the repair process through this growth factor release, including angiogenesis, inflammation, and migration of both keratinocytes and fibroblasts (Shaw & Martin 2009).

Next is the inflammatory stage, which was activated once the inflammatory cells move to the wound site. This stage involves the series of neutrophils, macrophages and lymphocytes infiltration (Guo & Dipietro 2010). This response is very quick and coincides with the key signs of inflammation, which are revealed by the edema and the erythema at the location of the lesion. Usually, cell response is established within the first 24 hours and can extend for up to two days. A quick activation of the immune cells in the tissue may also occur, as happens with mastocytes, gamma-delta cells, and Langerhans cells, which secrete chemokines and cytokines. Inflammation is a phenomena of localized and protective tissue response that is released by the lesion, causing tissue destruction (Gonzalez et al. 2016). Inflammatory cells play an important role in wound healing and contribute to the release of lysosomal enzymes and reactive oxygen species, as well as facilitate the clean-up of various cell debris. Inflammation resolved when macrophages clears the death cells and the third stage transition take place (Guo & Dipietro 2010).

The third proliferative stage main objective was to reduce the wound site through contraction and fibroplasia by creating a viable epithelial protection to trigger keratinocytes. The wound closure process includes several other process like formation of blood vessels, fibrous tissues and reepithelization (Gonzalez et al. 2016). Angiogenesis represents an important element in uncompromised wound healing due to its fundamental impact from the very beginning after skin injury until the end of the wound remodeling. This provisional wound microenvironment represents the starting point for new vessel formation and regeneration thereby ensuring the nutritive perfusion of the wound and the delivery of immune cells that remove the cell debris (Sorg et al. 2017).

Re-epithelialization of a wound by keratinocytes is achieved by a combination of migration and proliferation of cells in the surrounding area of the damage. Generally, the advancing cells of the wound epidermis migrate collectively after undergoing a number of changes to facilitate their movement (Shaw & Martin 2009). In the final stages of re-epithelialization, cells differentiate into epithelial cells that remain firmly attached to the basal membrane. Cell-cell and cell-extracellular matrix (ECM) interactions, growth factors, and cytokines released by various cell types stimulate keratinocytes to migrate over the provisional matrix deposited in the clot to cover the wound, while keratinocytes at the wound edges begin to proliferate and follow the migrating front(Cañedo-Dorantes & Cañedo-Ayala 2019). Concurrently, an active paracrine interaction between keratinocytes, fibroblasts, neutrophils, monocytes-macrophages, and endothelial cells increases the amount of cytokines, growth factors, and other biomolecules to promote the epithelial-mesenchymal interaction between keratinocytes and fibroblasts, where keratinocytes stimulate fibroblasts to release growth factors that in turn stimulate keratinocyte proliferation. Lastly, fibroblasts differentiate into myofibroblasts, increasing collagen deposition and initiating wound contraction (Hinz 2016).

The final stage of the process is the remodeling phase occur from day 21 up to 1 year of time followed injury. This phase is also known as maturation phase. In this phase, the granulation tissue stops through apoptosis of the cell and transferred for the scar tissues formation. At the same time, the deposition of collagen by fibroblasts continues until the tensile strength of the skin reached (Sinno & Prakash 2013). A mature wound is, therefore, characterized as avascular as well as acellular. During this period, the wound the components of the ECM undergo certain changes whereby the collagen III which was produced in the previous phase is now substituted by the collagen I which is stronger. Later on the myofibroblasts cause wound contractions by their multiple attachment to collagen and help to decrease the surface of the developing scar (Reinke & Sorg 2012).

### 2.2.2 Wound healing complication

The process of wound healing gets delayed due to several factors. The most common factors are lack of oxygen, infections, age, diseases, drugs, stress, nutrition and few others (Guo & Dipietro 2010). We knew that inflammation take places followed injury, prolonged inflammation condition can eventually affect the wound healing process (Maver et al. 2018). Besides, the release of white blood cells components like neutrophils will also leads to delayed wound healing when it produces enzyme protease and reactive oxygen species which damages the cells even though neutrophils helps the process in the other ways (Guo & Dipietro 2010). As stated by Maver et al. (2018), a proper wound healing treatment should help in overcoming above mentioned factors that delayed the wound healing process in order to provide proper healing therapy. Hence, as per studies and researches done, it has been showed that medicinal plant could be one of the alternative treatments because of their multi properties nature.

### 2.3 Medicinal plants in wound healing

Medicinal plants have been extensively used since decades ago worldwide as an alternative treatment for varieties of diseases. Approximately 500,000 species of plants around the world and only about 1% of it have been phytochemically studied demonstrating a great potential for the discovery of new compounds. Among those plants, some plants have been also explored for its wound healing activity. All that plants have their own mechanism in helping wound healing process via antimicrobial, antioxidant, anti-inflammatory proliferation and angiogenic properties (Georgescu et al. 2016). Stated below are some of the plants that have been tremendously studied and proved to have wound healing properties.

#### 2.3.1 *Aloe vera*

*Aloe vera* is a cactus-like plant from the family of Liliaceae, originated in South Africa. It has been shown to have benefits when used to accelerate wound healing. Almost 75 active ingredients including aloesin, aloe emodin, acemannan, aloeride, methylchromones, flavonoids, saponin, amino acids, vitamins, and minerals are available in aloe vera plant (Georgescu et al. 2016). Glucomannan, which is a mannose-rich polysaccharide, and gibberellin, which is a growth hormone, significantly increases collagen synthesis when the interacts with growth factor receptors on the fibroblast that stimulates its activity and proliferation. Aloe vera also changed the collagen composition and increases the degree of collagen cross-linking, hence accelerate the wound contraction and increase the breaking strength of scar tissue (Surjushe et al. 2008).

### 2.3.2 *Allium sativum*

*Allium sativum* or commonly known as garlic is a member of the Alliaceae family, has been widely recognized as a valuable spice and a popular remedy for numerous diseases and physiological disorders (Londhe 2011). *A. sativum* contains high levels of alliin, allyl cysteine, allyl disulfide, and allicin, which are known as powerful antioxidant and antiinflammatory agents. Hence, by upregulating the intracytoplasmic carbohydrate ratio, shortening the inflammatory stage of the healing process, improving angiogenesis, stimulating the proliferative stage, and finally accelerating collagen synthesis, *A. sativum* shorten the important stages in healing (Farahpour et al. 2017).

### 2.3.3 *Catharanthus roseus*

*Catharanthus roseus* L also known as *Vinca Rosea*, is native to the Caribbean Basin and has historically been used to treat a wide assortment of diseases. This plant belongs to the family of Apocyanaceae. There two major compounds in this plants which are the alkaloids and tannins (Nayak & Pereira 2006). Tannins help in wound healing process through several cellular mechanisms. It acts as antioxidant by chelating of the free radicals and reactive species of oxygen. Help in promoting wound contraction and increasing the formation of capillary vessels and fibroblasts (Singh et al. 2014).

### 2.3.4 *Moringa oleifera*

*Moringa oleifera* Lam. is commonly known as Drumstick tree are from the family Moringaceae is the native in Northwest India, Pakistan, Bangladesh and Afghanistan. However, it is now widely cultivated and has become naturalized in many locations in the tropics now distributed all over the world. Almost all parts of the plants have been used for various ailments (Lambole & Kumar 2012). It was reported that the leaves extract of *M. oleifera* contributes to wound healing process by stimulation of fibroblast proliferation. Besides, it was also identified that the extract contains compounds from the flavonoids group which act as antioxidant that is also helpful in wound healing process (Muhammad et al. 2013).

### 2.3.5 *Curcuma longa*

*Curcuma longa*, generally known as turmeric is an Indian dietary spice from the family of Zingiberaceae. It has been broadly used as traditional remedy for decades in indigenous medicine to treat different types of inflammatory conditions and other diseases (Thangapazham et al. 2016). Among many compounds that available in this, curcumin is the most active compound that has been widely studied for its biological activities. Similarly, curcumin has positive effect in wound healing where its stimulate the growth factors that involved in wound healing process and accelerate the wound healing (Tejada et al. 2016).

## 2.4 Bioactive compounds related to wound healing

Similar to the mechanism of medicinal plants in wound healing, its bioactive compounds also exhibit multiple characteristics in aiding wound healing process. Some compounds display individual effect and some compounds exert synergistic effect in the process. Overall, most of the compounds were from the family of alkaloids, essential oils, tannins, flavonoids, saponins, and others. Listed are the compounds that have been identified, isolated and evaluated for its wound healing activity (Tsala et al. 2013).

### 2.4.1 Quercetin

Quercetin, is the member of the flavonoids group that widely distributed in fruits and vegetables. The natural properties of it is act as an antioxidant and anti-inflammatory that help in protection of many diseases (Yin et al. 2018). Study suggested that quercetin help in wound healing process by increases fibroblast proliferation, decreases immune cell infiltration, and changes in signaling in fibrosis-associated signaling pathways (Jangde et al. 2018).

### 2.4.2 Vicenin

Two common types of vicenin was vicenin-1, a flavonol glycoside (Kandhare et al. 2016) and vicenin-2 di-C-glycosyl flavonoid (Silva et al. 2014) were the water soluble compounds of flavonoid group (Satyamitra et al. 2014). Typically, vicenin-2 was mostly studied for wound healing properties. It has been proven that, the vicenin-2 has potential wound healing properties via the mechanism of cell proliferation and migration (Muhammad et al. 2013).

### 2.4.3 Caffeic acid

Caffeic acid is the active compound extracted from honeybee propolis, belongs to the group of polyphenol. This compound has been well explored for its therapeutic properties and declared to own antioxidant, antimicrobial, anti-inflammatory and many other properties (Tolba et al. 2016). Caffeic acid help in wound healing process via antioxidant and anti-inflammatory activity where its stimulated the fibroblast to produce collagen and inhibit the reactive oxygen species generation (Song et al. 2008).

### 2.4.4 Apigenin

Apigenin is synthesized by numbers of plants is belong to the flavonoids group, which is a large family of polyphenolic compounds. Similar to other flavonoids type compounds, apigenin also have been proposed to have antioxidant, anti-inflammatory, antimicrobial, anticancer and other medicinal properties (Erdogan et al. 2017). It was reported that, apigenin help the epithelization of the wound and possess anti-inflammatory properties which is an essential factor in wound healing process (Manivannan 2016).

### 2.4.5 Rosmarinic acid

Rosmarinic acid is a polyphenol which is an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid. It is commonly found high level in Perilla oil and rosemary herb where it carried its name. It is also found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae (Nadeem et al. 2019). Rosmarinic acid has been reported to display anti-inflammatory and antioxidant characteristic which was the useful tool for the wound healing process. It was stated that, rosmarinic acid show potential wound healing activity as anti-inflammatory via suppression of the inflammatory enzyme cyclooxygenase-2 (COX-2) (Geller et al. 2010).

## 2.5 Chromatography

Chromatography is an essential biophysical technique that allows the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. The principle involve in chromatography is the separation of compounds from the stationary phase (the sample) with the help of mobile phase (solvent used in separation). There are many types of chromatography that available such as column chromatography, gas chromatography, ion-exchange chromatography, thin layer chromatography, gel permeation chromatography high-performance liquid chromatography (HPLC) and many more (Coskun 2016). Among listed chromatography techniques, HPLC is one of the most famous and extensively used chromatography.

HPLC is a specific form of column chromatography that is commonly used in biochemistry and analysis to separate, identify, and quantify the active compounds (Thammana 2016). HPLC compounds separation work by the help of pump and mobile phase that moves the compounds through packed material column (stationary phase) then detector and display the retention time of molecules. Retention time is the time specific compound is eluted and this retention time is completely dependent between the interactions of stationary phase, the molecules being analysed, and the solvent used (Malviya et al. 2010).

Later in research, the HPLC has been coupled with mass spectrometry which is then known as liquid chromatography-mass spectrometry (LC-MS). LC-MS has been one of the helpful technique in compounds identification with the development of electrospray ionization (ESI) that provide a simple and robust interface (Pitt 2009). The LC-MS work in way where first the molecules of interest are introduced into the ionization medium of mass spectrometer where the ionize to attain positive or negative charges. Then, the ions travel through the mass analyzer and reach the detector according to their mass/charge (m/z) ratio. After the ion-detector contact, signals are generated and recorded in computer system. The system then displays the signals graphically as a mass spectrum presenting the relative abundance of the signals according to their m/z ratio (Ho et al. 2003).

**2.6 Limitations with plant extracts**

Medicinal plants have been used extensively because of their multiple therapeutics properties which is due to its complex composition of various compounds (Schmidt et al. 2008). In comparison to the synthetics drugs, medicinal plants show more significant benefits with their properties by producing synergistic effect without any combination compounds (Carmona & Pereira 2013). However, according to a review on plant extracts, it was stated that extraction of plant uses different types of solvents which complicates the extraction and make it difficult for human consumption. Besides that, some plants develop toxic effect in certain circumstances (Armendáriz-Barragán et al. 2016).

Another common issue with extracts is the stability of the compounds which degraded especially during storage and causes the loss of the compounds (Thakur et al. 2011). Stability is the ability of a certain drug or compound to maintain its natural specifications such as purity, quality, efficacy and originality throughout its shelf life (Thakur et al. 2011). Stability assessment is important in providing data on the quality of the herbal plants which is often affected by the environmental causes (Atmakuri & Dathi 2010). The common factors affect the stability are the temperature, light, moisture that leads to oxidation, hydrolysis, degradation, evaporation and microbial contamination (Djordjevic 2017). As stated by Bansal et al. (2016), the stability study should be done by monitoring one or more compound as active or marker quantitatively. According to Food and Drug Association (FDA), this study can be done quantitatively using chromatographic condition which will provide fingerprint chromatogram of the available constituents (Xie et al. 2007).

## 2.7 Method of formulation preparations

Often, the limitations associated with plant extracts were overcome by formulating them based on their requirements. The common techniques used in preparation of formulation supercritical fluid technology (Durante et al. 2014; Girotra et al. 2013), nanotechnology (Rizvi & Saleh 2018; Sharma & Garg 2010), complexation (Kumar et al. 2008; Vikas et al. 2018), solid dispersions (Li et al. 2013; Tayyab Ansari et al. 2018) and others. Among those listed methods, complexation method is the most common and widely used method (Semalty 2014).

### 2.7.1 Cyclodextrins

The complex preparation done using cyclodextrin is a polymer from the oligosaccharide family which is cyclic in nature (Ikuta et al. 2013). It is the derivative obtained from enzymatic conversion of starch with amphiphilic properties with interior lipophilic cavity and exterior hydrophilic surface (Khadka et al. 2014). The chemical structure of cyclodextrin allow the loading of drugs or compounds into its cavity and then form a inclusion complex (Figure 2.2) (Kumar et al. 2013b). Three most common variations of cyclodextrin (Figure 2.3) are the α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin (Lu et al. 2014). Among all cyclodextrin derivatives, β-cyclodextrin is widely used complexation (Yang et al. 2017), while α-cyclodextrin was inappropriate for most compounds because smaller cavity size and γ-cyclodextrin is costly (Sambasevam et al. 2013).