Assessment of Potential Therapeutic Effect of Combined *Thymus serpyllum* and Ascorbic Acid Loaded Hydrogel Against Wounds.

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Thesis submitted in partial fulfillment of the requirement for the degree of Master of Sciences Healthcare Biotechnology

Supervisor: Prof. Dr. Attya Bhatti

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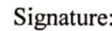
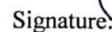
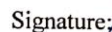
2. Name: **Dr. Sobia Manzoor** Signature: 

3. Name: **Dr. Jahangir Sarwar** Signature: 

Co-Supervisor's name: **Prof. Dr. Peter John** Signature: 

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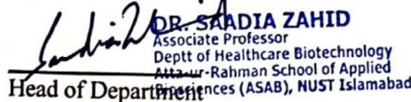

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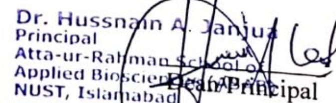

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Dedication

I dedicate my thesis to my beloved Parents

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Abbreviation

bFGF	basic Fibroblast Growth Factors
bM.E	blank Microemulsion
BSA	Bovine Serum Albumin
CEA	Cultured Epithelial Autografts
CoQ10	Coenzyme Q10
CRP	C-Reactive Protein
DFU	Diabetic Foot Ulcer
DPPH	1,1-diphenyl-2-picryl-hydrazyl
ECM	Extracellular Matrix
EGF	Epithelial Growth Factors
ESR	Erythrocyte Sedimentation Rate
FC	Folin-Ciocalteu
GA	Gallic Acid
GAE	Gallic Acid Equivalents
GG	Gellan Gum
GI	Gastrointestinal
HaCaT	Human Keratinocyte
HFD	High Fat Diet
HPMC	Hydroxy Propyl Methyl Cellulose

IC	Inhibition Concentration
IGF	Insulin Growth Factors
IPM	Isopropyl Myristate
KGF	Keratinocyte Growth Factors
M.E	Microemulsion with *T. serpyllum* extract and Ascorbic Acid
ME	Microemulsion
MMPs	Matrix Metalloproteases
N.C	Negative Control
NGF	Nerve Growth Factors
NPWT	Negative Pressure Wound Therapy
O/W	Oil in Water
P.C	Positive Control
PBS	Phosphate Buffer Saline
PDGF	Platelet-Derived Growth Factor
ROS	Reactive Oxidative Species
S.D	Standard Deviation
STZ	Streptozotocin
T2DM	Type 2 Diabetes Mellitus
TFC	Total Flavonoid Content

TPC	Total Phenol Content
VCO	Virgin Coconut Oil
VEGF	Vascular Endothelial Growth Factor
VWF	Von Willebrand Factors
W/O	Water in Oil

Abstract

Skin is the outermost layer covering 15% of the body and shields it against extrinsic factors, when get disrupted formed wound. It always remains a challenge for clinician as skin desecration open a gap for the microbes to invade causing morbidity and mortality. There are different treatment options available and are already in practice but limitations restrict their uses. To overcome the limitations, microemulsion-based hydrogel can be used with *Thymus serpyllum* and ascorbic acid as active ingredient. The microemulsion was formulated by using titration method. 40 formulations of microemulsion were prepared and short listed according to certain criteria, droplet size and zeta potential was measured as well. Qualitative and quantitative phytochemical analysis were performed for *Thymus serpyllum* and bioactive assays including antioxidant, anti-inflammatory and antimicrobial assays were performed for both plant extract and selected microemulsion. Skin irritability test was performed to know the safety of microemulsion for its tropical use. Selected microemulsion was tested for its ability to heal excision and diabetic wounds. All the results were statistically significant $p<0.05$. Microemulsion formed had droplet size 94.95 $\pm$ 7.1 nm and -14.6 $\pm$ 0.9 mV zeta potential. *T. serpyllum* seed extract contains essential phytochemicals The microemulsion showed more bioactivity as compared to plant extract. Skin irritability test was negative for microemulsion as no sign of skin dryness and color change was observed. The difference was significant for wound healing in different groups. Microemulsion showed more healing activity as compared to other groups in both excision and diabetic wounds. Microemulsion healed 100% wound in diabetic wounds and 99% excision wounds within 10 days.

Chapter 1: Introduction

Skin is the outermost layer covering 15% of the body and shields it against extrinsic factors. The skin performs different functions including protection, thermoregulation, sensation, excrete waste products (cellular waste through sweat glands) and synthesis of the vitamin D.

Skin consists mainly of two layers epidermis and dermis, which are further divided as shown in **Figure 1.1**. Epidermis is the outermost layer acting as barrier against the external environment, composed of keratinocytes (protein producing), melanocytes (pigment producing), Langerhans cells (antigen presenting cells) and Merkel cells (touch cells).

Keratinocytes make most of epidermis, the keratinocyte stems cells are present at the base of epidermis where they divide and differentiate, at the corneum they are keratinized and form the outer tough layer of skin where they not only serve as barricade against the environment assault and microbes but also halt the loss of moisture, heat and other important components of body. Besides the structural role keratinocytes also anticipate as immunomodulators, activate Langerhans cells and stimulate inflammation when injury occur but under normal circumstances inhibitory cytokines are released (Barbieri, Wanat, & Seykora, 2014).

Melanocytes are the pigment producing cells present in basal layer smaller in size than keratinocytes and the ratio of melanocyte to keratinocyte is 1:10. This equilibrium is maintained throughout the life span of human (Cichorek, Wachulska, Stasiewicz, & Tymińska, 2013).

Langerhans cells belong to family of dendritic cells inhabits the basal layer of epidermis. They act as antigen presenting cells in response to infection during injury. Although they are capable of vigorous immune response but have tolerance toward the commensal bacteria (Chomiczewska, Budźko, Kaczorowska, & Rotsztejn, 2009).

Merkel cells also known as touch cells as they interact with the nerve ending so have potential to sense the touch, pressure, heat or cold, basically all the senses related to touch (Gilaberte, Torres, Pastushenko, & Juarranz, 2016).

Another layer of skin is dermis which is fibrous structure consists mainly of elastic tissue and collagen. Blood vessels, nerve ending, glands and hair follicles in habit the dermis. Sweat glands help in thermoregulation and contribute to body odor while sebaceous gland aids in protecting skin from dryness. Collagen is the main portion of the dermis play structural role with in dermis along with the elastic tissues. There are different types of cells found in dermis including macrophages, adipocytes, fibroblasts, mast cells, Schwann cells and stem cells (Robert & Carrol, 2007).

Fibroblast is the dominant cell of the dermis and its function is to deal with the formation of the collagen, elastic fibers and material of extracellular matrix. Macrophages assist the immune system while the mast cells are responsible for inflammatory response, collagen remodeling and wound healing. Adipocytes play important role in supplying insulation against drastic temperature change, energy storage, aid in regeneration of hair follicles and wound healing (Brown & Krishnamurthy, 2021). Schwann cells has potential to repair and promote exon regeneration in response to injury to peripheral nervous system (Bray, Chéret, Yosipovitch, & Paus, 2019).

The pH for normal skin 5.5 meaning the skin is slightly acidic in nature. If skin came across something having pH outside of the normal one than there is potential threat to skin and more acidic and alkali can cause serious burn to skin.

Human skin has self-healing property due to presence of these different cells in epidermis and dermis but if the desecration is beyond the certain range and depth these cells get affected and are unable to perform their function then foreign help is required whether its medicine or transplant (Tavakoli & Klar, 2020).

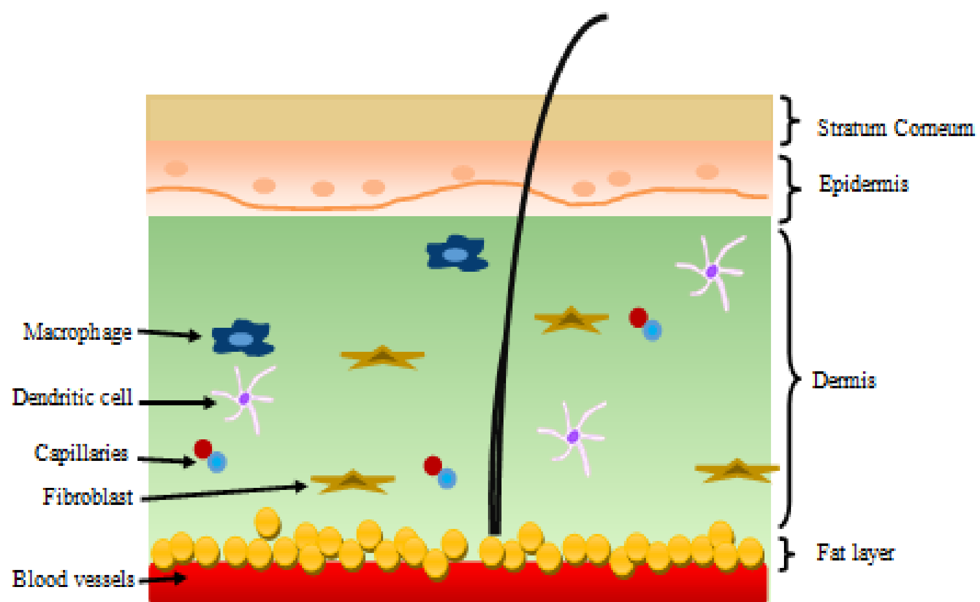


Figure 1.1. Skin Anatomy.

1.1 Wounds

Wound is defined as the disruption of the skin intentionally or unintentionally. It always remains a challenge for clinician as skin desecration open a gap for the microbes to invade causing morbidity and mortality.

1.1.1 Types

Wounds are classified in to different categories depending upon the different factors **Figure 1.2**.

- According to triggering factor injuries are caused by thermal (cold and hot), chemical (acid and alkalis) and mechanical force. The mechanical forces possess wide range to material that cause harm to skin such as the blunt forces which result in abrasions, contusions or laceration while the sharp forces cause either incision or excision wounds. The wounds occur due to firearms fall under this category as well (Chhabra, Chhabra, Kaur, & Gupta, 2017).
- According to exposure the wounds are either closed or open. The closed wounds are defined as the damage to tissues but the skin is not tear or split while open wounds are the one in which not only the tissues are damage but also the skin is cut open exposing the tissues beneath it.
- According to depth, wounds are divided into two types partial thickness and full thickness. In partial thickness wounds, epidermis is usually damaged sometimes partially harming dermis. But in the full thickness wounds the desecration is deep enough to expose the adipose tissue, tendons or even bones henceforth for such injuries more resources and time is required.
- According to healing time, there are wounds that get healed under the time required by the whole process to take place to restore the original functional and anatomical feature of integumentary system. Such wounds are known as acute while those who fail to follow the normal healing stage and time, fall under the term known as chronic wounds (Demidova-Rice, Hamblin, & Herman, 2012).
- According to risk of infection, wounds are classified into four classes (Herman & Bordoni, 2021).

- Class I or clean wounds: These are the surgical wounds that are free of contamination or inflammation, also this class of wounds don't involve the wounds in respiratory, alimentary, genital or urinary tract.
- Class II or clean-contaminated wounds: This class of wounds are clean but are prone to high risk of infection as the wounds are located in the areas like respiratory, gastrointestinal (GI), genital or urinary tract.
- Class III or contaminated wounds: These are the open or accidental wounds occur during the surgical procedure when there is major break in the sterile technique or there is some sort of spillage from the GI tract.
- Class IV or dirty/ infected wounds: The old wounds result from the unprofessional care and result from the microbes present before the surgical procedure.

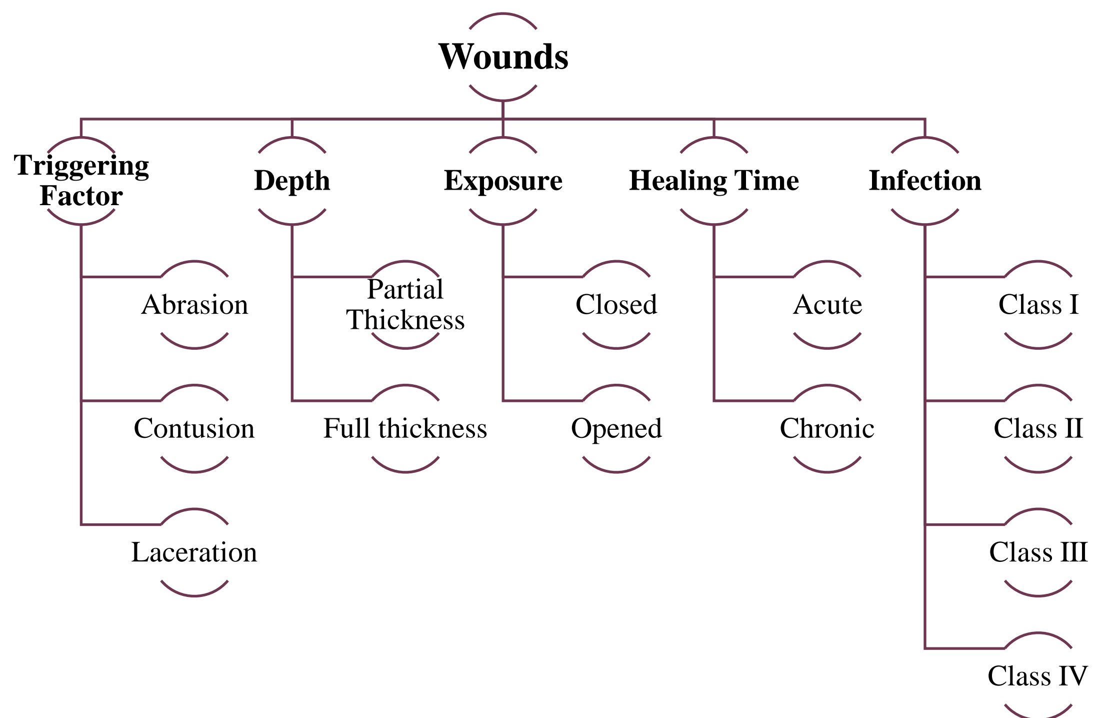


Figure 1.2. Classification of Wounds.

1.1.1.1 Chronic Wounds

Chronic wounds are defined as one fail to heal by normal process usually stuck at inflammatory phase, thus cost more making difficult for both patient and clinician to deal with it. All wounds are prone to change to chronic depending upon the depth, appearance and location. One of the reasons that make chronic wounds hard to treat is their association with other chronic diseases. So, the commonly observed chronic wounds include diabetic foot, pressure, arterial and venous ulcer (Bowers & Franco, 2020).

1.1.1.1.1 Diabetic Foot Ulcer

Diabetic foot ulcer (DFU) is common among the patients suffering from the diabetes mellitus. Its etiology is multifactorial commonly results from the uncontrollable glycemic load, peripheral vascular disease, dry skin, infirm footwear or poor foot care. These are present in areas of foot which are exposed to consistent trauma and Staphylococcus is the common cause of infection. DFU is develop from the callus formation which leads to sensory loss against the trauma thus, continuous trauma results in subcutaneous hemorrhage and ultimately deteriorate causing ulcer. The interprofessional help is required to deal with the diabetic foot ulcers, as in extreme conditions patient has to undergo amputation (Oliver & Mutluoglu, 2021).

1.1.1.1.2 Pressure Ulcer

Pressure ulcers are defined as the wounds developed when the pressure on the tissues is beyond 30-32 mm Hg and for long time. The ulcer developed when skin faces continuous shear forces between the skeleton and support like bed or chair. Usually, patients having the pressure ulcers are the one already suffering from the mental or physical health conditions. The ulcer developed from the first grade to fourth grade. It starts with the superficial skin discoloration, then the epidermis and dermis get damage, the deterioration didn’t stop here it get deeper to full thickness wound due to tissue necrosis resulting in exposing the bones and tendons (Bhattacharya & Mishra, 2015).

1.1.1.1.3 Venous Ulcer

Skin lesions occur near the area affected by the venous hypertension. They are commonly observed around the ankles. The venous obstruction or reflux cause the venous hypertension. Normally, the pressure in the legs should be dropped when walking but due to defect in the valves of vein the pressure sustains causing the ulcer formation. Age, gender and family history of vein related diseases are the factors

connected with the disease progress (Millan, Gan, & Townsend, 2019), (Grey & Harding, 2006).

1.1.1.1.4 Arterial Ulcer

As the name indicate the ulcer formed due to defect in the arterial blood supply. They are different from the venous ulcer as in arterial ulcer the tissue lacks blood flow while in venous ulcer the blood can't return to heart efficiently. Arterial ulcers appear as the punched out characterized by the prominent edges and wound base is pale without the granulation tissues. Its progress is associated with the atherosclerosis along with the other factors including smoking, diabetes mellitus, family history, obesity, hypertension and sedentary lifestyle. DFU may also fall under the category of arterial ulcers as they may be caused from the merge of trauma, neuropathy, and arterial insufficiency (Grey & Harding, 2006), (Hess C. T., 2010).

Although wounds are not considered as major issue like other communicable and non-communicable diseases but if they are not managed or treated properly, they can lower the living quality of patients.

1.2 Wound Burden

The wounds have been classified depending upon the triggering factor but chronic wounds are the one that hard to deal along with the infection that is one of the factors that lengthen the healing time.

Almost anyone from anywhere can suffer from wounds age is one of the factors on which the healing depends so it is more common in elderly population. About 7% of the country population is over 60 as of 2019 and it's expected to get double till 2050 (International, 2022). As the chronic wounds are also associated with the other diseases

so the chances of developing wounds and related problem is more prevalent in elderly population.

1.2.1 Prevalence of Wounds

According to data from different regions, trend of wounds depending upon the causes have been estimated and the data is from the year 2016 to 2019 **Figure 1.3** (Al-Hashemi, et al., 2019), (R A C & Samarawickrama, 2017), (Mukagendaneza, et al., 2019), (Younis, et al., 2018), (Sen, 2019), (Q Goh, et al., 2020).

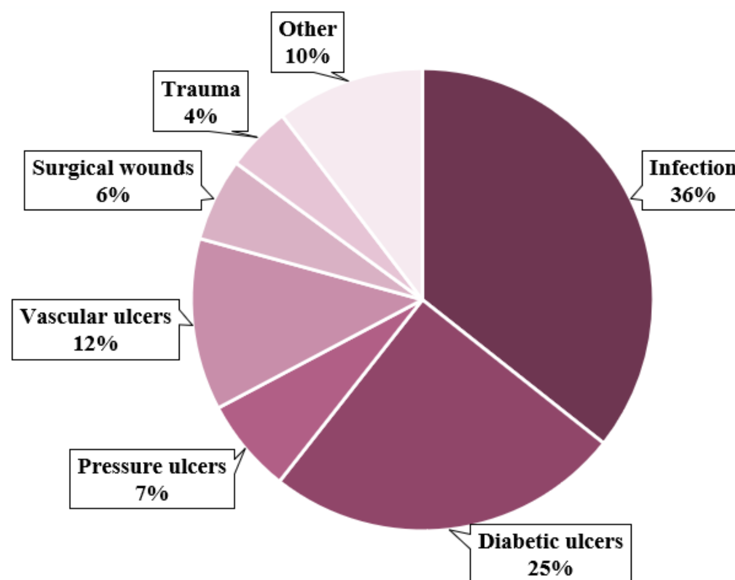


Figure 1.3. Wound Burden.

This chart shows the estimated prevalence of the wounds from the 2016 to 2019.

1.2.2 Treatment Cost

The cost for wound care depends upon causing factor and infection rate. The Medicare cost for the wounds is from $28.1 billion to $96.8 billion which include the costs for infection management as well and among different wound type diabetic ulcers (cost

$6.2, $6.9 and $18.7 billion) and surgical wounds ($11.7, $13.1 and $38.3 billion) fall under the high-priced category (Nussbaum, et al., 2018).

The market for wound care is increasing day by day. In 2014 the annual cost was $2.8 billion which rises up to $3.5 billion in 2021 and expected to exceed $15-$22 billion from 2022-2024 as driven by advancement in the field of wound care, an increase in incidence of chronic wound along with increase in geriatric population and government support (Settipalli, 2015), (Research, 2019).

According to IDF (International Diabetes Federation) 10th edition 537 million individual suffers from the diabetes and it is expected to increase in future along with the other problems related to it. But the one of the problems which is observed is foot ulcer and its prevalence is 4-10% and the possibility of getting is up to 25% (Amin & Doupis , 2016). The cost associated with the with is $9-$13 billion (Raghav, et al., 2018).

1.3 Healing Process

The healing process is divided into four phases but these phases may overlap with one another **Figure 1.4** (Wallace, Basehore, & Zito, 2021), (Heras, Igartua, Santos-Vizcaino, & Hernandez, 2020). The four phases as shown in **Figure 1.6** are

A. Hemostasis phase
B. Inflammatory phase
C. Proliferative phase
D. Maturation phase

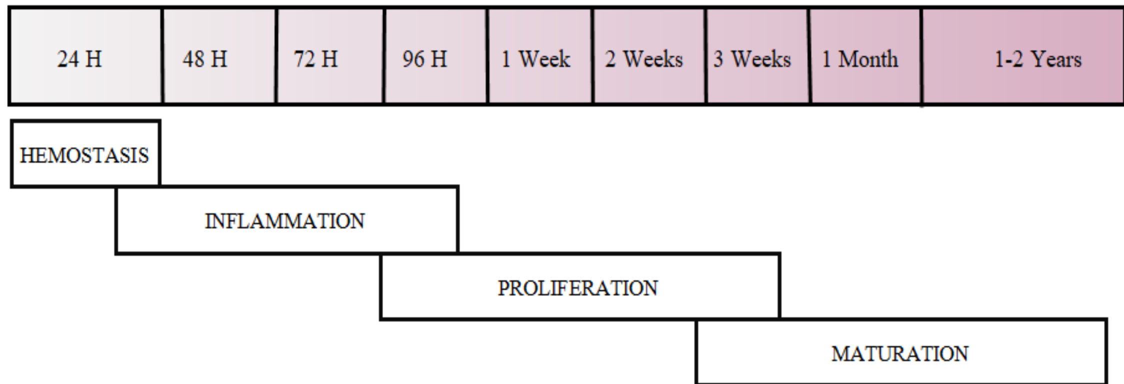


Figure 1.4. Timeframe of Wound Healing.

1.3.1 Hemostasis Phase

The priority of the body is to stop bleeding when any injury occurs. The hemostasis occurs in two steps primary and secondary hemostasis. Under normal physiological conditions, platelets flow in blood in inactivated form carrying the granules (alpha and dense) along with many receptors out of them two receptors play very important role in hemostasis. Also, the endothelial cells in the blood vessel secretes vasodilators, nitric oxide and prostacyclin to keep the blood in flow, but when an injury occurs primary and secondary hemostasis takes place to stop bleeding by fibrin clot formation **Figure 1.5**.

1.3.1.1 Primary Hemostasis

In this process platelet plug formation takes place which occurs in four steps

a. Vasoconstriction
b. Platelet adhesion
c. Platelet activation and degranulation
d. Platelet aggregation

When an injury occurs, the endothelium gets damaged releasing vasoconstrictor, endothelin instead of vasodilators thus resulting in constriction of vessel to restrict the blood flow. After vasoconstriction, next step is the platelet adhesion. The Von Willebrand Factors (VWF) act as glue, binding platelet to site of injury through the 1b glycoprotein receptor. Once the platelet gets attached, they get structurally modified and release the granules, alpha and dense. The 2b3a receptor get activated which is important in secondary hemostasis. The degranulation leads to secretion of the fibrinogen (secondary hemostasis), serotonin (vasoconstrictor), ADP (platelet activation) and Ca^{+2} (hemostasis).

1.3.1.2 Secondary Hemostasis

This action takes place to strengthen the platelet plug formed before. This is achieved by fibrin mesh network on the platelets plug. But fibrin is present in form of fibrinogen which is inactive form. Fibrinogen converts to fibrin with help of thrombin but it also needs to get activated from the prothrombin. Actually, the conversion of prothrombin to thrombin is through coagulation cascade by intrinsic and extrinsic mechanism. Once the fibrinogen converted to fibrin the plug gets stabilize (Gale, 2010), (Grover & Mackman, 2018). After the bleeding has stopped. The next phase will be inflammatory phase.

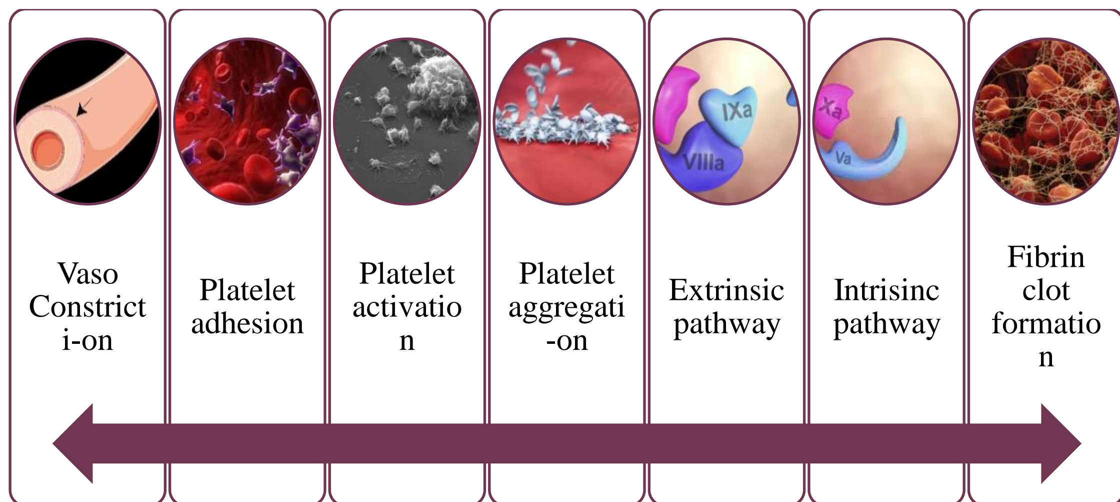


Figure 1.5. Steps Involved in Fibrin Clot Formation.

1.3.2 Inflammatory Phase

It is the important phase in wound healing as it prevents infection and control bleeding. The loss of blood allows the repairing proteins and factors along with the leukocytes to heal and clean wound. During this phase the leukocytes kill the pathogens and to keep the wounds free to infections which is observed as the redness and swelling at injury site. Innate and adaptive immune system is involved. When skin cells are exposed to danger, these signals are recognized by the toll like receptors leads to expression of chemokines, cytokines and antimicrobial peptides. At the beginning of the inflammation neutrophil will act and lingered for 4-5 days to clean the cell debris, pathogen and enhance inflammatory response. Monocytes convert to macrophages at third day of injury to further facilitate the inflammatory phase and they are important for transition of inflammatory phase to proliferative phase as they not only phagocytose the microbes, clear debris and produce proinflammatory mediators but also released growth factors and anti-inflammatory mediators along with the phagocytosis of neutrophil in order to switch to proliferative phase.

Both B and T cells play important role in healing activity. B cells not only produce antibodies but also growth factors and activate T-lymphocytes, which act as both the growth factors and the immunological effector cells in wound healing process Langerhans cells are present in the epidermis belong the family of dendritic cells and act first defense mechanism. They are present in numerous numbers at the site of injury at the beginning of wound healing process.

Inflammation is the defense mechanism but it is double edge sword. At the early stage of this phase proinflammatory mediators are produced to clear all the cell debris and kill pathogen, once neutrophil and macrophages perform their duties the proinflammatory mediator level decreases and anti-inflammatory level increases to facilitate the healing activity (Landén, Li, & Ståhle, 2016), (Wallace, Basehore, & Zito, 2021).

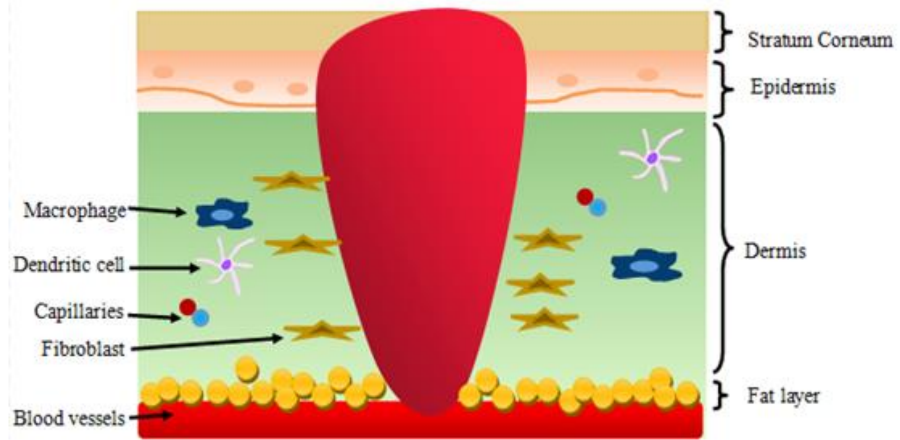
1.3.3 Proliferative Phase

After inflammatory phase, proliferative phase become dominant. It is associated with migration and regeneration of cells along with the vascular system restoration and granular tissue formation. Reepithelization is stimulated by secretion of different signals from the cells at injury site including cytokines and growth factors i.e., EGF (epithelial growth factors), IGF (insulin growth factors), NGF (nerve growth factors) and KGF (keratinocyte growth factors). When injury occurs, the keratinocytes left at the edge of wound will start to migrate and to cover the wound more keratinocytes will be produced from the basal layer after 2-3 days of laceration. The loss of contact and cell adhesion triggers the migration and once the cells adhere migration stops resulting in release of proteins from the keratinocytes to reconstitute the basement membrane.

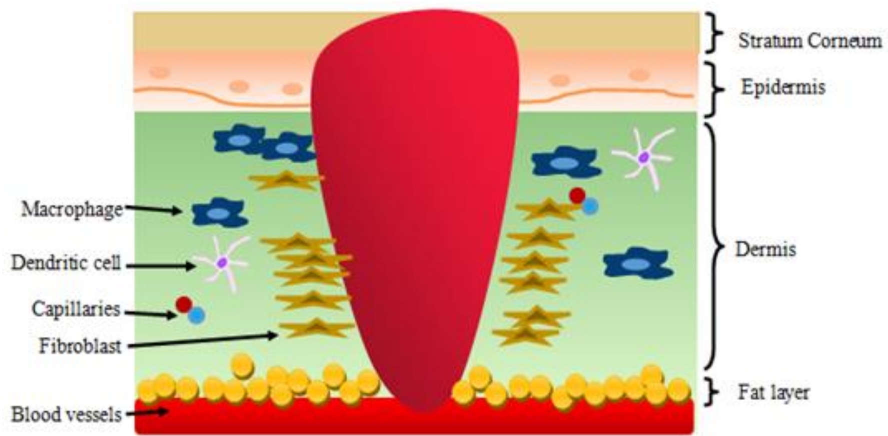
It is important to reestablish the vascular system to provide nutrients and oxygen to cells for healing. Angiogenesis will take place with the help of VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor) and bFGF (basic fibroblast growth factors). The circulating fibrocytes not only promote the healing but also produce cytokines, growth factors, act as antigen presenting cells and promote angiogenesis. They also aid in settling of collagen and other ECM (extracellular matrix) constitutes which provide scaffold for cell adhesion, migration, growth and differentiation to fill the wound gap. In other words, wound contraction take place due to conversion of fibroblast to myofibroblast following angiogenesis to construct the healthy granulation tissue (Landén, Li, & Ståhle, 2016).

1.3.4 Maturation Phase

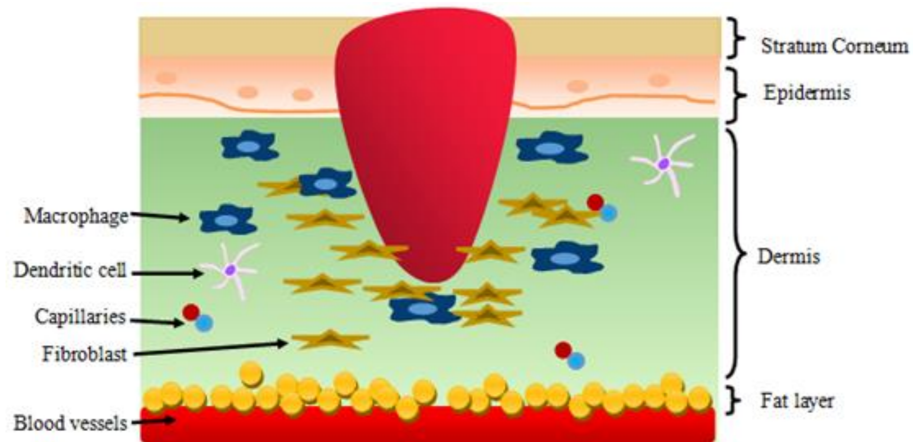
This phase is also known as remodeling phase as the collagen formed in the proliferative phase is type III and is converted to type I. The collagen formed in previous phase is uneven making wound thick but during this phase the collagen rearranged to form cross linking that will help to reduce the scar, result in strengthening the wound area. Collagen I is higher in tensile strength but it takes time to deposit in the ECM. The myofibroblast formed in the proliferative phase after performing their duties undergo apoptosis. The angiogenesis process will decline to get mature acellular and avascular environment. Other skin components like hair, glands will also recover but if the injury is severe then it’s difficult to recover them (Landén, Li, & Ståhle, 2016).



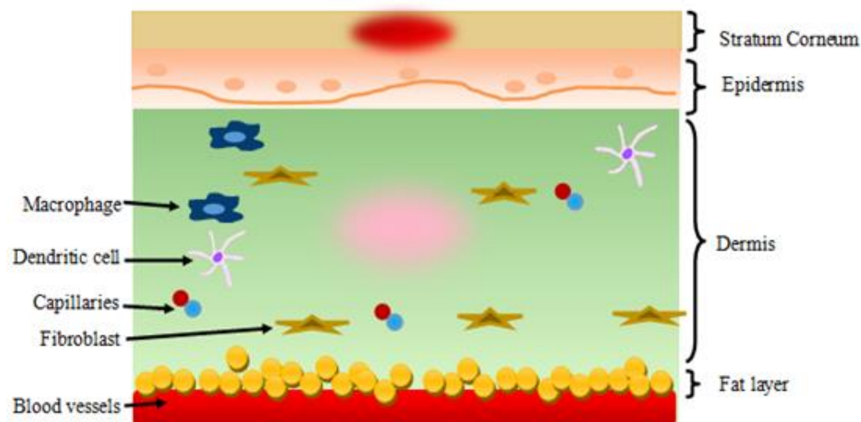
a: Hemostasis Phase.



b: Inflammatory Phase.



c: Proliferative Phase.



d: Maturation Phase.

Figure 1.6. Wound Healing Phases.

1.4 Risk Factors Affecting Wound Healing

Wounds heal within 4-6 weeks and there are many factors involved either local or systemic which affects the wound healing process.

1.4.1 Local Factors

1.4.1.1 Oxygenation

Oxygen is required by cells for metabolism to produce energy by mean of ATP, necessary for wound healing. It is important for infection prevention, angiogenesis, cell migration, differentiation and reepithelization. At the beginning of the healing, hypoxic environment is required to promote healing but prolonged hypoxia delays healing and responsible for chronic wounds. Hyperoxia increases ROS level which also exhibit beneficial effect against the pathogen but if it exceeds the limit can damage tissues. The optimum level of oxygen and for particular time is required to facilitate the cell regeneration (Guo & DiPietro, 2010).

1.4.1.2 Infection

Inflammation is required for pathogen removal but in absence of proper sterilization microbes and endotoxins can extend the elevated levels of proinflammatory cytokines dragging the inflammatory phase. This prolonged inflammation leads to high level of MMPs (matrix metalloproteases) which degrades ECM. Microorganisms formed biofilms which with passage of time form their microenvironment and become resistant to the antibiotics. Staphylococcus aureus, Pseudomonas aeruginosa and beta- hemolytic streptococci are usually present on either infected or uninfected wounds (Guo & DiPietro, 2010), (Hess T. , 2011).

1.4.1.3 Pressure

The pressure is one of the factors that affects the healing procedure. The obstruction of blood flow due to pressure on the wound site prevent the transport of nutrients and oxygen to the dividing cells thus delaying the healing (Hess T. , 2011).

1.4.2 Systemic Factors

1.4.2.1 Age

It has been observed that the wound healing process is delayed in older people than the younger one. It is due to change in inflammatory response like delayed T-cell infiltration, production of cytokines and growth factors, delayed angiogenesis and collagen deposition, ultimately leads to decreased wound strength (Guo & DiPietro, 2010).

1.4.2.2 Sex hormones

In comparison with the elderly females, aged males show delayed healing. Estrogen has been found to associated with gene that regulate matrix production, epidermal function and regeneration. Studies suggest that estrogen has ability to boost age related healing impairment in both male and female while androgen has negative impact on the healing process (Hardman & Ashcroft, 2008), (Hess T. , 2011).

1.4.2.3 Stress

Stress affects human health both psychologically and physically. Stress effects immune system by deregulating the levels of hormones like glucocorticoids which disturb the level of proinflammatory and cytokines that are required to initiate the wound healing. Thus, stress has direct impact on both endocrine and immune system (Gouin & Kiecolt-Glaser, 2011).

1.4.2.4 Diabetes

Wound healing process is complex but in diabetic patients the mechanism become even more difficult to deal with. Wounds in patients with other chronic morbidities are accompanied by the hypoxia. Hypoxic condition enhances the early inflammatory

reaction and also elevate the level of the free radicals (Tandara & Mustoe, 2004), (Woo, Ayello, & Sibbald, 2007).

Hyperglycemia inhibit the use of oxygen and nutrients by the cells thus adding the free radical load which exceeds beyond antioxidant capacity. Neuropathy, peripheral vascular disease poor immune system is observed in diabetic patients ultimately influencing the wound healing (Brem & Tomic-Canic, 2007).

1.4.2.5 Obesity

It increases the risk of other health associated problems like type II diabetes, cardiovascular disease, stroke, hyper tension, cancer and wound healing. High infection in wound is observed in obese patients as hypoperfusion and ischemia decreases the antibiotic delivery thus interrupting wound healing. The adipocytes act as caloric storage and produce various cytokines and hormone like factors which has negative impact on immune system and inflammatory response and henceforth, affect the wound healing (Wozniak , Gee, Wachtel, & Frezza, 2009).

1.4.2.6 Medications

Medications do interfere with the wound healing by altering the function of cytokines, platelet formation plug, inflammatory response and cell regeneration. The medications that have impact are NSAIDs, glucocorticoid steroids and chemotherapeutic drugs.

NSAIDs are widely used for antirheumatic, anti-inflammatory and as analgesic. They also have anti thrombotic properties and somehow have negative impact on the wound healing. It has been observed in animal model that use of ibuprofen is associated with decreased number of fibroblasts weak breaking strength, impaired angiogenesis and delayed epithelization (Guo & DiPietro, 2010).

Glucocorticoid steroids inhibit the hypoxia induced factor-1, thus result in inhibition of wound repair (Wagner, Huck, Stiehl, Jelkmann, & Hellwig-Bürgel, 2008). The chemotherapeutic drugs have the ability to prevent rapid cell division, cell migration and fibroblast. They not only prevent matrix production but also weaken the immune system as well (Franz , Steed, & Robson, 2007).

1.4.2.7 Nutrition

Body needs energy and several nutrients to function properly. Carbohydrate and fats serve as source of energy. Glucose provides the energy in form of cellular ATP while protein is related with capillary formation, proteoglycan synthesis, fibroblast proliferation, collagen production, wound maturation, enzymes and immune cells. Fatty acid provides the building blocks for tissue repair, also related to inflammatory cytokines gene expression and angiogenesis in wound sites. One of the fatty acids is omega-3 fatty acids related to enhance the immune activity.

Along with these major components, human body needs micronutrients and vitamin from outside source as most of the nutrients can’t be produced. Vitamin C, A and E have anti-inflammatory and antioxidant activities. Deficiencies of any of these vitamin leads to impaired healing, collagen formation and hyaluronate synthesis. Vitamins are also related to reduce the level of MMP which is a protease responsible for ECM degradation and vitamin C and E are responsible for anti-scar properties.

Micronutrients such as copper, iron and magnesium are associated with repair mechanism. Magnesium act as cofactor for many enzymes related to protein and collagen formation and copper is required for proper cross linking of the collagen. Iron is important not for blood hemoglobin but also for the collagen synthesis.

In short, all these nutrients act together for proper tissue regeneration and deficiency of these nutrients can lead to serious consequences (Campos, Groth, & Branco, 2008).

1.5 Diagnosis

Traditionally wounds are diagnosed on bases of observation but there are many methods to diagnose wound infection through hematology, radiology and microbiology. The different diagnostic methods are shown in the

Table 1.1 (Li, Renick, Senkowsky, Nair, & Tang , 2021). For microbiological investigation samples are collected in following forms.

- Tissue biopsy
- Wound swab
- Viable tissue from base of ulcers
- Wound fluid aspirate

Table 1.1. List of diagnostic techniques and their purpose for the wounds.

Diagnostic Investigations	Purpose
White blood cell count	Detect infection
C-reactive protein (CRP)	Detect inflammation related to infection
Erythrocyte sedimentation rate (ESR)	Detect inflammation related to infection
Blood culture	Detect and identify the causative organism.
Wound culture	Detect and identify the causative organism.
X-ray	Identify presence of osteomyelitis or abscess
Ultrasound	Identify presence of osteomyelitis or abscess

1.6 Treatment Options

There are variety of treatments available (**Table 1.2**) to deal with wounds but they fall under the category of the wound dressing, antimicrobial, physical treatment and grafting (Heras, Igartua, Santos-Vizcaino, & Hernandez, 2020).

1.6.1 Antimicrobial agents

The antimicrobial is defined as one that kill, reduce or inhibit the microorganism growth hence, it includes antiseptic, antibiotics and disinfectant. Except for disinfectant other microbial agents are used or applied on the living skin and wounds directly (Punjataewakupt, Napavichayanun, & Aramwit, 2019).

1.6.1.1 Antibiotics

The common problem faced by clinicians is bacterial infection which make it hard to deal with wound. In order to get rid of bacterial biofilms antibiotics are being administrated systemically or topically. Antibiotics that have been used frequently include Bacitracin, Neomycin, Silver sulfadiazine etc. But sometimes antibiotics hardly pass the biofilm formed on the wounds making topically application of antibiotics ineffective (Dumville, et al., 2017). Thus, the antiseptics are required.

1.6.1.2 Antiseptics

The commonly used antiseptics are Povidone-iodine, Sodium hypochlorite, Hydrogen peroxide and other antiseptics. Many antiseptics have been widely used by clinicians as they possess wide range of antimicrobial activity but they not only affect microbes but are also toxic to host tissues as well (Punjataewakupt, Napavichayanun, & Aramwit, 2019).

1.6.2 Physical Treatment

This includes debridement, negative pressure therapy and wave stimulation. Debridement is removal of dead tissue and foreign agents from the wounds. The methods that used are chemical enzymatic or surgical to manage wounds.

Negative pressure wound therapy (NPWT) is one of the traditional methods like debridement. This is close system which consists of foam dressing, the negative pressure is applied to the dressing to promote the wound healing. They are effective against the pressure ulcers either venous or arterial and diabetic foot ulcers as well in spite of these advantages they still can't be used by every individual especially those using anti thrombin, the NPWT will cause bleeding. The direct contact of this therapy with expose vascular system or tendons leads to many complications such as infection, pain or sometime bleeding (Lalezari, et al., 2016).

1.6.3 Wound Dressing

Simple bandages have been used for a very long time but their only purpose was to cover the wound but they do make wounds dry so, in order to keeps wound hydrated wound dressings with new formulations are designed which not only preserve hydration but also prevent infection and disruption of wounds (Dhivya, Padma, & Santhini, 2015).

1.6.3.1 Hyaluronic Acid Dressing

It is a biocompatible and biodegradable polysaccharide which supports cell growth, proliferation and migration thus is suitable for the wound treatments. But due to co-morbidities the hyaluronic acid grafts can't survival due to infections and thus effect wound repair

1.6.3.2 Biobrane Dressings

This is specially designed for burns but can be used for blisters and ulcers. They do reduce healing time but they are not capable of eliminating infections.

1.6.4 Grafting

The grafting is defined as the transfer of the tissues from one site (donor) to affected site (recipient). The grafting can be either done from patients own tissues or from other donors respectively called as autografts and allografts.

1.6.4.1 Autografts

Autograft is gold standard for treating wounds. In this method the full thickness of tissue sheet is taken from the donor site and placed on to the compromised site. Limitations linked to donor site morbidity, scarring and infection makes it an imperfect treatment option although it has been widely used.

1.6.4.2 Cultured Epithelial Autografts (CEA)

To compensate the disadvantages of the autografts, CEA has been introduced. The method involves the collection of the cells from the patient body then culturing the cells in vitro by supporting them with fibroblast cells and growth factors. Although donor site morbidity is less still there are limitations regarding the time required to culture the cells and also the attachment of graft (Heras, Igartua, Santos-Vizcaino, & Hernandez, 2020).

These different treatments are given depending upon the seriousness of the wounds but still they have limitations which urges the need to introduced new method to deal with wounds. Now a days microemulsion based hydrogel has been introduced to treat burn, diabetic and other wounds.

Table 1.2. Summary of current treatments with limitations.

Current Treatments	Limitations
Antimicrobial agents	
Antibiotics	Can't pass bacterial biofilms
Antiseptics	Toxic to host tissues as well
Physical treatment	
Debridement	Need expert
Negative pressure wound therapy	Cause bleeding and infection
Wound dressing	
Hyaluronic acid	Can't get settle on the wounds due to infection
Biobrane	Can't eliminate infection
Grafting	
Autografts	Donor site morbidity and infection
Cultured epithelial autografts	Time consuming, thus result in sepsis.

1.7 Hypothesis

Hydrogel based microemulsion with combination of both *Thymus serpyllum* and ascorbic acid is more effective for wound healing than the current treatment options.

1.8 Objectives

- To formulate microemulsion-based hydrogel.
- To evaluate the anti-inflammatory, antioxidant properties and antimicrobial activity of microemulsion.
- To validate wound healing rate of excision and diabetic wounds.

Chapter 2: Review of Literature

2.1 Wounds

Wound is explained as the disruption of the epithelial surface and the connective tissues beneath it. The wound healing stages consists of four phases. There are several factors involved that has effect on the healing process. Thus, the wounds are grouped in to two types:

- **Acute wounds** are defined as the one that undergoes normal healing process after burn, surgery or trauma within 30 days approximately.
- **Chronic wounds** are described as one that are unable to heal properly and took more than one month approximately due to disease or underlying pathologies, for example diabetes, obesity or infection (Dunnill, et al., 2015).

Out of many factors the one that will be focused are reactive oxidative species (ROS), bacterial infection and inflammation that lead to improper wound healing.

2.1.1 ROS and Wounds

Oxygen is one of the important elements required for yield of ATP, that is required for tissue regeneration. Normal level of ROS is required as the they are essential for protection of wounds initially as they have role in thrombus formation, recruit the neutrophil, monocytes or other immunocyte to prevent the pathogen attack and also help in fibroblast and keratinocytes proliferation and migration. In other words, ROS play role in four phases of wound healing. However, its only effective as long as the levels are normal but the level rises the healing will be impaired and the situation gets under the oxidative stress.

ROS can lead to sustained secretion of proinflammatory cytokines leading to introduction of the matrix metalloproteases degrading ECM proteins, damage the function of both keratinocyte and dermal fibroblast. Due to negative influence of high level of ROS in wound healing and its essentiality in chronic wounds, antioxidants can be used as strategy to prevent the excess of ROS (Dunnill, et al., 2015).

2.1.2 Bacterial Infection in Wounds

Bacterial infection plays crucial role in the development of the chronic wound thus delay the healing process. As the result of presence of microorganism as natural microflora of skin the wounds get contaminated. Colonization of microbes on wound is characterized by the presence of increase in number of the microbe without the immune response from host. But some wound infections depend upon the virulence and pathogenicity of microbes result in clinical symptoms like pain, erythema, pus and cellulites (Jones, et al., 2005).

A study reported that almost 70% of the patients with wounds suffer from the infection while the 30 % have saprophytic microbial flora. The microbes isolated are both gram positive and gram-negative bacteria, involved in causing infection. the most common among them *Staphylococcus aureus* rate is more as compare to *Escherichia coli*, *Pseudomonas aeruginosa* and others. Antibiotic resistance is one of the problems toward modern medicine and this mechanism is common in both gram-positive and gram-negative bacteria. In gram-positive bacteria the enzymatic degradation by beta-lactamases, by decreasing susceptibility and affinity of antibiotic or other related action while in gram-negative, the change in hydrophobic features of outer membrane make them resistant (Munita, Bayer, & Arias, 2015), (Miller, 2016), (Breijyeh, Jubeh, & Karaman, 2020), (Jubeh, Breijyeh, & Karaman, 2020), (Bessa , Fazii, Giulio, & Cellini, 2013).

2.1.3 Inflammation Affecting Wound Healing

Wounds especially chronic, don't follow the normal healing cascade. Chronic wounds often get locked in the inflammatory phase that debar proliferation of the cells and tissues of wounds thus affecting wound healing. Microbial attack, oxidative species and comorbidities also contribute to get wounds stuck in the inflammatory phase. In acute wounds high levels of mitogenic activity is observed but is absent in chronic wounds. The hostile microenvironment is amplified often due to multifactorial stimuli result in disturbance of delicate balance between proteases, proinflammatory cytokines chemokines and their inhibitors that is observed in acute wounds (Schultz & Mast, 1999).

The immoderate infiltration of neutrophil plays critical role in chronic inflammation and act as biological marker for it. Excess of neutrophil result in overproduction of oxidative species leading to ECM damage, plasma membrane ultimately causing cell senescence. The damage to ECM products further develops inflammation which combine with the compromised cellular and systemic host response, leads to sustain deleterious cycle.

Thus, the anti-inflammatory compound or element is required to unlock the inflammatory phase and help the wound healing cascade to proceed to next phase (Zhao, Liang, Clarke, Jackson, & Xue, 2016).

2.2 *Thymus serpyllum*

Thymus serpyllum L. commonly known as wild thyme, Breckland thyme or creeping thyme, belong to family Lamiaceae as shown in **Table 2.1**. It is a perennial shrub naturally grows in Pakistan known as jangli ajwain shown in **Figure 2.1**. It consists of

long stem with oval shaped leaves while the flowers grow usually during summer at the tip of the stem and form spherical verticillaster.

Table 2.1: Classification of wild thyme.

Kingdom	**Plantae**
Clade	Angiosperms
Class	Dicotyledonae
Order	Lamiales
Family	Lamiaceae
Genus	*Thymus*
Species	*serpyllum*

Figure 2.1. Wild Thyme.

This plant has been extensively used for its medicinal properties for many centuries. The whole plant has been used for its therapeutic properties either fresh or in dried form. Wild thyme is capable of healing due to the significant amount of the essential oil collected from the upper portion of the plant when its flowers. Because of therapeutic properties such as anti-oxidant, anti-inflammatory, anti-microbial and antitumor properties and the emergence of multidrug resistance microorganism along with increased demand of natural products *T. serpyllum* become the excellent source in synthesizing many products related to pharmaceutical, chemical, cosmetic and food industries (Jarić, Mitrović, & Pavlović, 2015).

2.2.1 Ethnobotany

T. serpyllum had been used since ancient Egypt, for curative purposes. Even in 1st and 2nd century it was still known for its pharmacological value especially in treating stomach ache, asthma and congestion in throat (Heilmeyer, 2007). In European Ages it had been used to help in sleep and to fend off nightmare. In ancient pharmacological manuscript the use of *T. serpyllum* had been mention for treating headache, laryngitis, as antitussive and diseases of digestive system. It is reported that in 16th and 17th century it was used to treat epilepsy and malaria (Adams, Schneider, Kluge, lKessler, & Hamburger, 2012). It had been used worldwide as antiseptic, disinfectant, sedative and antispasmodic.

In Northern Pakistan, it has been reported to be used as an anthelmintic. *T. serpyllum* also aid in treating wounds, eczema, reduce swelling or apply as antiseptic and may other diseases (**Table 2.2**).

Table 2.2. List of traditional recipes of *T. serpyllum* for different diseases.

Disease	Traditional Recipe	Reference
Eczema	Infusion prepared or tea	(Benítez, González-Tejero, & Molero-Mesa, 2010)
Reduce swelling		(Kozuharova, Lebanova, Getov, & Benbassat, 2013)
Wounds		(Mati & Boer, 2010)
Flu and fever	Tea from aerial part of plant	(Jabeen, Ajaib, Siddiqui, & Ulfat, 2015)
Bronchitis		(Qureshi & Bhatti, 2009)
Gastrointestinal problems		(Jabeen, Ajaib, Siddiqui, & Ulfat, 2015)
Respiratory problems		
Liver and kidney problems		(Qureshi R. , Ghufran, Sultana, Ashraf, & Khan, 2007)
Laxatives		
Blood purification		

2.2.2 Pharmacological Properties

Many studies have been conducted on the chemical composition and yield of the plants belong to Thymus genus especially *T. serpyllum*. The chemical composition and yield

depend upon the geographic region, harvesting season, development stage and climate conditions.

The content of essential oil of *T. serpyllum* is 0.3% or 3ml/kg in Serbia (Stanisavljević, et al., 2012) while the sample taken from Pakistan shows yield of 0.48% (Ahmad, et al., 2006). Similarly, the oil content of *T. serpyllum* is 4.5-7.4ml/kg obtained from the five region in Armenia (Paaver, Orav, Arak, Mäeorg, & Raal, 2008).

According to PDR (Physician Desk Reference) for Herbal Medicines, the essential oil of *T. serpyllum* consists of these following compounds including carvacrol, thymol, α-terpineol, isobutyl acetate, and γ-terpinene. Both thymol and carvacrol belongs to group of phenolic monoterpenoids that possesses powerful antiseptic properties. These components are subjected to quick metabolism as they don't undergo biotransformation and are excreted out of body within 24 hours. Therefore, the amount of these two should be 40% or more. But the number of chemical components is different in *T. serpyllum* from different regions. *T. serpyllum* grows in Pakistan has mainly 53.3% of thymol and 10.4% of carvacrol (Ahmad, et al., 2006) but the thyme grows in Gilgit Valley consists of 44.4% of carvacrol and 14% of o-cymene (Hussain, et al., 2013). As it is evident that due to variability in chemical composition of wild thyme, the essential oil composition can't be used as chemotaxonomic marker.

However, the chemical elements have extensive importance not in culinary but also in field of cosmetics, medicine and industries as well (**Table 2.3**). The monoterpenic phenols inhibit the lipid peroxidation and exhibit powerful anti-microbial properties on variety of microorganism (Čančarević, Bugarski, Šavikin, & Zdunić, 2013). There are number of compounds that act as natural anti-oxidants and anti-inflammatory as well.

Table 2.3. List of compounds with the pharmacological activities.

Compounds	Pharmacological Activities	References
Thymol	Antiseptic	(Jarić, Mitrović, & Pavlović, 2015) (Nikolić, et al., 2014) (Meeran, Javed, Al Taee, Azimullah, & Ojha, 2017)
	Anti-proliferative	
	Anti-microbial	
	Anti-inflammatory	
	Anti-cancerous	
Carvacrol	Anti-cancerous	(Jaafari, Mouse, Mostapha, & M'barek, 2007) (Jarić, Mitrović, & Pavlović, 2015)
	Anti-bacterial	
p-cymene	Anti-bacterial	(Ultee, Bennik, & Moezelaar, 2002)

2.2.2.1 Antioxidant Activity

The comparison of anti-oxidant activities between Thymus genus has been demonstrated that *T. serpyllum* has more free radical scavenging ability than T. linearis. Thymol is the one that act as anti-oxidant than the carvacrol as it has been established that the species with high concentration of thymol has strong radical scavenging ability than the one with high level of carvacrol.

The anti-oxidant capacity of any compound can be tested by neutralizing free radicals of DPPH (1,1-diphenyl-2-picryl-hydrazyl) by donating hydrogen atom to it therefore, reducing DPPH (S., et al., 2014), (Jarić, Mitrović, & Pavlović, 2015). The natural antioxidants are more significant than the synthetic one.

2.2.2.2 Antimicrobial Property

It has been reported that species from Thymus genus exhibit anti-microbial activity. The carvacrol present as the essential component in oil has biocidal properties which cause bacterial membrane destruction. Carvacrol and other significant elements in the extract act in synergetic manner to exhibit the anti-microbial properties (Jarić, Mitrović, & Pavlović, 2015).

The ethanolic and aqueous extract of wild thyme shows inhibitory effect on Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli. The last two are involve in forming biofilm on the wound bed (Bessa , Fazii, Giulio, & Cellini, 2013). According to the research it is reported that *T. serpyllum* has bactericidal property but not bacteriostatic effect. The best anti-microbial activity can only be achieved when components of oil act in synergy.

2.2.2.3 Anti-inflammatory Activity

For many years people of Gilgit-Baltistan are using wild thyme for various purpose such as antiseptic, expectorant, anthelmintic and anti-inflammatory (Qureshi R. A., Ghufran, Gilani, Sutana, & Ashraf, 2007). In Chinese literature the analgesic effect of *T. serpyllum* has been reported. The experiment on mice model with ethanolic, aqueous and ether extract was conducted in order to justify the anti-inflammatory, analgesic and antipyretic effect of *T. serpyllum* (Jarić, Mitrović, & Pavlović, 2015), (Alamgeer, et al., 2015).

Such studies explain the traditional use of *T. serpyllum* as anti-inflammatory, anti-oxidant and anti-microbial for many ages.

2.2.2.4 *Thymus* for Wound Healing

Wounds are not the major problem but once it gets infected or become chronic, they become life threatening. The species of Thymus gene exhibit healing properties and had been used for many centuries for its anti-microbial, anti-inflammatory, analgesic and anti-oxidant properties as well. Few of the studies have been conducted to validate the traditional use of thyme against wounds.

The extract of *Thymus sisyleus* shows positive result on cell proliferation and scratch wound healing of fibroblasts than the control thus has potential to be used for treating skin lesions and injuries in order to overcome the issues related to modern therapies like high cost, increased bacterial resistance and time taken manufacturing (Ustuner , et al., 2019). Similarly, thyme oleoresin from *Thymus vulgaris* shows great wound healing activity on human keratinocytes (HaCaT) cell line in comparison with the control (Anitha, Subeeksha, & Lakshmi, 2018). Thymus oil has also been reported to be effective against the thermal injury and overcome the nitic oxide level in burn tissues to promote healing (Dursun, Liman, Ozyazgan, Güneş, & Saraymen, 2003). There is another study which indicates that Thymus vulgaris promotes wound healing by upregulating new vessel formation and collagen deposition (Panah, Hesaraki, & Farahpour, 2014).

In all of these studies it has been observed that all these Thymus species have one thing in common that is their anti-microbial, anti-inflammatory and anti-oxidant properties which make them capable of wound healing.

2.3 Ascorbic Acid

Ascorbic acid (AA), commonly known as Vitamin C, is water soluble vitamin. The vitamin fall under the category of micronutrient and nutrient is one of the factors that affect wound healing. Vitamin C deficiency leads to gum bleeding, corkscrew hair, skin fragility and wound healing which is characterized by scurvy. This exhibits the importance of ascorbic acid in collagen synthesis, wound healing and skin health. High concentration of ascorbic acid is present in normal skin 3-64 mg/100g, but the level of vitamin C is lower in damaged or aged skin. The amount of vitamin C is more in epidermis than dermis.

Ascorbic acid has been reported to be involved in collagen formation. It acts as co-factor to hydroxylases proline and lysine, required to maintain and stabilize the tertiary structure of collagen molecule shown in **Figure 2.2**. Fibroblasts in the dermis are responsible of collagen formation result in generation of basement membrane and collagen of dermis (Hinek , Kim , Wang , Wang , & Mitts, 2014), (Pullar, Carr, & Vissers, 2017).

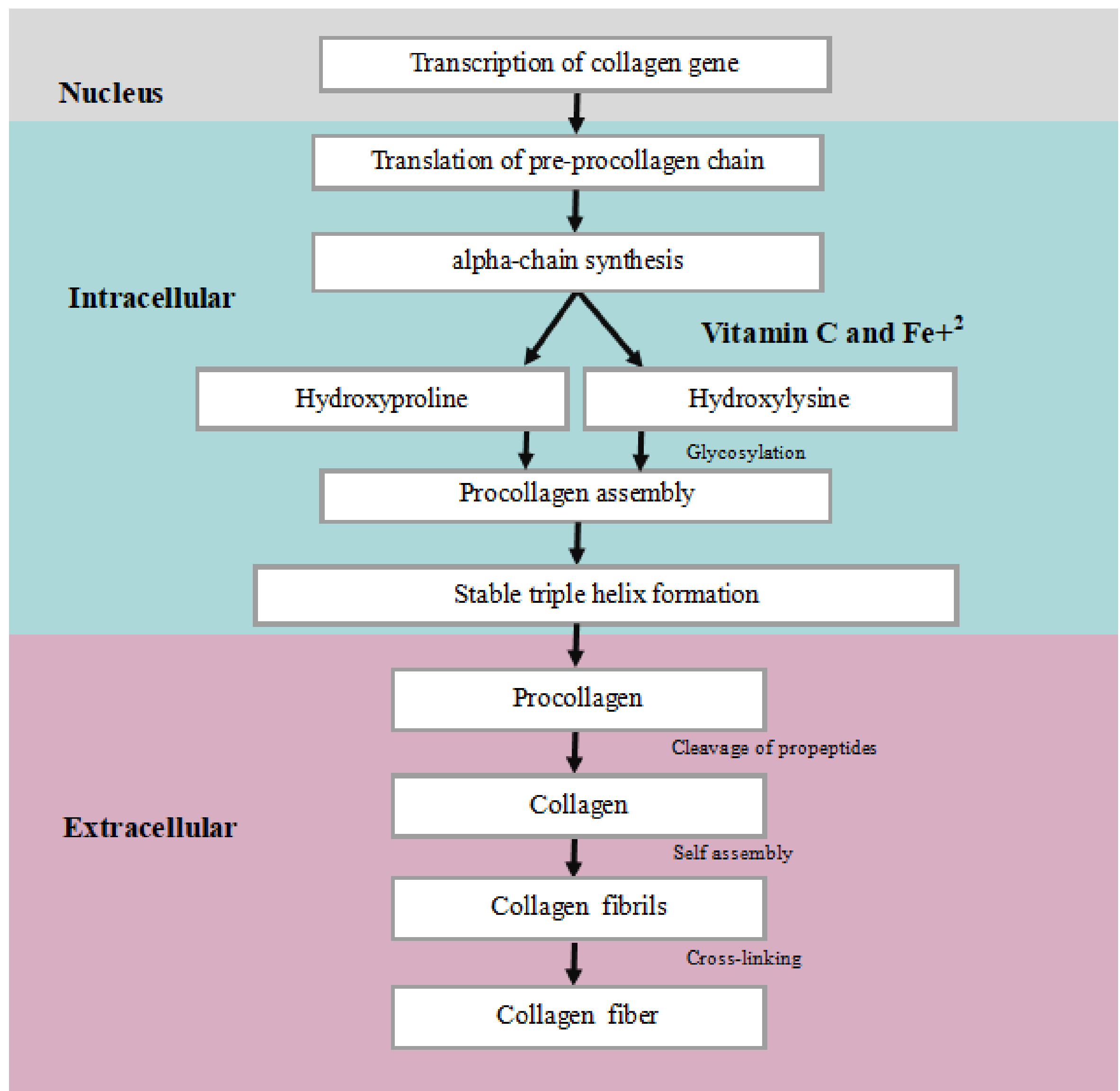


Figure 2.2. Collagen Formation.

Role of ascorbic acid and ferrous in collagen formation.

Vitamin C also has ability to scavenge the free radicals produced as the result of the environmental pollutants and the ultraviolet radiation exposure so the high concentration of vitamin C in epidermis provide protection to skin. Thus, vitamin C has both anti-oxidant and healing properties as well.

2.3.1 Prevalence of Ascorbic Acid Deficiency

Although for many years lots of people suffer from the vitamin C deficiency but there is no up to date data for the prevalence of insufficiency of the ascorbic acid therefore the **Table 2.4** shows data from different years of different countries with varied number of sample size (Rowe & Carr, 2020).

Table 2.4: Countries with ascorbic acid deficiency.

Countries	**Age**	**Deficiency (% <11 µmol/L) %**
UK (1994-1995)	>65 years	14
United States (2003-2004)	>20 years	8.4
United States (2016)	12-19 years	3.3
New Zealand (2010-2013)	50 years	2.4
India (2004-2006)	>60years	59
India (2012-2013)	11-18 years	6.3
Uganda (2008-2009)	15-49 years	56

2.3.2 Ascorbic Acid Against Wounds

The effect of ascorbic acid has been tested for many years against different types of wounds. Vitamin C deficiency is common making them at a risk of impaired wound healing. The patients were given ascorbic acid which not only improve the post-surgery

wound healing process but also lessen stay time at hospital and other wound healing treatment cost (Bikker, Wielders, Loo, & Loubert, 2016).

A study was conducted to determine the effect of high dose ascorbic acid on patients with severe burn and the results were satisfactory. As ascorbic acid has anti-oxidant property, it reduces oxidative stress in endothelial cells and tighten endothelial barriers. The high dose of ascorbic acid is linked with the lessened mortality in severe burn patients when used with the minimum amount of 10g within first 2 days of admission (Nakajima, et al., 2019).

Sepsis and oxidative stress work together to make wound healing defective. Sepsis is caused by both gram positive and gram-negative bacteria sometime fungi or both. It aggravated inflammatory response, hypotension which may lead to vasodilatory shock ultimately resulting in reduced oxygen delivery to tissues. It has been reported that mitochondrial dysfunction causes cellular energies failure during septic condition, while the mitochondrial dysfunction results from ATP depletion, excessive production of reactive oxidative species (ROS), release of pro-apoptotic proteins and disturbance in Ca^{+2} homeostasis (Prauchner, 2017). The vitamin C is natural anti-oxidant thus, help in preventing sepsis and cellular destruction (Anand & Skinner, 2018).

When the skin gets damage, the tissues produce collagen to fill the gap but the fiber composition of collagen produced is different. Normally, the collagen is produced in random basketweave pattern but in scar the collagen is in single direction and the scar tissue is devoid of normal function. The uptake of ascorbic acid more than the dose recommended by the WHO is effective against all collagen related pathologies (Hujoel & Hujoel, 2022). The strength of scar tissues depends upon the availability of vitamin

C, the scar will have low tensile strength if there is deficiency is ascorbic acid (Bourne, 2007). The intake of ascorbic acid helps to restore 80% of the original tensile strength.

From the studies conducted it has been proved that ascorbic acid is necessary to promote wound healing and redeem the original state of tissues. But, the availability of vitamin C to skin is little than the amount ingested due to body control mechanism. Ascorbic acid has been tested on human skin fibroblast and they showed promising results in synthesis collagen without affecting other protein synthesis (Pinnell & Madey, 1998).

2.4 Microemulsion

Microemulsions (MEs) are combination of oil and water stabilize by surfactant and sometimes by mixture of surfactant and cosurfactant. It appeared to be transparent and thermodynamically stable system with the droplet size 10-100nm. MEs are classified as oil in water (O/W), water in oil (W/O) and bicontinuous system. The MEs system has low interfacial tension thus low viscosity. As ME is dispersion of two immiscible liquids so, it has unique solubilization properties. It carries wide range of drugs due to its both hydrophilic and lipophilic nature thus, attracted attention as potential delivery system (Sabale & Vora, 2012).

As compare to the traditional creams, gels and ointments the main advantage ME has over them is its unique solubilization ability, size and improves stratum corneum hydration which aids in drug permeation and skin flux. The only limitation that microemulsion has is its low viscosity but it can be overcome by formulating microemulsion based hydrogel by using gelling agents such as xanthan gum, Carbopol and hydroxy propyl methyl cellulose (HPMC) (Chen, et al., 2007).

At molecular level, microemulsions are perfectly balance system where surface energies and entropy energies are opposing each other. Entropy is related to the dispersion of the droplet while surface energy is associated with the number of droplets present. Minor change in chemical composition can shift this equilibrium may lead to dramatic changes in behavior of system (Karunaratne, Pamunuwa, & Ranatunga, 2017).

2.4.1 Pseudo-Ternary Phase Diagram

Pseudo-ternary phase diagrams present the microemulsion classification. It is constructed by titration method. The surfactant mix (surfactant+ cosurfactant) and oil are mixed in fixed ratios then water is added dropwise. The point at which the solution become turbid is noted down. Then the pseudo phase diagram is drawn. The diagram is basically equilateral triangles and the corners typically represents the components (oil, water or surfactant) or binary mixture of components (surfactant+ cosurfactant) (Lawrence & Rees, 2000). Each side is divided into 100 equal parts. Therefore, in order to plot a ternary blend, the mass fraction of components should be calculated as percentage by weight (**Figure 2.3**) (Berkman & Güleç, 2021). Creating ternary pseudo phase diagram is time devouring process yet, it is an essential step in microemulsion formulation as it helps to find the region where the microemulsion exists and the effect of variety of surfactant mix weight ratios on the stability of microemulsion area.

After spotting region of microemulsion with help of phase diagram, the microemulsion is formulated in accordance with the desire ratios of components. Then the different tests are conducted and one of the stable microemulsion formulation is selected among many. To study the microemulsion in detail methods including rheology, conductivity, dynamic light scattering and electron micrography used (Karunaratne, Pamunuwa, & Ranatunga, 2017).

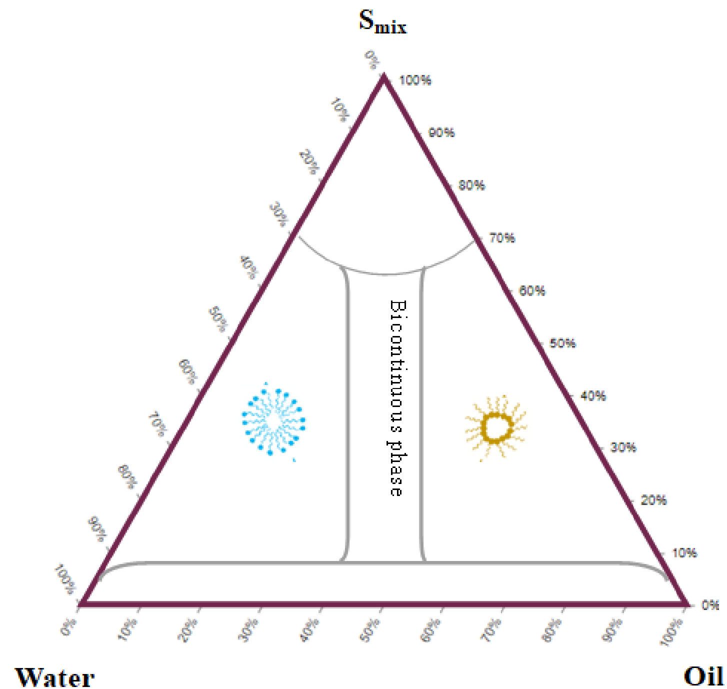


Figure 2.3. Pseudo Ternary Phase Diagram.

Represents phase behavior of microemulsion depending upon the mole ratio of oil, water and Smix (surfactant + cosurfactant). O/W microemulsion droplet (left) and W/O microemulsion droplet (right). The middle is bicontinuous phase.

2.4.2 Applications

Microemulsion has applications in variety of fields for instance food, cosmetics, lubricants, drug delivery, nanoparticle synthesis, biotechnology and chemical reactors. Microemulsion shows pseudo biphasic behavior, which makes them capable of solubilizing highly hydrophilic compounds in oil-based (W/O) systems and highly lipophilic substances in water-based (O/W) system. Presence of nanosized droplets, slow release, protection of active material and ability to penetrate through biological membranes make microemulsion significant in different fields. A brief explanation regarding role of microemulsion in pharmaceutics and cosmetics.

2.4.2.1 Cosmetics

In recent years the use of microemulsion system in cosmeceutics and cosmetics has emerged in many products. The term cosmeceutics is actually a combination of cosmetics and pharmaceutics and is defined as the skincare products that consists of properties more than cosmetics but don't completely to pharmaceutical products. The cosmeceutics usually includes the skincare products related to acne, aging, hyperpigmentation and others.

2.4.2.2 Pharmaceutics

The small size of the microemulsion put them at the advantage of using as delivering system thereby ameliorating drug permeability, solubility and shelf life. The major advantage of microemulsion as delivering system is its ability to carry both hydrophilic and lipophilic drugs effectively through W/O or O/W system respectively (Lawrence & Rees, 2000), (Karunaratne, Pamunuwa, & Ranatunga, 2017). It has been tested and demonstrated that the smaller the size the better its ability to deliver the key component. Nanoemlusion of size 200nm shows great transdermal permeability (Su, et al., 2017).

2.4.2.2.1 Topical Drug Delivery

Topical application of drug is one of the best non-invasive routes to deliver drug to treat various diseases. A microemulsion based hydrogel has been formulated for topical delivery of bifonazole, using HPMC K100M as the gelling agent, against the fungal skin infection. Bifonazole is the broad-spectrum antifungal drug, lipophilic in nature. It has been topically administrated for 2-3 weeks once a day to treat Athlete's foot but absorption through skin is low and the half- life is about 1-2 hours. Therefore, the microemulsion based hydrogel solve all these issues and increase the absorption rate and also the duration of action.3 Similarly, many microemulsion systems have been

developed for topical delivery of many drugs and active compounds as mentioned in **Table 2.5**.

Table 2.5: List of Microemulsion formulations for different purpose.

Sr.No.	Active Compounds	Properties	Microemulsion Formulation	References
1	Salicylic acid	Antimicrobial and fungicidal	Isopropyl Myristate (IPM) and Tween 80	(Badawi, Nour, Sakran, & El-Mancy, 2009)
2	Oxyresveratrol	Against herpes simplex virus infection	IPM, Tween 80 and Isopropanol.	(Sasivimolphan , et al., 2012)
3	Clotrimazole	Antimycotic	IPM, Tween 80 and n-butanol	(Hashem, Shaker, Ghorab, Nasr, & Ismail, 2011)
4	Eucalyptus oil	Antibacterial	Eucalyptus oil, Tween 80 and Ethanol	(Elfiyani, Amalia, & Pratama, 2017)
5	Efavirenz	Bioavailability	Capryol 90, Tween 20 + Cremophor EL and Transcutol HP	(Tandel, Patel, & Jani, 2015)

6	Clotrimazole	Antimycotic	Clove oil, Brij-35 and ethanol or 1-propanol	(Siddique, et al., 2021)
7	Griseofulvin	Antifungal	Oleic acid, Tween 80 and ethanol	(Iradhati & Jufri, 2017)
8	Dexamethasone	Anti-inflammatory	Almond/ Olive/ Linseed/ Nutmeg oil, Egg lecithin and Isopropyl alcohol	(Chandra, Sharma, & Irchhiaya, 2014)
9	Gallic acid	Anti-inflammatory, anti-oxidant, antiviral and antibacterial.	Olive oil, Tween 20 and Polietilen glikol 400	(Böncü, Yücel, Karatoprak, & Takır, 2021)
10	Curcumin	Anti-oxidant, anti-inflammatory and wound healing	Grape seed oil, Tween 80+Plurol® Diisostearique CG and Ethanol	(Scomoroscenco, et al., 2021)

2.4.3 Microemulsion Against Wounds

Due to advantages of microemulsion over the other delivery system and formulations for wounds, microemulsion gets the limelight to treat different types of wounds whether burn, diabetic, or other wounds. Fusidic acid is antibiotic agent which belong to steroids

category but doesn't have corticosteroids which exhibit anti-inflammatory or immunosuppressive properties. For skin infection and related problems doctor recommend topical application of fusidic acid to be effective. Therefore, the fusidic acid loaded microemulsion gel has been prepared for burns. The study helps to indicate that microemulsion gel with the fusidic acid is more effective in exhibiting its antibacterial properties and healing ability in comparison against the commercially available fusidic acid cream (Okur, et al., 2020).

In another study, a unique combination of the microemulsion-based hydrogel is formulated with the virgin coconut oil (VCO) and gellan gum (GG) for the material of wound dressing. The wound dressings available may have toxic material leads to other skin related diseases. The material used for making this wound dressing is non-toxic and natural one. GG is biodegradable, biocompatible and nontoxic while VCO is natural product, with antioxidant activity and rancidity free. From i*n vivo* studies it is observed that the wound dressing with GG and VCO shows commending results. No skin irritation was observed during treatments and the healing condition of wound was satisfactory. The formulated wound dressing provides the moist and suitable for the re-epithelization thus, enhance wound healing (Muktar , et al., 2021).

Another microemulsion formulation, loaded with the coenzyme Q10 (CoQ10) was prepared and tested on the human keratinocyte (HaCaT) cell lines. The results were positive for cell proliferation assay and wound healing scratch assay (Ryu, et al., 2020).

Not only synthetic compounds but also the plant extract is also been loaded in microemulsion to aid natural compounds to display their ability as wound healer. Curcumin is the compound found in *Curcuma longa* which possesses anti-oxidant activity and detoxify the enzymes. Microemulsion gel loaded with curcumin was

formulated to reduce the wound closure time. The curcumin is effective for wound healing but the limitations like bioavailability and stability put hinderance in way of using it as potential treatment for wounds. Thus, microemulsion gel act as delivery system to help curcumin not only to display its anti-oxidant property and help in healing but also aid in keeping the wound moist to promote wound closure (Guo, Pu, Liu, Lo, & Yen, 2020).

The microemulsion based hydrogel has been prepared with hexane extract of *Moringa oleifera* seeds. The study helps to confirmed that microemulsion gel prepared by the hexane extract of *M. oleifra* shows positive results regarding the cell proliferation, epithelization and granular tissue formation thus, reducing healing closure time (Ali , et al., 2021).

The individual components *T. serpyllum*, ascorbic acid and hydrogel based microemulsion have been reported to be used effectively against the wounds. Thus, synergetic effect of these three will give a positive response towards wound healing.

Chapter 3: Methodology

3.1 Plant Extraction

The extract of *Thymus serpyllum* was prepared by the Soxhlet extraction method. The seeds of *T. serpyllum* were taken (**Figure 3.1**) and grind to fine powder. The large particles were stained out and the fine powder with uniform size particles was weigh about 25g and the porous thimble was filled with the seed powder. Subsequently, 250 ml of absolute ethanol, as solvent, was added to round bottom flask, that is then connected to the Soxhlet extractor as shown in **Figure 3.2**.

Figure 3.1. *Thymus serpyllum* Seeds.

After extraction the solvent is removed by evaporating the solvent at room temperature (25ºC). scratch the extract and store it at 4ºC to maintain its medicinal properties.

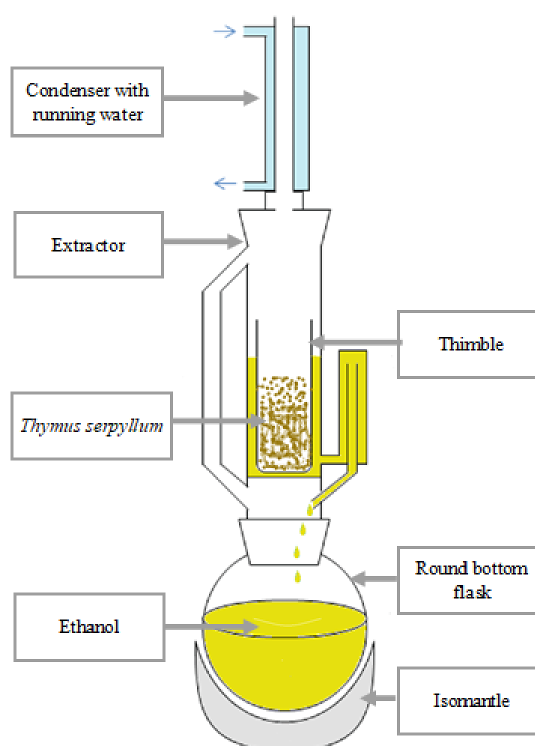


Figure 3.2. Soxhlet Apparatus Setup.

3.2 Phytochemicals

After extraction, the crude plant extract was tested for qualitative analysis of the following phytochemicals which are required for treating wounds. The function along with the observations are shown in the **Table 3.1** (Beseni, et al., 2017), (Hossain, AL-Raqmi, AL-Mijizy, Weli, & Al-Riyami, 2013).

3.2.1 Phenols

To check the availability of the phenol, 1ml of *T. serpyllum* extract was taken in test tube then few drops of $FeCl_3$ was added in it.

3.2.2 Flavonoids

Took 1ml extract of *T. serpyllum* inn test tube then added 1ml of 10% $Pb(C_2H_3O_2)_4$ to check the availability of flavonoid.

3.2.3 Saponins

For testing the saponins, 5ml of wild thyme seeds extract was taken in the test tube then 5ml of warm water was added then shook it vigorously for froth formation.

3.2.4 Anthraquinones

3ml extract of wild thyme seeds was taken then 3ml of benzene was added with the 5ml of 10% NH_3 to confirm the presence of the anthraquinones.

3.2.5 Steroids

To check the presence of the steroids, 2ml of chloroform was added to 2ml of extract followed by 2ml of concentrated sulfuric acid.

3.2.6 Cardiac glycosides

In order to test the cardiac glycosides 2ml extract of was taken and 2ml of acetic acid, few drops of ferric chloride along with 1ml of concentrated sulfuric acid.

Table 3.1. Phytochemical analysis.

Metabolites	Functions	Observations
Phenols	Anti-microbial, anti-inflammatory, anti-oxidant, analgesic, anesthetic, disinfectant and antiseptic (Zhang, Cai, Cheng, & Zhang, 2022)	Bluish Black color
Flavonoids	Anti-inflammatory, anti-viral, anti-microbial and anti-oxidant (Kumar & Pandey, 2013)	Yellow precipitate
Saponins	Anti-inflammatory, reduce skin inflammation and edema (Barbosa, 2014)	Froth appearance
Anthraquinones	Anti-inflammatory and anti-microbial (Feng & Wang, 2020)	Pink, Violate or Red coloration
Steroids	Anti-inflammatory, anti-eczematic, anti-diabetic and analgesic (Dembitsky, Savidov, Poroikov, Gloriozova, & Imbs , 2018)	Reddish brown color at upper layer
Cardiac glycosides	Collagen synthesis, anti-tumor activity, against cardiac failure (El-Shemy, 2017), (El-Okdi, et al., 2008)	Violet layer with brown to reddish color

3.3 Total Phenolic Content

3.3.1 Principle

Total phenolic content (TPC) is quantitative analysis assay for phenol in the plant extract. It is founded on the Folin-Ciocalteu (FC) method. The FC have

phosphotungstic/ phosphomolybdic acid complexes. The principle depends upon the electron transfer in alkaline condition from the phenolic compound to produce the blue chromophore formed by the complex of phosphotungstic/ phosphomolybdenum and the absorption is directly related with the concentration of phenolic compound. The reduced FC reagent is detected with the spectrophotometer within in the range from 690-710 nm. The increase in temperature is related to the to achieve the maximum color in less time. Usually, gallic acid (GA) is used as the standard and the result are demonstrated in mg/ml as gallic acid equivalents (GAE) (García, et al., 2015).

3.3.2 Procedure

Gallic acid was taken as standard and the stock was prepared (1mg /ml). The concentrations were prepared for the standard (25, 50, 75, 100 µg/ml) while for the test sample 5mg/ml stock was taken. 0.4ml of FC reagent was added in 1ml of test sample and standard of different concentrations. 4ml of 7.5% of aqueous sodium carbonate solution was added after 5 minutes. Following this water was added to make up to 10ml of the total volume. The prepared mixture was incubated for 2 hours in dark at room temperature. The absorbance was checked at 700nm through spectrophotometer. For the determination of TPC linear regression is used from a gallic acid calibration curve standard (Beseni, et al., 2017) and to measured TPC in mg GAE/g of extract following calculations were done.

$$y = \text{slope } x \text{ intercept (from gallic acid)}$$

$$x = (y - \text{intercept})/\text{slope}$$

$$x = \text{in mcg/ml}$$

$$x \text{ or } c = \text{in mg/ml}$$

$$C = c(V/m)$$

Where, C is TPC, c is extract in mg/ml, V is volume of extract used in ml and m is mass of extract in grams

3.4 Total Flavonoid Content

3.4.1 Principle

For determination of total flavonoid content (TFC) aluminium chloride ($AlCl_3$) colorimetric method was used. The basic principle is that either with C-3 or C-5 hydroxyl and C-4 keto group of flavonols or flavones $AlCl_3$ forms acid stable complexes and the absorbance can be measured at 415nm or 510nm depending upon the chemical used to make different complexes (Ahmed & Iqbal, 2018).

3.4.2 Procedure

Rutin was taken as standard and the stock was prepared in methanol (1mg/ml). The concentrations were prepared for standard (25, 50, 75, 100 µg/ml) while for the test sample 5mg/ml stock was taken. 100 µl of 10% aluminium chloride was added in the 100 µl of test sample and standard of different concentrations. Then 100 µl of 1M of potassium acetate and 2800 µl of distilled water. The resultant mixture was left at 25 $\pm$ 1°C for 30 minutes. The absorbance was measured at 415nm by spectrophotometer. For the determination of total flavonoid content (TFC) linear regression is used from a rutin calibration curve standard (Beseni, et al., 2017) and to measured TFC in mg rutin equivalent /g of extract following calculations were done.

y=slope x intercept (from rutin)

x= (y-intercept)/slope

x= in mcg/ml

x or c= in mg/ml

$$C=c(V/M)$$

Where, C is TFC, c is extract in mg/ml, V is volume of extract used in ml and m is mass of extract in grams

3.5 Toxicity Test

3.5.1 Principle

The destruction of the red blood cells due to toxic compounds result in release of the hemoglobin in the surrounding medium. This assay work on this principle, the toxicity of drug is measured by using this sensitive method by measuring the extent of hemolysis.

3.5.2 Procedure

The procedure was modified (Zohra & Fawzia, 2014), (Greco, et al., 2020). The solutions required were prepared beforehand. 0.2M of PBS was prepared in water with pH 7.5 and 0.85% of saline was prepared by dissolving 0.85 g of NaCl in 100 ml of water. The different concentrations of the extract were prepared in PBS by serial dilution method i.e., 5000, 500, 50, 5 and 0.5 µg/ml. 20 ml of triton X was mixed in 80 ml of water through sonication to get 20% of it.

The fresh blood was used after removing plasma by centrifugation at 4000 rpm. The RBC (red blood cells) pellets were washed 3 to 4 times with the saline solution, which was added equal to volume of the plasma. After that, 2 % of the RBC solution was prepared by using the PBS. 100 µl of different concentrations of extract prepared were added in 190 µl of blood solution. Similarly, for negative control PBS was added in place of extract while for positive control triton X was added. Then it was incubated for 30 minutes at 37ºC. Following incubation, it is then centrifuged for 6 minutes at 2500x

g. The absorbance was measured at 520 nm with spectrophotometer. The hemolysis percentage was measured by using the following formula

(Sample- negative control)/ (positive control- negative control) *100

Where, negative control was PBS in blood and positive control was 20% triton X in blood.

By using the linear regression equation $\%H_{50}$ (hemolysis percentage) was calculated to know how much of the concentration is required to cause 50 % hemolysis.

3.6 Microemulsion Formulation

3.6.1 Materials

Tween 20 (polyoxyethylene sorbitan monolaurate), ascorbic acid, castor oil (cold pressed), ethanol, *T. serpyllum* seeds, distilled water and deionized water.

3.6.2 Solubility

Solubility determination of the active ingredient is necessary to know in which component it should be dissolved. It is also helpful to know the effect of the active ingredient on microemulsion structure. If its soluble in all the components then chance of structure difference is less.

3.6.2.1 Procedure

The excess amount of the extract and the ascorbic acid was added in 1 ml of each water, oil (castor oil), surfactant (tween 20) and cosurfactant (ethanol) and the mixture formed was kept at 37 $\pm$ 1°C for 72 hours. Then the mixture was centrifuged for 10,000rpm for 10 minutes and the supernatant was filtered and the amount of both extract and ascorbic acid was determined by using spectrophotometer at 405 nm and 280 nm for extract and ascorbic acid respectively.

3.6.3 Construction of Pseudo-Ternary Phase Diagram

Pseudo ternary phase diagram helps to find the microemulsion region and it was constructed by the water titration method at 25 $\pm$ 1°C temperature. A total of nine different phase diagrams were constructed with 1:1, 1:2, 3:1, 4:1, 6:1, 8:1, 5:4, 7:6 and 11:10 weight ratios of tween 20 to ethanol respectively and for each diagram, the specific weight ratios of castor oil to Smix (tween 20 and ethanol) were taken as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Then water was added in dropwise manner until the mixture become turbid under constant stirring. The values were note down and then the pseudo ternary diagram was drawn with the final concentration of the components obtained in percentage w/w. With help of Chemix School software the pseudo phase diagram was constructed. With the help of these phase diagram the suitable concentrations of the components were selected and the microemulsion was prepared (Akram, et al., 2019).

3.6.4 Preparation of Microemulsion

The microemulsions were formulated by using different ratios combination of components according to the microemulsion area in the diagram. The formulations prepared were by adding Smix (tween 20 and ethanol), castor oil and water to make up to 100% (w/w). This microemulsion is blank means without the extract and ascorbic acid and known as bM.E.

3.6.5 Microemulsion with *T. serpyllum* and Ascorbic Acid

For the microemulsion with the *T. serpyllum* and ascorbic acid, 4.5 mg of extract and 15 mg of ascorbic acid was dissolved in 1 ml of the components in which they have high solubility. This one is simply called M.E.

3.7 Microemulsion Evaluation

A total of 40 formulations were prepared from the selected phase diagrams region and further selections were made on the basis of these following parameters and all the experiments were performed in triplicate (Tandel, Patel, & Jani, 2015).

3.7.1 Centrifugation

The microemulsion formulations were centrifuged for 30 minutes at 13,000 rpm to check the physical stability of the prepared formulations. The formulations that were stable and didn’t show the phase separation were taken for further parameters.

3.7.2 Freeze Thaw Cycle

The formulations were put through six cycles between -20 $\pm$ 1°C and 25 $\pm$ 1°C at each temperature for less than 48 hours. The stable ones were studied further.

3.7.3 Heat Cool Cycle

The formulations were put through six cycles between 4 $\pm$ 1°C and 45 $\pm$ 1°C at each temperature for less than 48 hours. The stable ones were studied further.

3.7.4 Dilution Test

The formulated microemulsions were diluted in ratios 1:10 and 1:100 with water in order to observe any phase separation (Tandel, Patel, & Jani, 2015).

3.7.5 Dye Solubility

The water-soluble dye such as bromothymol blue (pH indicator) was added in the microemulsion system to get clear view of the microemulsion system.

3.7.6 Transparency

The percentage transparency was measured for the microemulsions in triplicate by using the spectrophotometer at 620 nm using distilled water as blank. The absorbance was converted to the % transparency.

$$\%T = \text{antilog}\ (2 - \text{absorbance})$$

The above-mentioned evaluation methods were performed only for bM.E but from pH onward evaluation methods were performed for both bM.E and M.E.

3.7.7 Measurement of pH

The pH of the formulations was measured by using the pH meter at room temperature and all the measurements were conducted in triplicates.

3.7.8 Droplet Size and PDI

Droplet size is the mean diameter of the microemulsion while polydispersity index (PDI) helps to know the homogeneity of the droplets of microemulsion. Lower the value of PDI higher the homogeneity similarly, higher the PDI higher the variation in the droplet size.

The droplet size and the PDI of the microemulsion were measured with the Malvern ZetaSizer at $25 \pm 1^{o}C$. For testing, 10 µl of the microemulsion was diluted in the 1 ml of the deionized water and the results obtained were in triplicate.

3.7.9 Zeta Potential

Zeta potential is the physical property of the particle in the material surface, macromolecule or suspension. It helps in prediction of long-term stability. if charge is either negative or positive the system is stable but if the value is 0mV then the system is not stable and the phase separation will occur.

The zeta potential of the microemulsion were measured with the Malvern ZetaSizer at $25 \pm 1^{o}C$. For testing, 10 µl of the microemulsion was diluted in the 1 ml of the deionized water and the results obtained were in triplicate.

3.7.10 Viscosity

Viscosity of the selected formulation was tested by using spindle 64 for 1 minute at rotational speed of 0.5 rpm at 25 $\pm$ 1°C. A total of 10ml was used for measuring the viscosity and the instrument used was Brookfield Rheometer DVIII.

3.7.11 Stability

In order to test the stability of microemulsion, it was kept at 25 $\pm$ 2°C and 4 $\pm$ 2°C for 60 days and then pH was measured and centrifugation was performed along with single phase maintenance.

3.8 Bioactivity Assays

All the tests were performed for standards, *T. serpyllum* extract and M.E and in triplicates.

3.8.1 Antioxidant

3.8.1.1 DPPH Scavenging Assay

3.8.1.1.1 Principle

The antioxidant assay based on the free radical method by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate). This method is based on the principle of electron transfer to produce the violet color solution in methanol. The free radical produced is stable at room temperature and is reduced when anti-oxidant is present resulting in colorless solution (Garcia, et al., 2012).

3.8.1.1.2 Procedure

39.4 mg of DPPH was dissolved in 1000 ml methanol to prepared the 0.1 mM solution of DPPH. Then ascorbic acid, extract or microemulsion was dissolved in methanol in equal w/v (weight by volume) ratio (1mg/1ml). The concentration of test sample and standard were taken as 10, 20, 30 and 40 µg/ml. For 10, 10 µl of the solution was taken

from the stock, then 990 µl of methanol was added and concentrations were prepared in v/v (volume by volume). Total volume that was prepared was 200 µl which was consisted of 150 µl of different concentration of testing material in methanol and 50 µl of DPPH solution. Following this, it was then incubated for 1 hour, then the absorbance was checked using spectrophotometer at 550 nm. The percentage inhibition was measured by using the following formula.

[(absorbance of control-sample)/control] *100

Where, control was DPPH solution in methanol while blank was methanol.

By using the linear regression equation (from the scatter plot) IC_{50} (inhibition concentration) was calculated to know how much of the concentration is required to inhibit 50 % of the activity.

3.8.2 Anti-Inflammatory Assay

3.8.2.1 Protein Denaturation

3.8.2.1.1 Principle

This anti-inflammatory test was based on the inhibition of albumin protein denaturation. Protein is denatured and loses its tertiary structure as result of external stimulus. The BSA (bovine serum albumin) is heat denatured and the denaturation is shown by the turbidity of the BSA (Leelaprakash & Dass, 2011).

3.8.2.1.2 Procedure

Stock solution of test sample and standard was prepared in w/v (1 mg/1 ml) in water while for microemulsion concentrations were prepared with methanol. Aspirin was taken as standard for this test. The total of 5 ml µg/ml mixture was prepared which consisted of 0.2 ml of 1% aqueous solution of BSA, 2.8 ml of 0.2 M PBS (phosphate buffer saline) and 2 ml of the standard and the test sample of different concentration

i.e., 0.1, 1, 10 and 100 µg/ml. The mixture was incubated at 37 ± 1°C in water bath for 15 minutes and then at 70 ± 1°C for 5 minutes. Then the mixture was allowed to reach room temperature then the absorbance was measured at 620 nm. The percentage inhibition was measured by using following equation

100*[1-(absorbance of sample/absorbance of control)]

Where, control was PBS

The blank was the test mixture without BSA for standard, extract and microemulsion of different concentrations.

By using the linear regression equation (from scatter plot) IC_{50} (inhibition concentration) was calculated to know how much of the concentration is required to inhibit 50 % of the activity.

3.8.3 Anti-Microbial Activity

3.8.3.1 Principle

Disk diffusion method is based on the principle that disks saturated in the antibiotic are laced on the agar inoculated with the test bacterium strain and the antibiotic in the disk get diffuse outward forming the concertation gradient.

3.8.3.2 Procedure

The stock of antibiotic and plant extract was prepared in DMSO (dimethyl sulphoxide). 10 µg/ml of ampicillin (antibiotic) was used while 1 mg/ml and 2 mg/ml of plant extract was used along with 20 µl of DMSO as control. Antimicrobial activity was checked against *Staphylococcus aureus* and *Escherichia coli* bacteria. The disk of 6mm were saturated with the standard and sample. On other hand 0.5 g of microemulsion was loaded on the disk to know it activity against the two bacteria. The colonies were mixed

in normal saline (0.085%) and the absorbance was 0.5 measured at 600nm taking normal saline as blank which follows McFarland.

The plates were lawned with inoculum and then the disks were placed at equal distance. The plates were sealed with paraffin. The prepared plates were incubated at 37 $\pm$ 1°C for 16-20 hours then the reading was noted (Hudzicki, 2009).

3.9 Animal Model

All the healthy female BALB/c mice were procured from the ASAB animal house lab after getting permission from the Ethic Review Committee of the department.

3.9.1 Skin Irritability

All the components used Tween 20, castor oil, ethanol and xanthan gum are considered to be safe to use and the doses used for each component are much less than their toxic effect (Tesoro). But still there are many components that can cause the skin irritation. Therefore, skin irritability test was conducted to confirm the safety of the microemulsion .

For the irritability test, 8-9 weeks old female albino mice were taken and the method performed was according to the Soliman et.al. The mice were divided into three groups: control group, standard group and microemulsion group. The control group didn’t receive any treatment while the standard group received 0.8% formalin solution and the last group was treated with the microemulsion. The dorsal side of the mice were shaved 12 h before the test. For 3 days consecutively, 0.5ml formalin and 0.5g of formulated M.E were applied to observe any signs of skin irritation such as edema and erythema (Soliman, Malak, El-Gazayerly, & Rehim, 2010).

3.9.2 Excision Wounds

For excision wounds, a total number of 12, 8-12 weeks old healthy albino female mice were procured. The mice were then acclimatized to the environment. The animals were given anesthesia with chloroform and the hairs were removed from $4cm^2$ area. The wounds were caused by using the biopsy punch with 6mm diameter. They were divided into six groups as in **Table 3.2** (Masson-Meyers, et al., 2020).

Table 3.2: Groups for excision wounds.

Group no.	Group names
1	Control (no treatment)
2	Standard
3	Plant extract
4	bM.E
5	M.E

The wound contraction percentage was measured by using the following equation:

$$\%\text{Wound contraction} = \frac{\text{area of wound on day 0} - \text{area of wound on day n}}{\text{area of wound on day 0}} \times 100$$

3.9.3 Diabetic Wounds

The 8-12 weeks old albino BALB/c female mice were given 75% high fat diet (HFD) for 5 weeks continuously. The streptozotocin (STZ) was injected intraperitoneal for 4 consecutive days, 3 weeks after HFD. The HFD was continued till the treatment time. The dose of STZ given was 40mg/kg. The STZ was weighed and mixed in sodium citrate buffer maintained at pH 4.5. STZ degrades even in acidic solution therefore, STZ

should be injected within 5 minutes of mixing it with buffer. Following injections 10% sucrose was given to the animals along with the HFD. After 10th day of administrating STZ the fasting blood glucose level was checked to confirm the type 2 diabetes mellitus in mice (T2DM). The blood glucose level was checked twice a week during treatment to confirm the condition (**Figure 3.3**) and then they were divided in to groups as mentioned in **Table 3.3** (Parveen, Hussain, & Khan, 2018). For the wounds, same procedure was followed as for excision wounds.

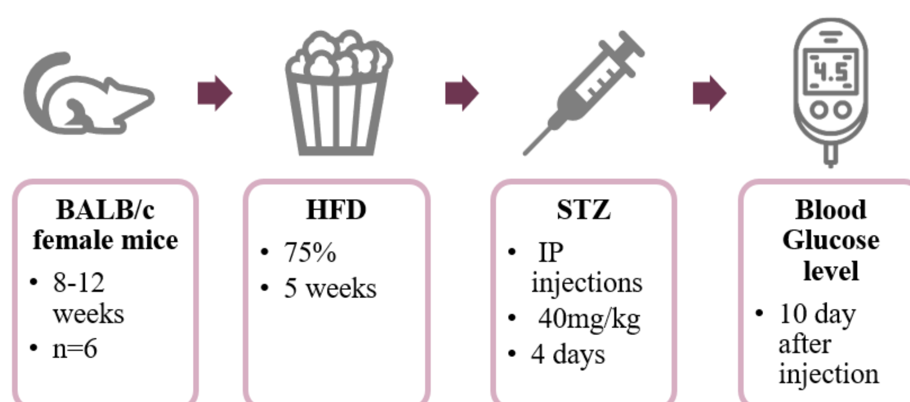


Figure 3.3: Overview of diabetes model.

Table 3.3: Groups for diabetic wounds.

Group no.	Group names
1	Control (no treatment)
2	Standard
3	M.E

Chapter 4: Results

4.1 Plant Extraction

After extraction, through Soxhlet method the extract (**Figure 4.1**) was air dried and stored at 4 $\pm$ 1°C.

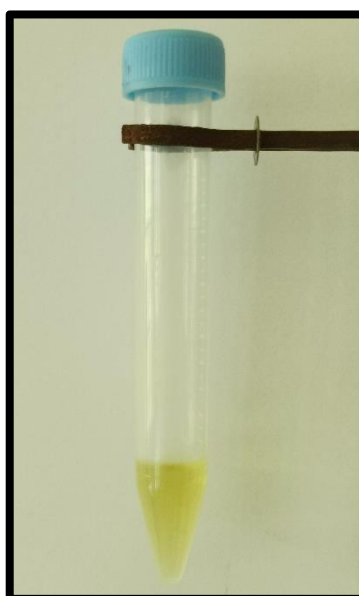
Figure 4.1: *T. serpyllum* ethanolic extract

4.2 Phytochemicals

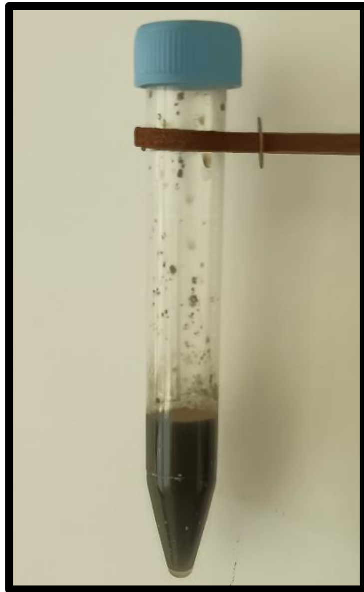
In order to determine the presence of different metabolites qualitative analysis were performed and the results are shown in **Table 4.1** and **Figure 4.2**.

Table 4.1: Phytochemicals result.

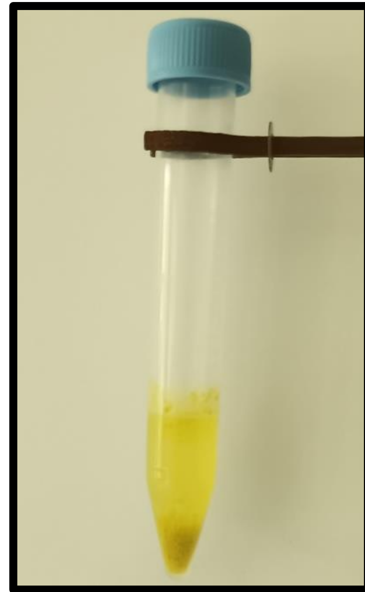
Metabolites	Results
Phenols	+
Flavonoids	+
Saponins	-
Anthraquinones	-
Steroids	+
Cardiac glycosides	+



a: Before



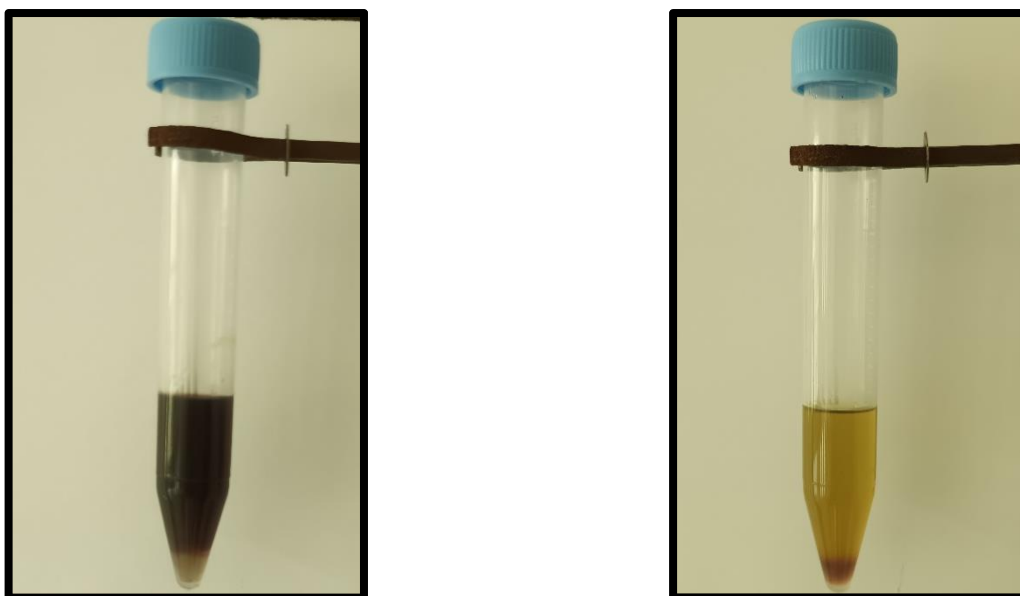
b: Phenol



c: Flavonoid

d: Saponins

e: Anthraquinones



f: Steroid **g:** Cardiac glycosides

Figure 4.2: Phytochemical Analysis Result.

Statistical Analysis

The standard deviation and mean of all the results were calculated by using Microsoft Excel. For analysis of results independent t-test one sample t-test and ANOVA (one-way analysis of variance) was applied by using SPSS. If confidence interval was 95% and $p<0.05$, the results were statistically significant.

4.3 Total Phenolic Content

The graph is plotted (**Figure 4.3**) to estimate total phenolic content by gallic acid and for seeds extract it was expressed as mg gallic acid equivalent per gram of extract. The total phenolic content of *T. serpyllum* was 226.3 $\pm$ 10.56 mg/g.

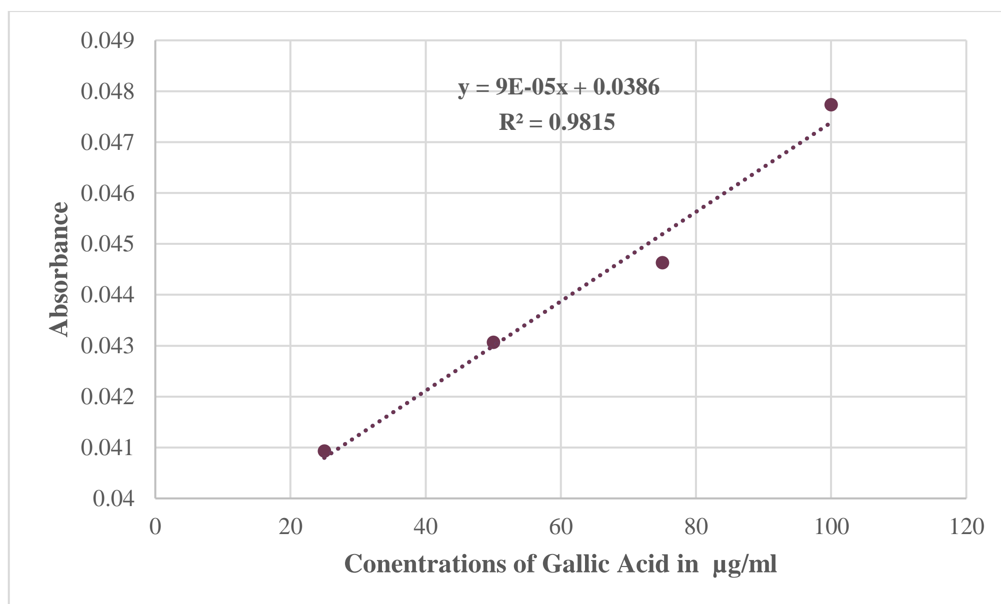


Figure 4.3: TPC Standard Curve.

4.4 Total Flavonoid Content

The graph is plotted (**Figure 4.4**) to estimate total flavonoid content by rutin and for seeds extract it was expressed as mg rutin equivalent per gram of extract. The total flavonoid content of *T. serpyllum* was 50.29 $\pm$ 3.4 mg/g.

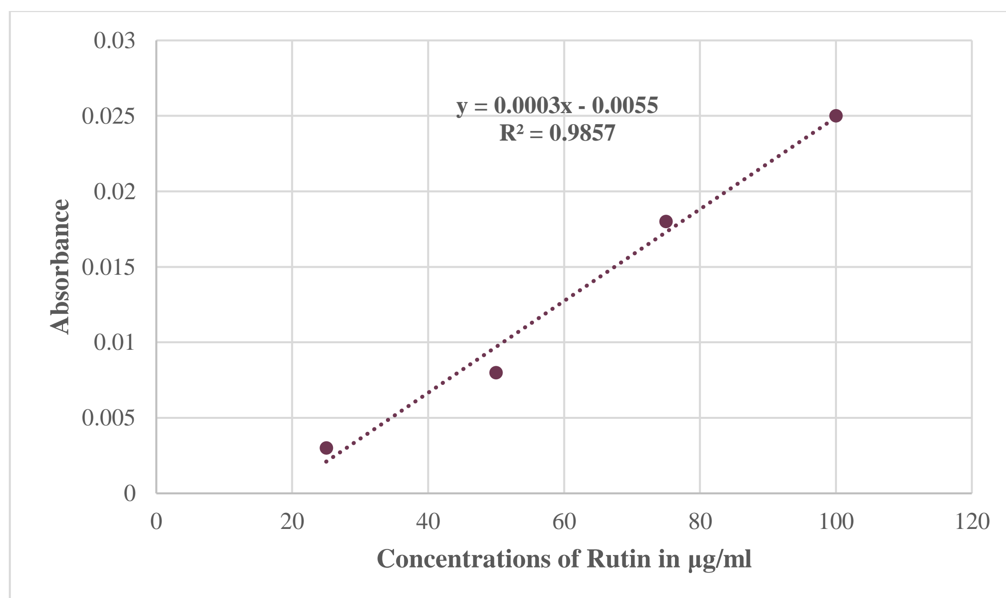


Figure 4.4: TFC Standard Curve.

4.5 Toxicity Test

The toxicity of *T. serpyllum* extract was found out by blood hemolysis in the **Figure 4.5** and **Figure 4.6**. The $\%H_{50}$ was calculated by using linear regression. The IC_{50} of *T. serpyllum* extract was 35854 µg/ml or 35.854 mg/ml.

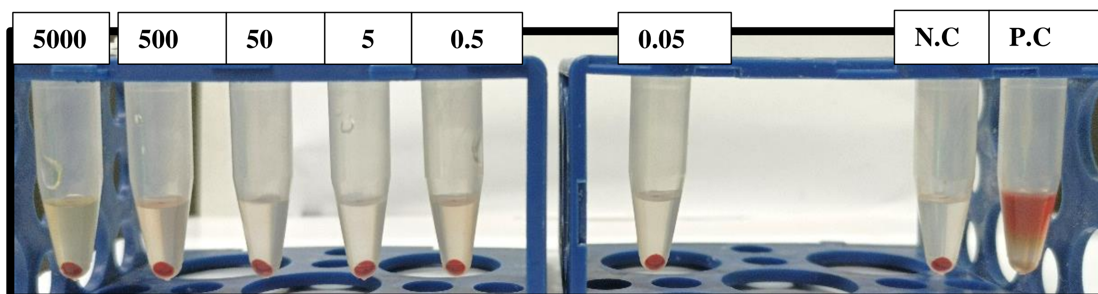


Figure 4.5: *T. serpyllum* Blood Hemolysis Test.

The concentration ranges from 5000- 0.05 µg/ml while N.C was negative control while P.C was positive control.

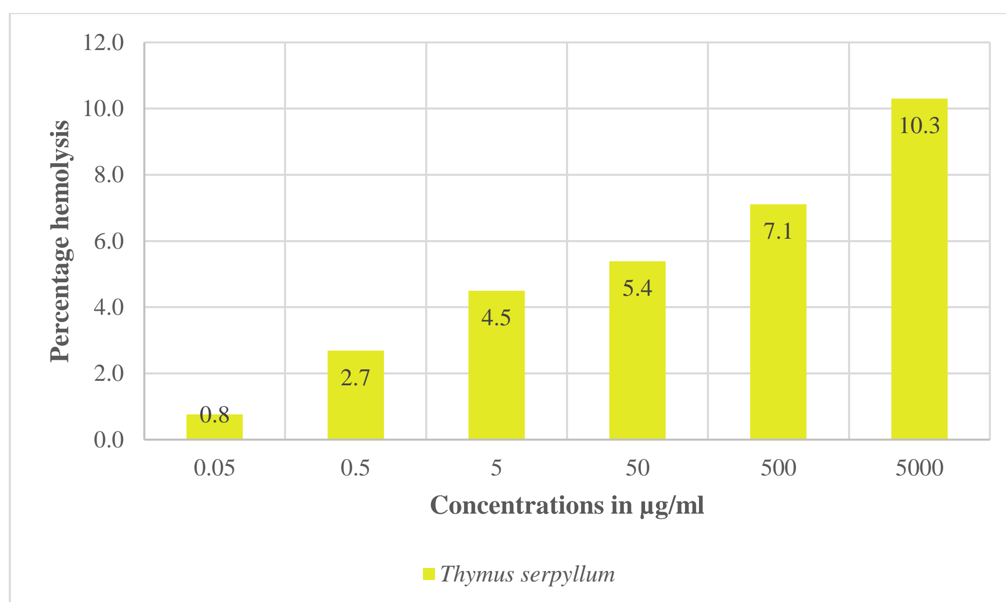


Figure 4.6: Percentage Hemolysis by *T. serpyllum.*

4.6 Microemulsion Formulation

4.6.1 Solubility

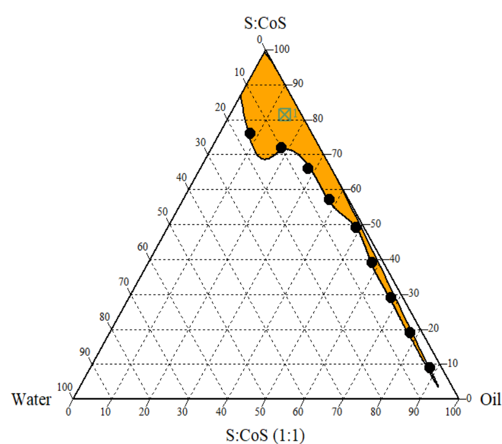
The solubility of both extract at 405 nm and ascorbic acid at 280 nm in different components of microemulsion was arranged in **Table 4.2**.

Table 4.2: Solubility in M.E's components.

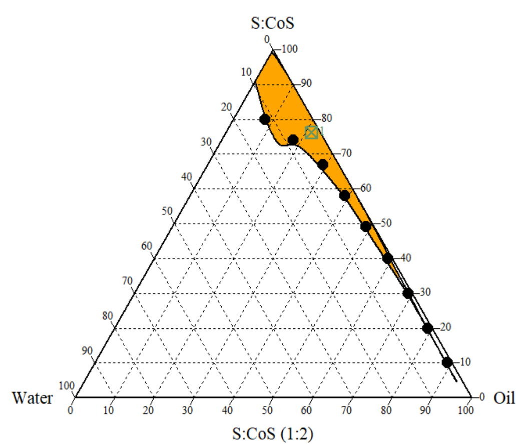
M.E's components	*T. serpyllum* mg/ml + S. D	Ascorbic acid mg/ml + S. D
Ethanol	4.98 + 0.26	2.67 + 0.25
Tween 20	0.53 + 0.02	0.02 + 0.005
Castor oil	0.07 + 0.008	0.04 + 0.01
Water	2.34 + 0.25	37.26 + 0.69

4.6.2 Construction of Pseudo-Ternary Phase Diagram

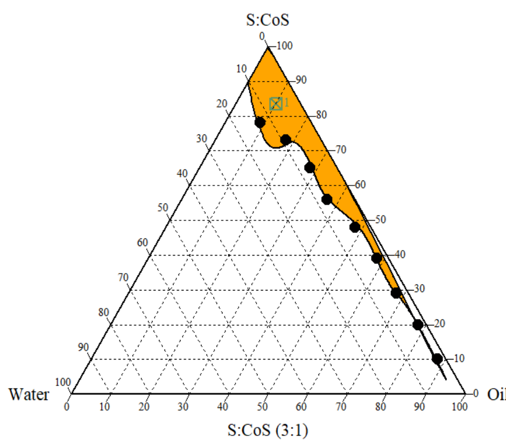
The pseudo ternary phase diagram (**Figure 4.7**) shows that one with the 7:6 and 11:10 ratios had the large microemulsion area therefore, these were taken for the preparation of the microemulsion whereas surfactant is Tween 20 and cosurfactant is ethanol.



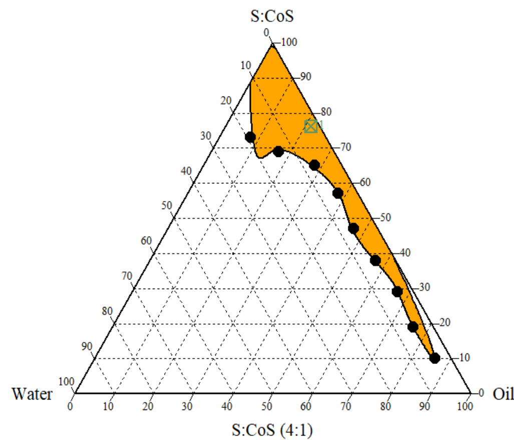
a: S:CoS (1:1)



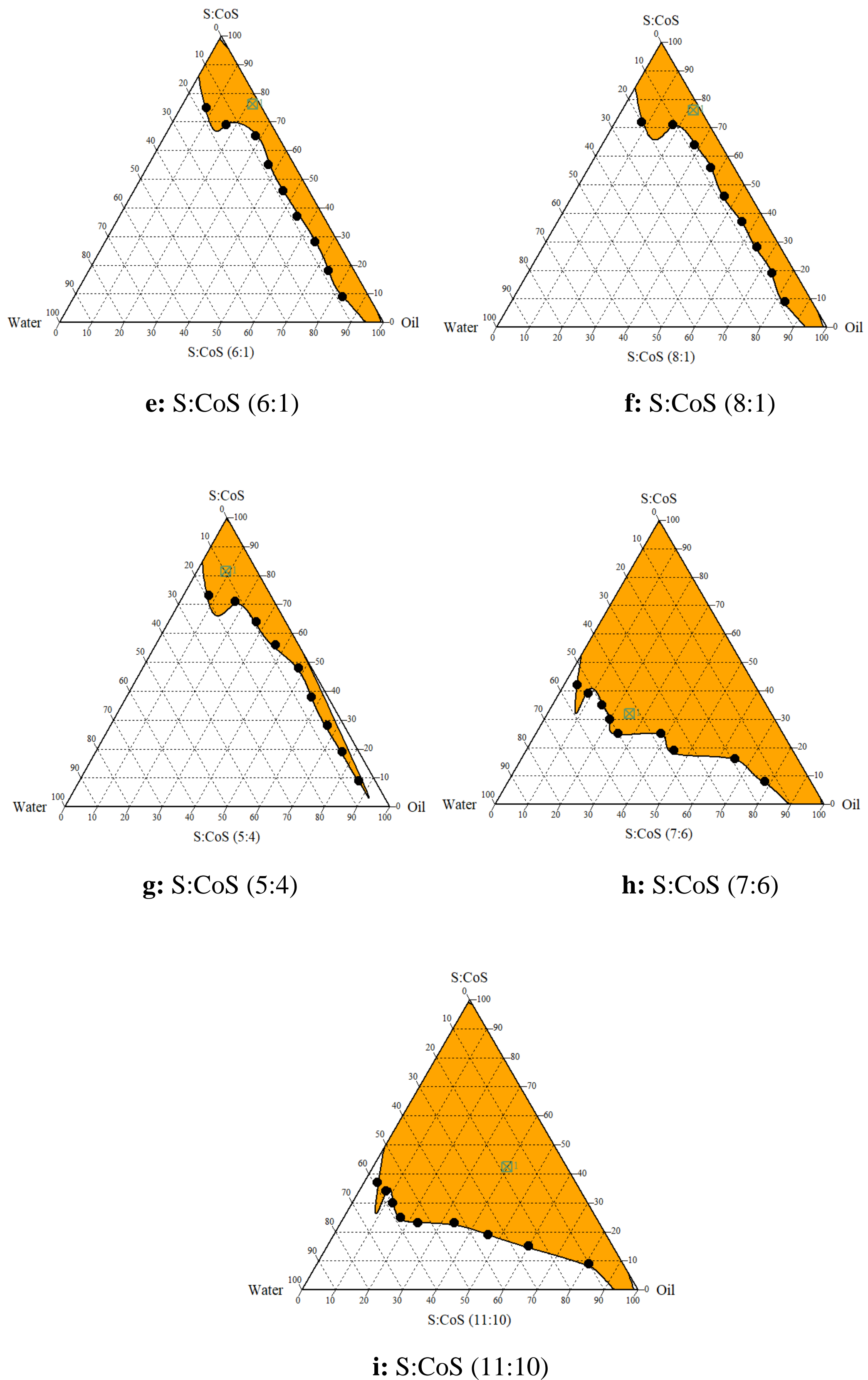
b: S:CoS (1:2)



c: S:CoS (3:1)



d: S:CoS (4:1)



e: S:CoS (6:1)

f: S:CoS (8:1)

g: S:CoS (5:4)

h: S:CoS (7:6)

i: S:CoS (11:10)

Figure 4.7: Pseudo-Ternary Phase Diagram.

4.6.3 Preparation of Microemulsion

The microemulsions were formulated from the 7:6 and 11:10 and the total of 40 formulations were prepared (**Table 4.3**).

Table 4.3: Microemulsion formulations.

Microemulsion Formulations			
F1	F11	F21	F31
F2	F12	F22	F32
F3	F13	F23	F33
F4	F14	F24	F34
F5	F15	F25	F35
F6	F16	F26	F36
F7	F17	F27	F37
F8	F18	F28	F38
F9	F19	F29	F39
F10	F20	F30	F40

4.6.4 Microemulsion with *T. serpyllum* and Ascorbic Acid

The **Table 4.2** showed *T. serpyllum* and ascorbic acid had maximum solubility in ethanol and water respectively. The resultant M.E was clear as well just like the bM.E with slight color of the extract.

4.7 Microemulsion Evaluation

The **Figure 4.17** gave summary of microemulsion evaluation process.

4.7.1 Centrifugation

Before centrifugation (**Figure 4.8**), all the formulations had single phase but after centrifugation (**Figure 4.9**), only the stable one maintained the single phase shown in **Table 4.4**.

Table 4.4: Microemulsions with single phase.

M.E Formulation	Phase	M.E Formulation	Phase
F12	Single	F28	Single
F13	Single	F29	Single
F14	Single	F30	Single
F15	Single	F33	Single
F19	Single	F34	Single
F21	Single	F35	Single
F23	Single	F37	Single
F24	Single	F38	Single
F25	Single		

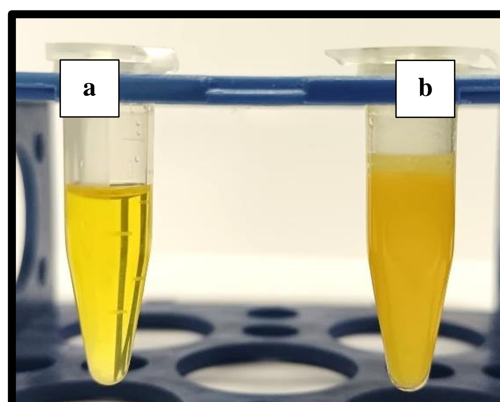


Figure 4.8: Before Centrifugation.

Both **a** and **b** had single phase.

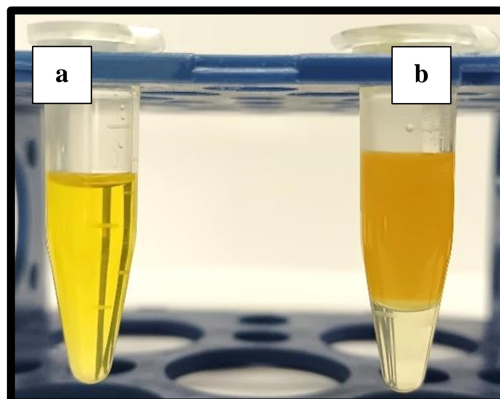


Figure 4.9: After Centrifugation.

a had single phase but **b** had double phase.

4.7.2 Freeze Thaw Cycle

After six cycles these formulations i.e., F12, F13, F14, F15, F19, F21, F23, F24, F25, F28, F29, F30, F33, F34, F35, F37 and F38 were further studied (**Figure 4.10**).

4.7.3 Heat Cool Cycle

After six cycles these formulations i.e., F12, F13, F14, F15, F19, F21, F23, F24, F25, F28, F29 F30, F33, F34, F35, F37 and F38 were further studied for dilution test (**Figure 4.10**).

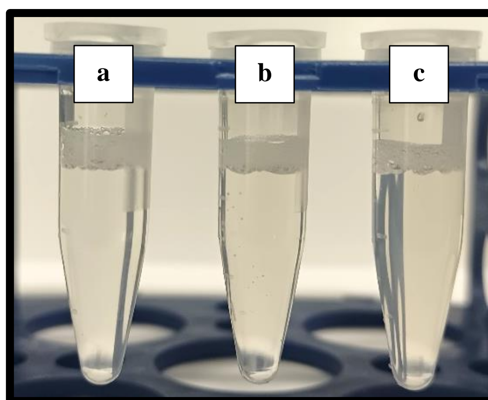


Figure 4.10: Freeze Thaw and Heat Cool Cycle.

After freeze thaw and heat cool cycle **a**, **b** and **c** remained clear.

4.7.4 Dilution Test

Following freeze thaw and heat cool cycle, clear formulations (**Figure 4.11**) were F12, F13, F14, F15, F19, F21, F23, F24, F25, F28, F29 and F30.

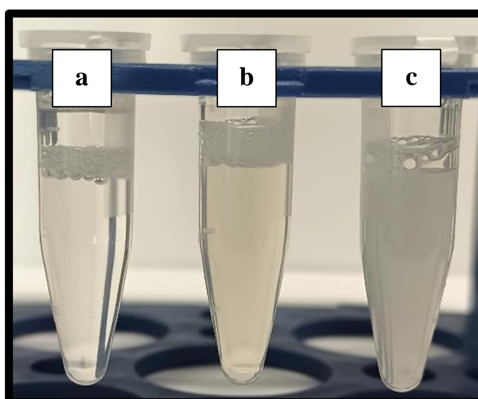


Figure 4.11: Dilution Test.

When dilution test was performed **a** was transparent, **b** was translucent and **c** was opaque.

4.7.5 Dye Solubility

The microemulsion formed had O/W system which was confirmed by adding water soluble dye as the dye flow all over and didn't form microscopic clumps as shown in **Figure 4.12** which can be observed if system was W/O.

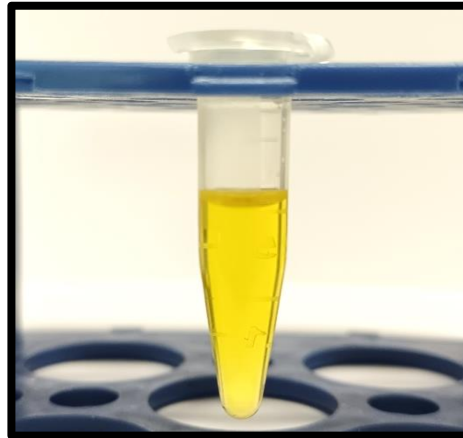


Figure 4.12: O/W Phase.

4.7.6 Transparency

After calculation, one with the percentage transparency greater than 90 were selected as shown in **Table 4.5** and all the results were significant as $p<0.05$.

Table 4.5: Transparency of selected microemulsion.

M.E Formulation	% Transparency ± S. D
F12	92.05 ± 0.22
F13	91.27 ± 0.10
F14	91.62 ± 0.38
F15	90.96 ± 0.17
F19	90.52 ± 0.26
F21	92.45 ± 0.41
F28	95.28 ± 0.22
F29	97.50 ± 0.12
F30	90.78 ± 0.17

4.7.7 Measurement of pH

Out of 9, only 2 formulations had pH 4.5-6, in both blank and microemulsion with the ingredient ($p<0.05$) as shown in **Table 4.6**.

Table 4.6: pH of selected bM.E and M.E with active ingredients.

M.E Formulation	pH ± S. D (bM.E)	pH ± S. D (M.E)
F28	5.92 ± 0.01	5.57 ± 0.006
F29	5.58 ± 0.01	5.16 ± 0.031

4.7.8 Droplet Size and PDI

The droplet size and PDI of both the bM.E and M.E were presented in the **Table 4.7** and shown in **Figure 4.13** and **Figure 4.14** respectively. The droplet size of the bM.E and M.E were 77.49 nm and 94.95 nm with low PDI 0.644 and 0.349 respectively which depicts the uniformity among droplet size of microemulsion in both bM.E and M.E. The change in size and PDI can be due to *T. serpyllum* and ascorbic acid but it was revealed by sample t-test that these changes were non-significant (p>0.05).

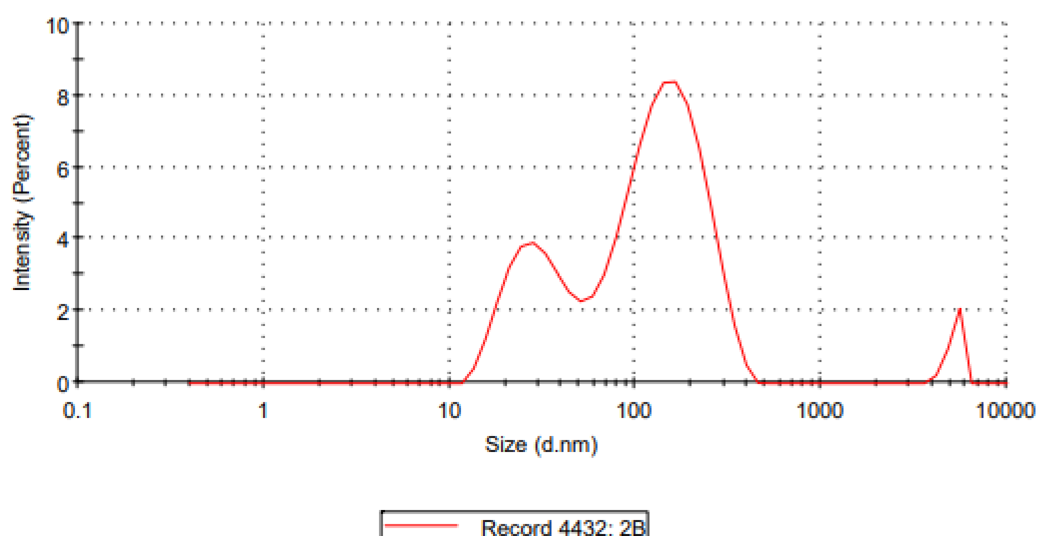


Figure 4.13: Size Distribution by Intensity of bM.E.

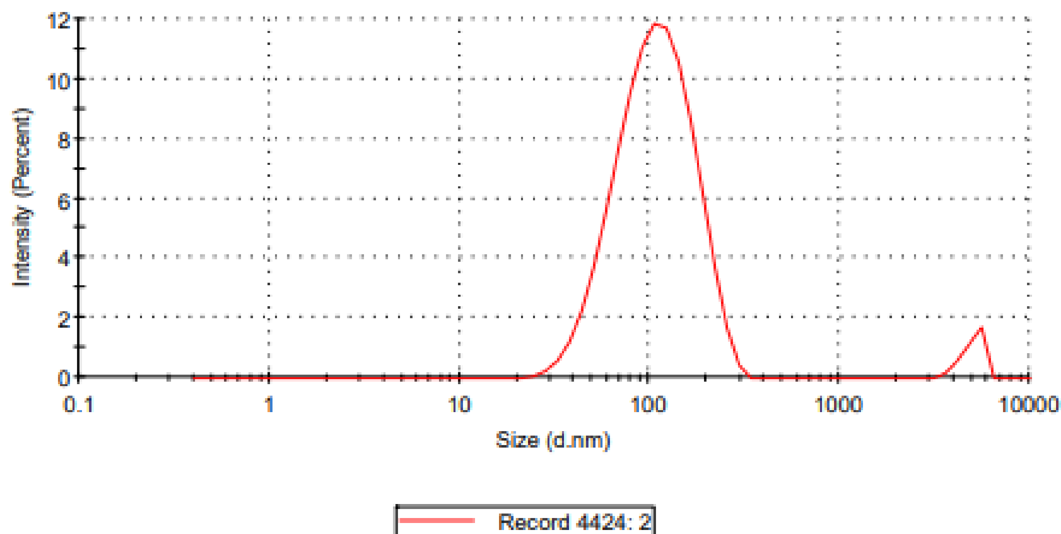


Figure 4.14: Size Distribution by Intensity of M.E.

4.7.9 Zetapotential

The zetapotential of both the bM.E (**Figure 4.15**) and M.E (**Figure 4.16**) was non-significant ($p>0.05$) and were presented in **Table 4.7**.

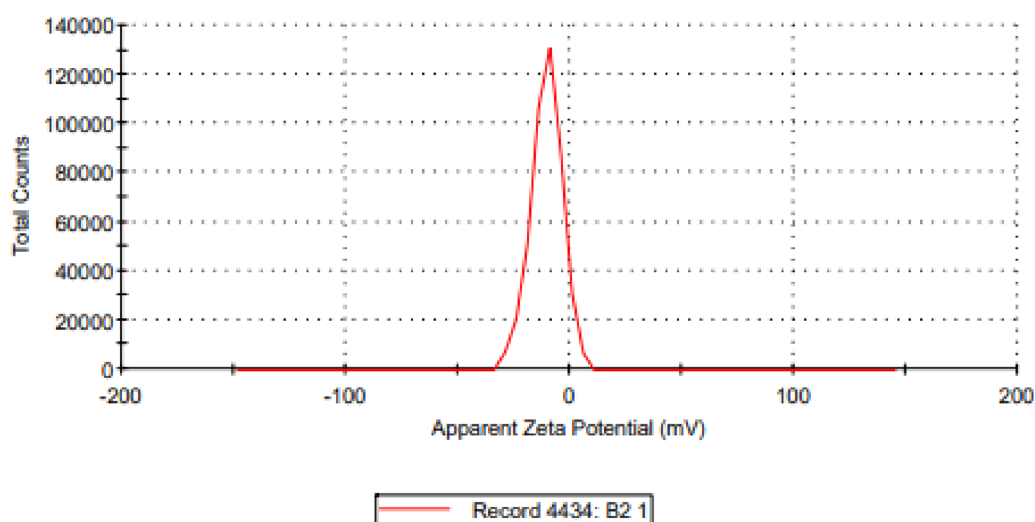


Figure 4.15: Zeta Potential Distribution of bM.E.

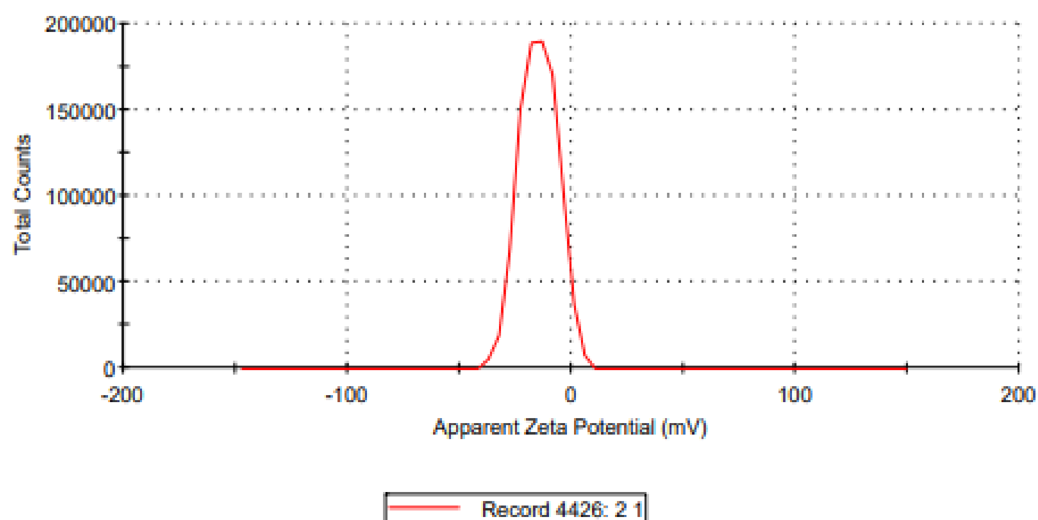


Figure 4.16: Zeta Potential Distribution of M.E.

Table 4.7: Droplet size, PDI and zetapotential of bM.E and M.E.

Test	bM.E	M.E
Droplet size nm $\pm$ S.D	77.49 $\pm$ 11.6	94.95 $\pm$ 7.1
PDI $\pm$ S.D	0.644 $\pm$ 0.09	0.349 $\pm$ 0.02
Zetapotential mV $\pm$ S.D	-10.4 $\pm$ 0.2	-14.6 $\pm$ 0.9

4.7.10 Viscosity

The viscosity of selected formulation was 100 $\pm$ 3.8cP

4.7.11 Stability

The microemulsion was stable for 60 days and the differences were non-significant between microemulsion formulated 60 days before and the one after storing at 4°C and 25°C.

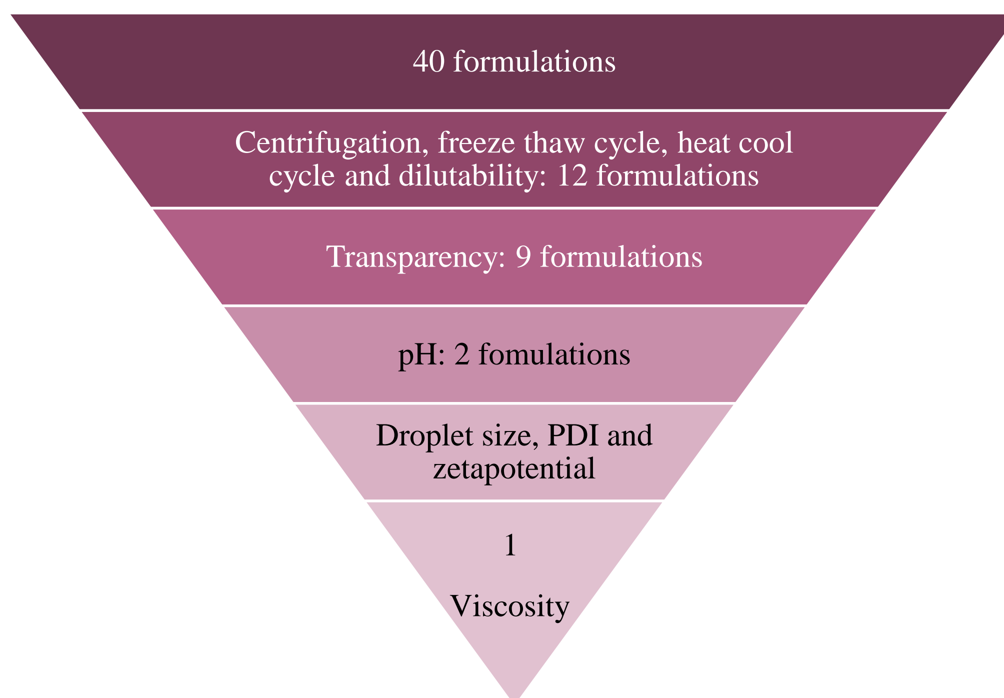


Figure 4.17: Summary of M.E Selection.

4.8 Bioactivity Assays

4.8.1 Antioxidant

4.8.1.1 DPPH Scavenging Assay

The DPPH scavenging activity of standard (**Figure 4.18**), *T. serpyllum* extract (**Figure 4.19**) and microemulsion were compared in **Figure 4.20**. The IC_{50} was calculated by using linear regression. The IC_{50} of standard was 22.4 µg/ml while at the 20 µg/ml of concentration the *T. serpyllum* extract and microemulsion showed 78% and 89.3% of the inhibition respectively. Independent t-test displayed that microemulsion had higher antioxidant activity as compared to both standard and plant extract ($p<0.05$).

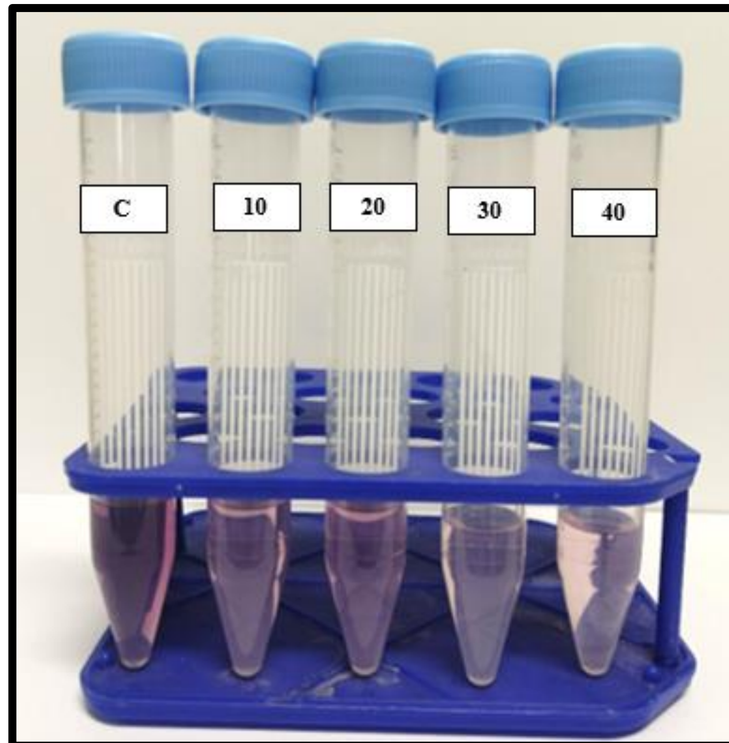


Figure 4.18: DPPH of Standard.

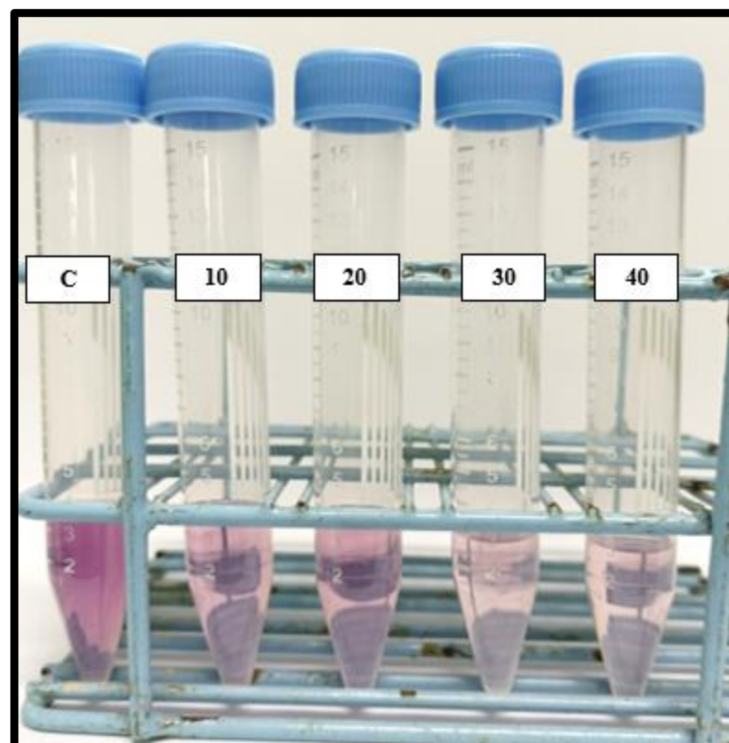


Figure 4.19: DPPH of *T. serpyllum.*

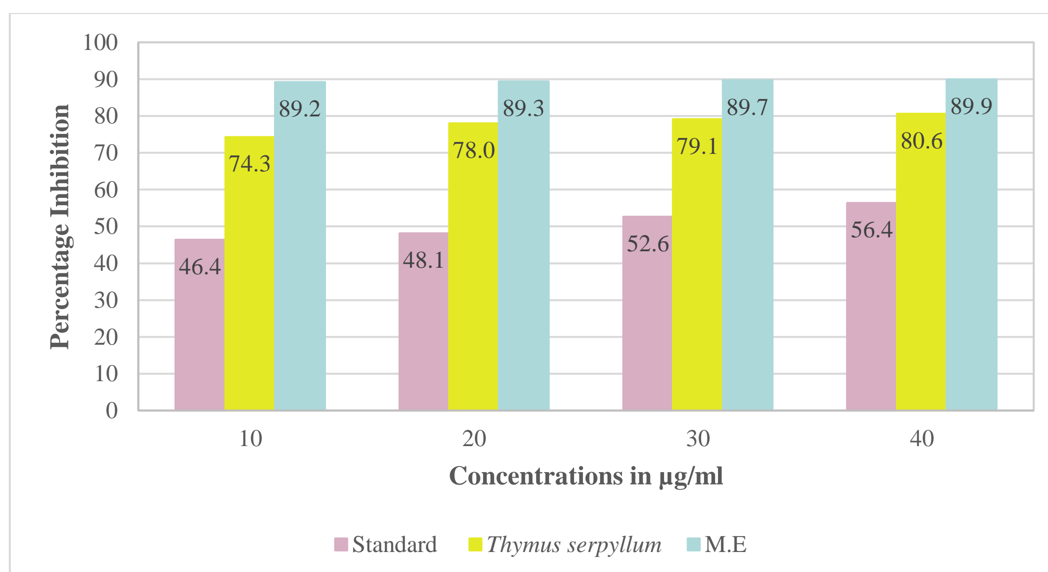


Figure 4.20: Antioxidant Activity of Standard, *T. serpyllum* and M.E.

4.8.2 Anti-Inflammatory Assay

4.8.2.1 Protein Denaturation

The anti-inflammatory activity through protein denaturation of standard, *T. serpyllum* extract and microemulsion were compared in **Figure 4.21**. The IC_{50} was calculated by using linear regression shown in **Table 4.8**. The difference between standard and plant was non-significant ($p>0.05$) while with microemulsion the difference is significant.

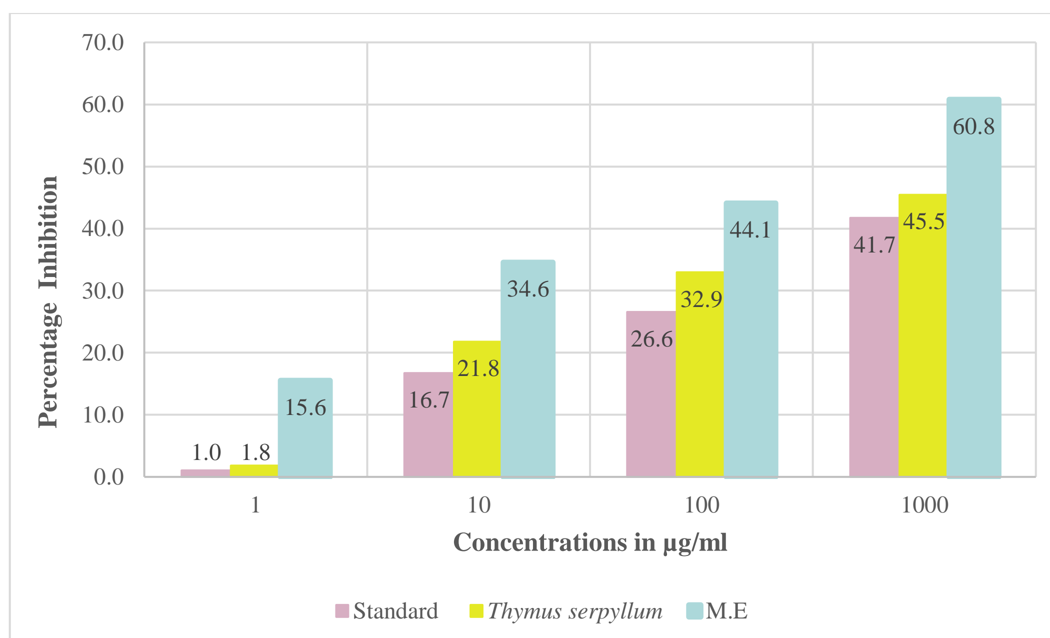


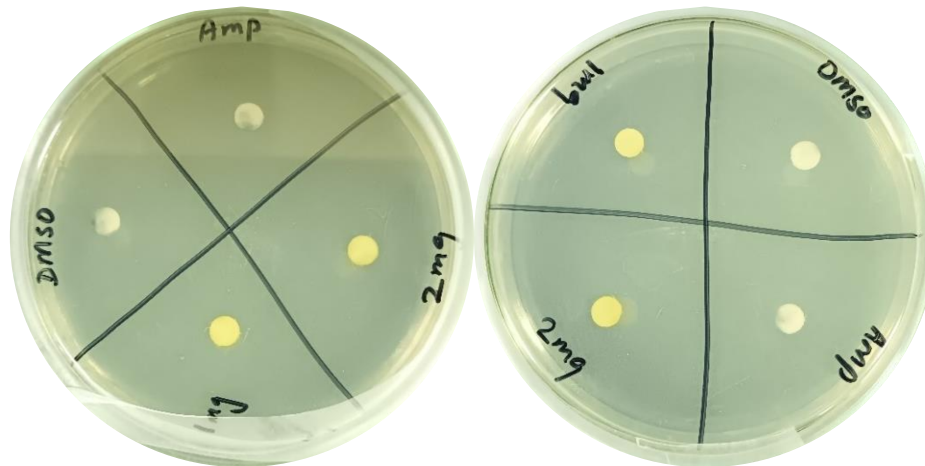
Figure 4.21: Anti-Inflammatory Activity of Standard, *T. serpyllum* and M.E.

Table 4.8: IC_{50} of standard, extract and M.E.

IC_{50} of Standard	IC_{50} of *T. serpyllum*	IC_{50} of M.E
1247 µg/ml	1111 µg/ml	628 µg/ml

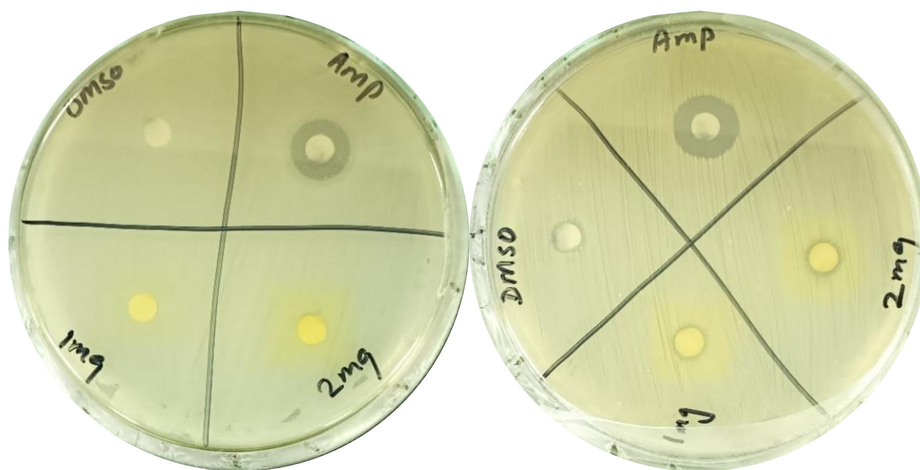
4.8.3 Anti-Microbial Activity

The anti-microbial activity of standard, *T. serpyllum* extract and microemulsion were checked after 20 h of incubation as shown in **Figure 4.22** and **Figure 4.23** respectively through disk diffusion method and the **Table 4.9** shows the reading.



a: *E. coli* **b:** *S. aureus*

Before Incubation.



a: *E. coli* **b:** *S. aureus*

After Incubation.

Figure 4.22: Culture Plates for *T. serpyllum* and Controls.

a: *S. aureus* **b:** *E. coli*

Before Incubation.

a: *S. aureus* **b:** *E. coli*

After Incubation.

Figure 4.23: Culture Plates for Microemulsion and Control.

Table 4.9: Readings of zone of inhibition.

Disk	*S. aureus* (diameter ± S.D)	*E. coli* (diameter ± S.D)
Ampicillin 10 µg/ml	13 ± 1 mm	13 ± 1 mm
T. serpyllum extract 1mg/ml	0	0
T. serpyllum extract 2mg/ml	0	0
Microemulsion 10 µg	10 ± 1 mm	0

The difference was non-significant ($p>0.05$) between zone of inhibition formed by microemulsion and ampicillin on *S. aureus.*

4.9 Animal Model

4.9.1 Skin Irritability

In comparison to normal skin **Figure 4.24**, no visible sign of erythema and edema on the skin of mice were observed but when microemulsion was applied, the skin was appeared to be smooth and soft as shown in **Figure 4.25**. On other hand, when formalin was applied on skin of mice, they felt irritation with discoloration and dryness of skin was also observed as seen in **Figure 4.26**.

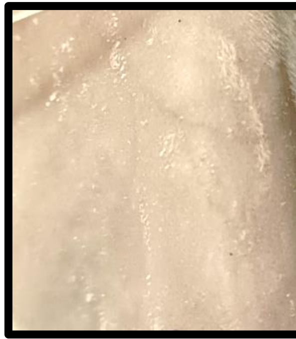


Figure 4.24: Normal Skin Before any Application.

Figure 4.25: Microemulsion Applied on Skin.

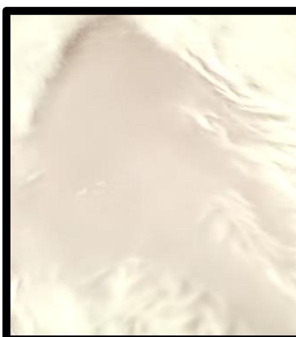


Figure 4.26: Formalin Applied on Skin.

4.9.2 Excision Wounds

Excision wounds were observed for 11 consecutive days (**Figure 4.27**). Day 0 is the when the wound was inflicted and first treatment was given while Day 1 is 24 hours after treatment. The wound closure rate / percentage was measured by using the formula mentioned in **Table 4.10** and was presented in **Figure 4.28**.

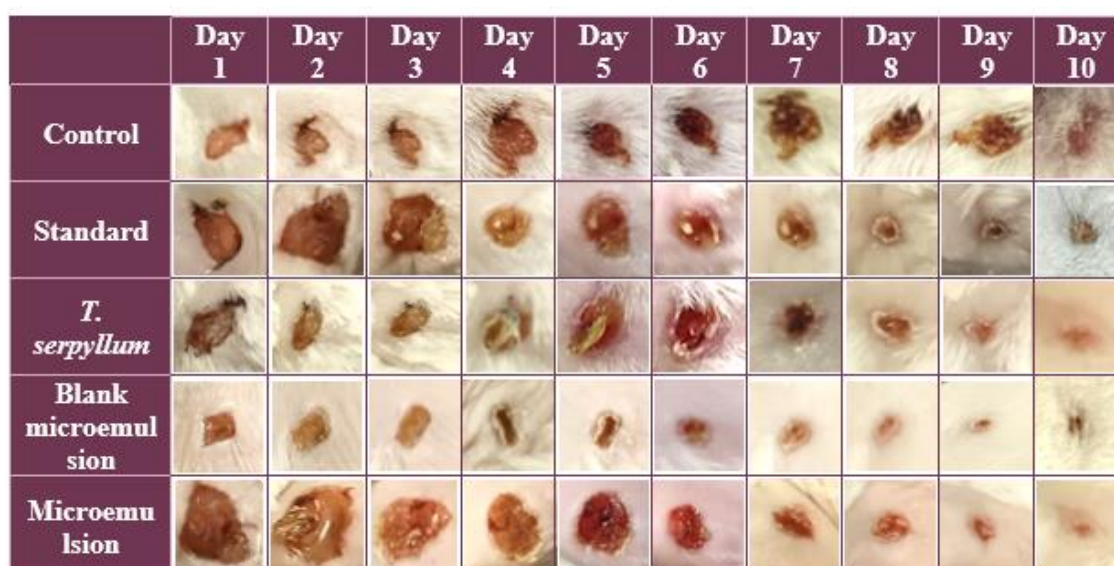


Figure 4.27: Wound Contraction on Post Wounding Days.

Table 4.10: Wound Contraction rate in excision wounds.

Days	Group 1 ± S.D %	Group 2 ± S.D %	Group 3 ± S.D %	Group 4 ± S.D %	Group 5 ± S.D %	Significance p<0.05
1	17.3 ± 0.02	8.5 ± 1.7	18.9 ± 0.09	5.4 ± 0.57	22.5 ± 2.03	0.01
2	25.4 ± 1.45	31.7 ± 0.92	35.9 ± 1.30	21.5 ± 0.73	36.0 ± 0.13	0.000
3	40.2 ± 1.25	45.3 ± 0.07	43.7 ± 0.10	40.9 ± 0.05	42.2 ± 0.04	0.000
4	53.5 ± 3.15	51.6 ± 0.14	50.9 ± 1.11	40.9 ± 2.44	48.1 ± 1.07	0.000
5	59.5 ± 2.10	62.9 ± 0.27	57.7 ± 1.04	49.5 ± 2.08	59.0 ± 2.40	0.000
6	65.1 ± 2.74	62.9 ± 2.07	63.9 ± 0.27	64.7 ± 0.87	72.9 ± 1.07	0.000
7	70.2 ± 0.73	72.7 ± 1.74	77.4 ± 0.02	68.1 ± 0.35	76.9 ± 0.48	0.000
8	70.2 ± 1.10	77.1 ± 1.54	83.9 ± 1.05	77.2 ± 3.52	84.0 ± 0.25	0.000
9	86.7 ± 0.09	81.0 ± 0.56	92.4 ± 1.10	84.8 ± 2.77	89.7 ± 2.07	0.000
10	92.5 ± 0.07	87.9 ± 3.07	97.7 ± 0.61	96.2 ± 1.35	99.3 ± 1.57	0.000

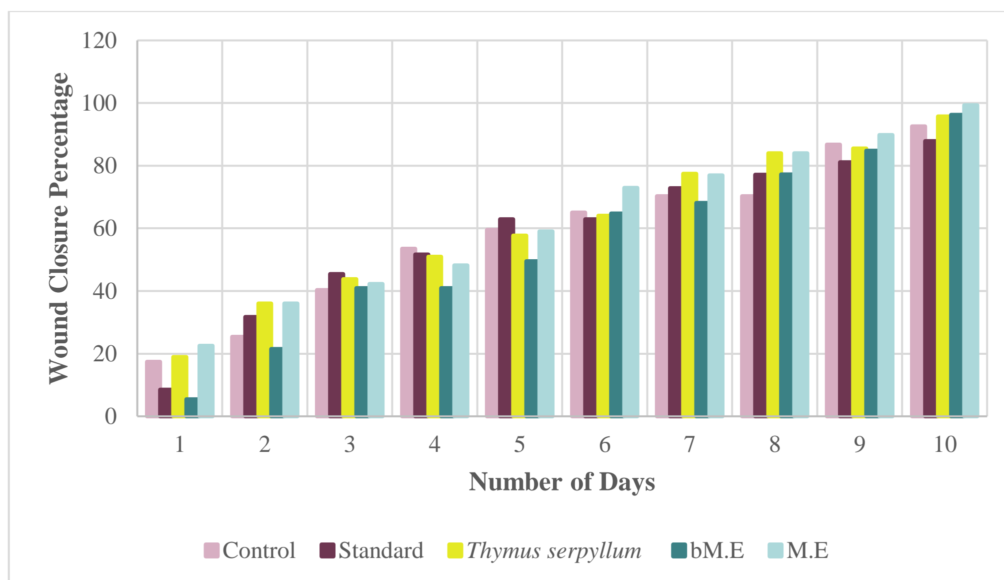


Figure 4.28: Wound Closure Rate among Different Groups.

4.9.3 Diabetic Wounds

Diabetic wound was observed for 11 days as well (**Figure 4.29**). The wound closure rate / percentage was measured by using the formula mentioned in **Table 4.11** was presented in **Figure 4.30**.

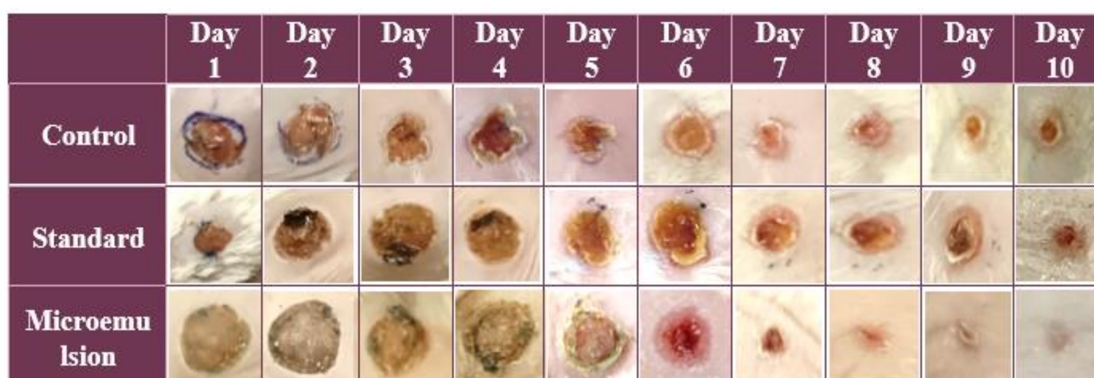


Figure 4.29: Wound Contraction on Post Wounding Days.

Table 4.11: Wound contraction rate in diabetic wounds.

Days	Group 1 $\pm$ S.D %	Group 2 $\pm$ S.D %	Group 3 $\pm$ S.D %	Significance $p<0.05$
1	0 $\pm$ 0.12	15.3 $\pm$ 0.11	21.7 $\pm$ 0.18	0.195
2	10.7 $\pm$ 0.73	36.0 $\pm$ 1.04	28.3 $\pm$ 1.04	0.079
3	30.5 $\pm$ 0.02	36.0 $\pm$ 1.54	34.7 $\pm$ 0.05	0.002
4	47.8 $\pm$ 2.42	36.0 $\pm$ 0.03	40.8 $\pm$ 3.52	0.007
5	62.6 $\pm$ 1.32	48.1 $\pm$ 0.19	40.8 $\pm$ 0.73	0.016
6	62.6 $\pm$ 0.04	59.0 $\pm$ 2.40	57.2 $\pm$ 2.42	0.001
7	62.6 $\pm$ 1.45	72.9 $\pm$ 0.73	85.2 $\pm$ 1.7	0.008
8	77.6 $\pm$ 0.92	80.6 $\pm$ 3.12	94.6 $\pm$ 0.02	0.004
9	88.8 $\pm$ 2.74	84.0 $\pm$ 0.27	98.6 $\pm$ 2.08	0.002
10	95.0 $\pm$ 0.05	92.1 $\pm$ 1.71	100 $\pm$ 1.74	0.001

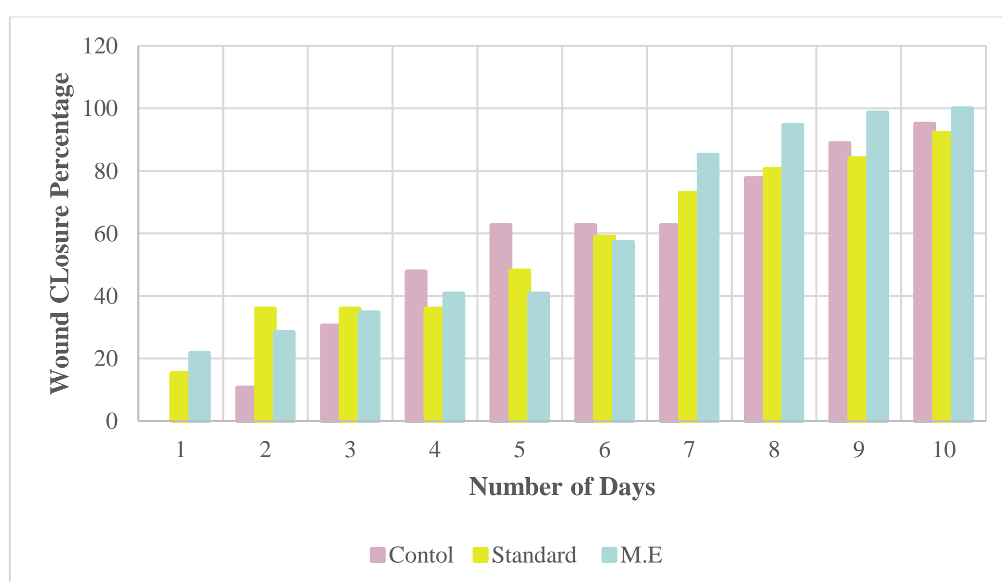


Figure 4.30: Wound Closure Rate among Different Groups.

Chapter 5: Discussion

Skin is the outermost layer covering 15% of the body and shields it against extrinsic factors. It has self-healing property due to presence of these different cells in epidermis and dermis but if the desecration is beyond the certain range and depth these cells get affected and are unable to perform their function then foreign help is required whether its medicine or transplant (Barbieri, Wanat, & Seykora, 2014). Wound is defined as the disruption of the skin intentionally or unintentionally. It always remains a challenge for clinician as skin desecration open a gap for the microbes to invade causing morbidity and mortality. Different treatments options are available for wounds such as wound dressing, antimicrobial, physical treatment and grafting (Heras, Igartua, Santos-Vizcaino, & Hernandez, 2020). Despite of being used for several years these treatments have their own limitations and the increase in demand for natural products due to their nontoxic and useful effects a new treatment method is required for treating wounds. This treatment method is microemulsion-based hydrogel with combination of both *Thymus serpyllum* and ascorbic acid which might be more effective for wound healing.

Oxygen is one of the important elements required for yield of ATP, that is required for tissue regeneration. Normal levels of ROS are beneficial for wound healing but if the level rises healing will be impaired and the situation gets under the oxidative stress (Dunnill, et al., 2015). Bacterial infection is one of the problems faced by clinicians as it results in symptoms like pain, erythema, pus and cellulites (Jones, et al., 2005). Microbial attack, oxidative species and comorbidities also caused wounds to stuck in the inflammatory phase. The hostile microenvironment is amplified often due to multifactorial stimuli result in disturbance of delicate balance between proteases, proinflammatory cytokines chemokines and their inhibitors that is observed in acute

wounds (Schultz & Mast, 1999). Therefore, the anti-inflammatory compound or element is required to unlock the inflammatory phase and help the wound healing cascade to proceed to next phase (Zhao, Liang, Clarke, Jackson, & Xue, 2016).

Thymus serpyllum has been used for curative purposes and its essential oil consists of compounds such as carvacrol, thymol, α-terpineol, isobutyl acetate, and γ-terpinene exhibiting anti-oxidants, anti-microbial and anti-inflammatory properties (Čančarević, Bugarski, Šavikin, & Zdunić, 2013). On other hand ascorbic acid help in collagen formation and reduce the scar formation. Microemulsion is new method of topical delivery of drug as the size of particle is 10-100nm which can easily cross the stratum corneum in skin. Therefore, these components were selected for their ability to help in wound healing.

According to Jarić, et al polar compounds are found in *T. serpyllum* extract and the phytochemical analysis seed extract showed presence of phenols, flavonoids and few other metabolites. Sarfaraz et al. reported TPC and TFC of different *Thymus* species and according to findings TPC and TFC value of *T. serpyllum* were 22.14 mg/g and 1.77 to 8.72 mg/g respectively (Sarfaraz, Rahimmalek, & Saeidi , 2021) while TPC and TFC ethanolic extract of seed were 226.3 mg/g and 50.29 mg/g respectively the obvious difference can be due to plant extract from different parts and initially concentration of extract taken as 5 mg/ml while Sarfaraz et al. took 1 mg/ml of extract for TPC and TFC.

The LD50 of *T. serpyllum* was 4.7 mg/g as reported by Dilaser (Stahl-Biskup) as sub chronic toxicity was observed. The toxicity test performed showed 10.3% hemolysis at 5 mg/ml. but at 500-0.05 mg/ml hemolysis had been observed which may be due to RBC damage occur when processing blood. Here in this experiment extract is in direct

contact with blood but when toxicity was performed in vivo the extract has to pass barriers before come in contact with the blood. But still the hemolysis is less than 50%.

Microemulsion formulations prepared were shortlisted on basis of the criteria mention by Tandel et al. The selected formulations were checked for droplet size which was 77.49nm and 94.95 nm (bM.E and M.E) while the microemulsion of size about 200 nm had been formulated by Soliman et al. (Tandel, Patel, & Jani, 2015), (Soliman, Malak, El-Gazayerly, & Rehim, 2010). In another study it was mentioned that nanoemulsion of size greater than 80 nm and equal or less than 200 nm showed moderate penetration through skin and stratum corneum (Su, et al., 2017). Therefore, microemulsion with extract and ascorbic acid matches with above research and the size is less than 100 nm that help to maintain its thermodynamical stability. As for zeta potential it needs to be greater or lesser than 0 mV so the chance of phase separation reduces and the formulated microemulsion had enough zetapotential to avoid phase separation.

The ethanolic extract of *T. serpyllum* seeds showed more antioxidant activity than the activity of essential oil reported (Jarić, Mitrović, & Pavlović, 2015). According to Jarić et al. essential oil of wild thyme had IC50: 34.8mg/ ml which is lower than that of ethanolic extract of seeds. The microemulsion formulated showed more antioxidant activity as compared to extract and the difference was significant ($p<0.05$).

Alamgeer, et al. reported anti-inflammatory activity of ethanolic, ether and aqueous extract of wild thyme and according to this ether extract showed significant result as compared to other extracts (Alamgeer, et al., 2015). The results of tested seed extract were similar to the report of Alamgeer, et al. as the 1 mg/ml of extract showed 45.5% of inhibition. Similarly, microemulsion showed 60.8% inhibition and difference

between plant and microemulsion were significant ($p<0.05$). It was concluded that microemulsion has anti-inflammatory property and the reason behind is the presence of castor oil that has been known for its anti-inflammatory properties according to Vieira, C, et al.

The aqueous and ethanolic extract of T. serpyllum showed antimicrobial activity against *S. aureus, E. coli, P. aeruginosa* and *B. subtilis* as reported by Jarić, et al. But the seed extract showed no zone of inhibition for both *S. aureus* and *E. coli* upto 1 mg/ml and 2 mg/ml of extract. The reason could be low concentration of the extract for not showing antimicrobial activity. On other hand microemulsion showed antimicrobial activity against *S. aureus* which is one of the major bacteria causing wound infection

Skin irritability was performed to check whether microemulsion fell under GRAS category or not. As mentioned by Soliman et al. edema and erythema were observed in group treated with formalin solution and few of the microemulsion also showed irritation (Soliman, Malak, El-Gazayerly, & Rehim, 2010). As compared to this, microemulsion formulated with plant extract and ascorbic acid showed no sign irritation in fact, the microemulsion improve skin and maintain its moisture while formalin on other hand didn't show any erythema and edema sign either but the skin become rough and dry along with the change in color of skin was also observed.

Closure rate of both excision and diabetic wounds was observed to be more in microemulsion group as compared to other groups. The increase in wound closure rate was observed after 5 or 6 days of treatment. In excision wounds, effect of *T. serpyllum* was also observed on wound healing and at day 7, 8 and 9 the wound healing activity of it is better than the standard and in control group. Blank microemulsion also tried to help in wound healing but the results are not effective. The formulated microemulsion

with extract and ascorbic acid showed significant results even on diabetic wounds. The wound healing function of microemulsion has been reported by Okur, et al. The report showed burn wound healing for 10 days and results for microemulsion were effective against burn wounds. The result of this report is on coherence with one formulated for diabetic and excision wounds.

Conclusion

In this work, the bioactivity and wound healing capacity of microemulsion was evaluated. The finding showed significant wound healing rate in both excision and diabetic wounds. This finding could provide promising use of microemulsion-based hydrogel with *thymus serpyllum* and ascorbic acid for its application as therapeutic agent against wounds. The formulated microemulsion system showed improved antioxidant, antimicrobial and anti-inflammatory activity This system showed 99% and 100% wound closure rate in both excision and diabetic wounds respectively within 10 days of treatment.

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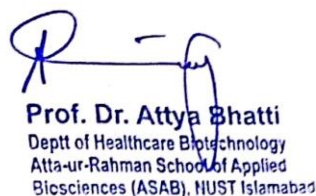

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