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DEVELOPMENT AND OPTIMIZATION OF A TOPICAL NANOEMULSION FORMULATIONFORWOUND HEALING

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DEVELOPMENT AND OPTIMIZATION OF A TOPICAL NANOEMULSION FORMULATION FORWOUND HEALING

Master Thesis

By

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DECLARATION

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from the planning of the thesis till writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments written which could not be obtained by this thesis study and these references were written into the reference list, I had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis.

Abdulkader Rawas

Signature

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LIST OF ABBREVIATIONS

API:	Active Pharmaceutical Ingredient
CFU/ml:	Colony Forming Units Per ml
CMC:	Critical Micelle Concentration
DCMS:	Decyl Methyl Solfoxide
DDDS:	Dermal Drug Delivery System
DLS:	Dynamic Light Scattering
DMAC:	Dimethyl Acetamide
DMF:	Dimethyl Formamide
DMSO:	Dimethyl Sulfoxide
ECM:	Extracellular Matrix
ED:	Erectile Dysfunction
EO:	Essential Oil
ESBL:	Extended-Spectrum B Lactamase
FDA:	Food and Drug Administration
GC-MS:	Gas Chromatography-Mass Spectrometry
GIT:	Gastrointestinal Tract
Gr-:	Gram Negative
Gr+:	Gram Positive
GRAS:	Generally Recognized As Safe
GSH:	Glutathione
GST:	Glutathione-S-Transferase
HDL:	High Density Lipoprotein
HEE:	High-Energy Emulsification
HLB:	Hydrophilic-Lipophilic Balance
HPLC:	High Performance Liquid Chromatography
HSV:	Herpes Simplex Virus
IL:	Interleukins
KHz:	Kilohertz
kV:	Kilovolt
LCTs:	Long-Chain Triglycerides
LDL:	Low Density Lipoprotein
LEE:	Low-Energy Emulsification

LP:	Lipid Peroxidation
MCTs:	Medium-Chain Triglycerides
ME:	Microemulsion
MHB:	Mueller-Hinton Broth
MIC:	Minimum Inhibitory Concentration
MRSA:	Methicillin-Resistant Staphylococcus Aureus
mV:	Millivolt
NE:	Nanoemulsion
NLCs:	Nanostructured Lipid Carriers
nm:	Nanometer
NPEs:	Natural Penetration Enhancers
NSAIDs:	Nonsteroidal Anti-Inflammatory Drugs
O/W:	Oil In Water
OEO:	Oregano Essential Oil
OSCs:	Oregano Sulfur Components
PBS:	Phosphate Buffer Saline
PCS:	Photon Correlation Spectroscopy
PDGF:	Platelets Derived Growth Factor
PDI:	Polydispersity Index
PEG:	Polyethylene Glycol
PG:	Propylene Glycol
PIE:	Phase Inversion Emulsion
PIT:	Phase Inversion Temperature
psi:	Pound Per Square Inch
PTA:	Phosphotungstic Acid
REO:	Rosemary Essential Oil
rpm:	Revolutions Per Minute
RSC:	Free-Radical Scavenging Capacity
SC:	Stratum Corneum
SEM:	Scanning Electron Microscopy
SLNs:	Solid-Lipid Nanoparticles
TDDS:	Transdermal Drug Delivery System
TEM:	Transmission Electron Microscopy
TEWL:	Transepidermal Water Loss
TNF:	Tumor Necrosis Factor

TTO:	Tea Tree Oil
W/O:	Water In Oil
ZP (ζ):	Zeta Potential
µg:	Microgram
µl:	Microliter
µm:	Micrometer

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Danışman: Doç. Dr. Yıldız Özalp.

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ÖZET

Amaç: Yaralarda bakteri üremesinin engellenmesi, iyileşme sürecinin kısaltılması ve hızlandırılması için aktif madde olarak uçucu yağlara dayalı bir nanoemülsiyon formülasyonunu oluşturmak ve optimize etmek.

Materyal ve Metot: Kekik ve Biberiye yağlarının *S. Aureus* türleri üzerindeki antibakteriyel aktiviteleri karşılaştırıldı. Ultrasonikasyon yöntemi ile nanoemülsiyon formülasyonları hazırlandı; yüzey aktif madde olarak Kolliphor PS® 80 ve Kolliphor RH® 40, farklı yüzey aktif**:** yağ oranı kullanıldı. Sulu faz olarak saf su kullanılmıştır. Çalışmayı optimize edecek en uygun formülasyonu bulmak için, hazırlanan formülasyonlar üzerinde farklı çalışmalar yapılmıştır.

Bulgular ve Sonuç : Kekik yağı çok az, minimum inhibitör konsantrasyonuyla Biberiye yağına göre daha fazla antibakteriyel aktivite ve *S. Aureus* türlerinde daha geniş inhibisyon bölgesi gösterdi. Optimum nanoemülsiyon formülasyonu; aktif madde ve yağ fazı olarak Kekik yağı, yüzey aktif madde olarak Kolliphor RH® 40 kullanılarak elde edildi. 4:1 yüzey aktif madde yağ oranı belirli bir sonikasyon süresinde ve genlik yüzdesinde uygulandı. Damlacık boyutu, zeta potansiyeli ve polidispersite indeksi sonuçları optimize edildi.

Anahtar Kelimeler: Topikal Formülasyon, Yara iyileştirme, Nanoemülsiyon Ultrasonikasyon, Kekik yağı.

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SUMMARY

Aim: To formulate and optimize a nanoemulsion formulation based on essential oil as an active ingredient for the improvement and acceleration of the wound healing process through the inhibition of the bacterial growth in the wounds.

Material and Method: Oregano and Rosemary oils were compared fortheir antibacterial activity on *S. aureus* species. Eight nanoemulsion formulations were prepared by Ultrasonication method and the surfactants used were Kolliphor PS® 80 and Kolliphor RH® 40 with different surfactant:oil ratio. Purified water was used as an aqueous phase.Different studies were conducted on the prepared formulations to find out the optimum formulation that will represent the study.

Finding and Results: Oregano oil showed greater antibacterial activity over Rosemary oil with a very small minimum inhibitory concentration and wider inhibition zone on *S. aureus* species.The optimum nanoemulsion formulation was achieved by taking Oregano oil as an active ingredient and oil phase, Kolliphor RH® 40 as a surfactant, and the ratio was 4:1 surfactant to oil ratio at a specific sonication time and amplitude percentage. The droplet size, zeta potential, and polydispersity index results were optimized.

Key words: Topical formulation, Wound healing, Nanoemulsion, Ultrasonication, Oregano oil.

CHAPTER ONE

1. INTRODUCTION AND AIM

1.1 Wound of The Human Skin

Skin is the largest organ of the human body, it protects the body and the internal organs from the external environment that could be filled with microorganisms. The uppermost layer of the skin; the stratum corneum (SC), plays an important role in this protection and also prevents dehydration by preventing the water from coming out of the body.

Any cut or rupture of the skin that leads to loss of these functions will refer to wound. Wound healing is a critical process that consists of four overlapped phases, these phases should progress properly in order to close the wound and restore the skin's normal functions. Bacterial wound infection is a problematic issue that will lead to the alteration of wound healing. The prevention of bacterial growth and the enhancement of tissue regeneration are key factors for the improvement of the wound healing process and restoration of the skin's integrity.

1.2 Essential Oils and Their Role in Wound Healing

Essential oils (EOs) are aromatic volatile compounds extracted from the aromatic plants as secondary metabolites. They have a variety of functions in many aspects of human life. The pharmacological activities of the essential oils gave them the importance to be used therapeutically in different fields. Two of the most remarkable oils in antimicrobial and antioxidant fields are oregano and rosemary essential oils. These two oils proved in many literatures their antimicrobial and antioxidant activity. Therefore, by controlling the inflammation of the wounds and by the inhibition of the bacterial growth along with the prevention of wound's oxidation through applying one of these two oils; the whole process of the wound healing will be accelerated and tissue regeneration will be enhanced.

1.3 Topical Formulations and Nanoemulsions

The treatment of the wounds is usually achieved by the topical application of a specific formulation on the wound area and the ingredients will work to speed-up the healing process. The application of essential oils directly on the wounds could be irritating and uncomfortable to be used, therefore, we incorporated the essential oil in a nanoemulsion system (NE). Nanoemulsion is a type of nanotechnology that got the interests of the researchers in the last two decades due to the outstanding advantages offered by this system. Nanoemulsion system is composed of oil, water, surfactant, and sometimes co-surfactant/co-solvent. The active substance will be dissolved in the oil phase and then mixed with water, the whole system will be stabilized and homogenized by the surfactants. Nanoemulsions are prepared by two methods; High-Energy Emulsification (HEE) methods and Low-Energy Emulsification (LEE) methods, each method has a specific process to achieve droplets with nano-sized.

Specific characterizations are then conducted of the formulated nanoemulsion formulation to ensure the optimization of the nanoemulsion formulation. The most critical characterizations among the others are the droplet size, zeta potential, and polydispersity index.

1.4 Aim and Scope

The aim of the study was to formulate and optimize a nanoemulsion formulation based on essential oil as an active ingredient for antimicrobial purposes for the improvement and acceleration of the wound healing process through the inhibition of the bacterial growth in the wounds and speeding-up the tissue regeneration of the skin.

CHAPTER TWO

2. GENERAL INFORMATION

The formulation of topical essential oils-based nanoemulsion for wound healing requires many fabrication steps to be formulated and ready for the intended use, so going directly to the formulation will be a little mysterious leaving many question marks asking about many topics. So, to understand the whole story about our formulation and what exactly we are dealing with, and even more, to build up good information starting by getting knowledge about the nature of our skin, continuing withthe wound healing processes along with many other points till reaching the aim of our study; this story should be started with discussion of the following points.

2.1 Integumentary System of The Human Body

The human body is a well-organized structure made up of many biological systems that carry out specific functions and work together as a team depend on each other to perform the daily tasks of our life that can't be achieved by a single part alone.For example, how we know that this surface is hot? First, we will touch it by our hand (integumentary system), then our brain will send signals to the hand that this is hot and you should take your hand away from this surface (central nervous system).Another example, when we start to eat something, the journey of food digestion (digestive system) starts by mouth, passing through the esophagus, reaching the stomach, and eventually ended by thesmall and large intestine, then after the digestion process proceeded, the kidneys will absorb the nutrients and excrete the waste products (urinary system) and so on, all these processes from the previous two examples should be done by the assistance of heart, blood, arteries, and veins (circulatory system).

Among all these systems, the largest one is a system that covers the whole area of our body and gives it the shape, appearance, and specific features, this system is the integumentary system. Integumentary systemconsists of skin and hair in addition to

nails. Our concern will be focused on the skin because it forms approximately most of the integumentary system, furthermore, most of the functions are done by it.

2.1.1 Structure and layers of the skin

The human skin, generally, is the largest organ among all other organs in the body, it weighs around 3-5 kg. The thickness of the skin differs according to the area and the function supporting this area, for example, the thickness of the eyelid is only 0.5mm, while the thickness of the palms of our hand or the soles of our feet is approximately 3-4mm, and as a consequence, the functions that support the eyelid will be different from that in palms and soles. However, the skin, in general, is around 1-2 mm thick(McLafferty et al., 2012). Regardless of thickness, the barrier activity and immunological functions are the same inall kinds of skin. The human skin is a very well organized and engineered structure made up of three distinguished layers: epidermis, dermis, and hypodermisFigure 2.1. Each layer of these layers has its own functions and plays a vital role in the accomplishment of everyday functions of the skin. The following explanation will show brief information about the main differences in structure and functions between these three layers:

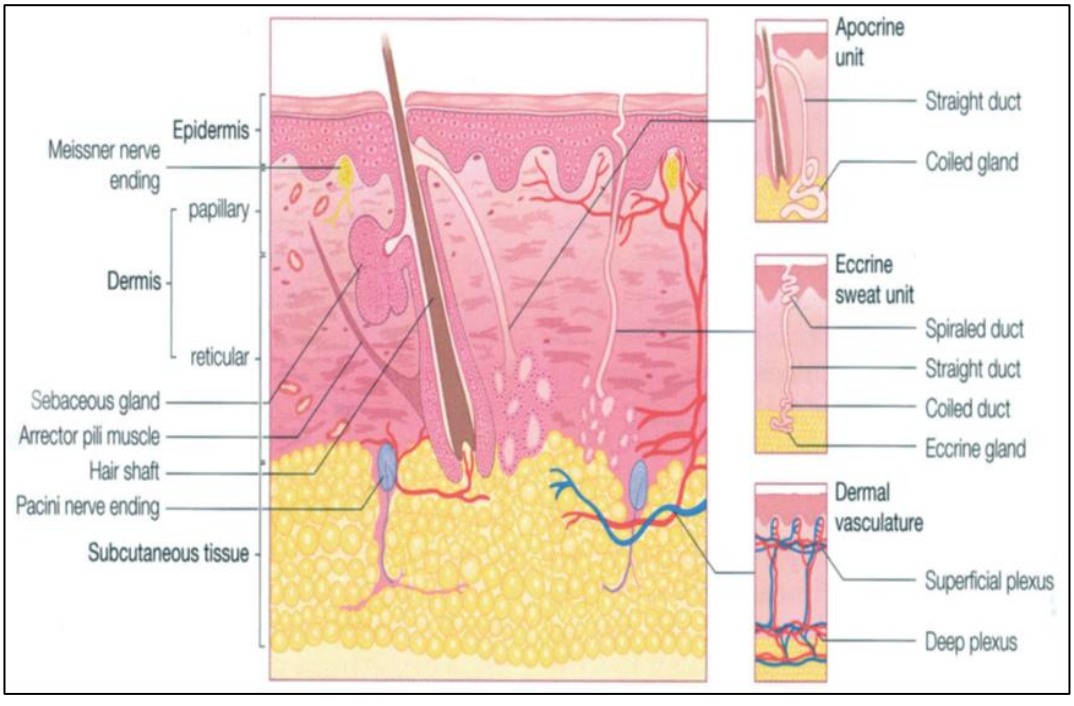


Figure 2.1:Cross section of the skin shows the three layers of the skin with accessory structures.(Kolarsick et al., 2009).

2.1.1.1 Epidermis

The epidermis is the first and the outermost layer of the skin consisting of stratified, keratinized squamous epithelium. The epidermis is avascular (free of any blood vessels) and it is composed of four or five epithelial layers of cells according to its position in the body. The area that has four layers makes up most of the skin and it is considered as ``thin skin``, these layers, from thebottom to the top are the stratum basale, stratum spinosum, stratum granulosum, and the uppermost layer; the stratum corneum. The only areas that have ``thick skin`` are the palms of our hand and the soles of our feet, they have the same layers of thin skin but in addition to the fifth layer, the stratum lucidum, which lies between stratum corneum and stratum granulosum(Betts et al. 2013). There are four main types of cells existing in the epidermis layer, Keratinocytes, Melanocytes, Langerhans, and Merkel cells. Keratinocytes form the majority of the cells that found in the epidermis, these cells store and manufacture keratin, which is the protein that gives the rigidity for hair, nails, and skin, and supports the skin with the required protection against external environment from heat, chemical factors, and microorganism. Melanocytes are the cells that are responsible for the production of melanin, the pigment that gives the color for the skin and hair, in general, the higher the melanin production, the darker the skin or hair, melanin also gives protection to the skin when it exposed to the UV light that may cause harmful effects. Langerhans cells have assistant functions in the immune responsein which they recognize microbes, attract them, and eventually destroy them by the presence of antigen that they have. Merkel cells have sensory neuron in their structure and therefore, they have receptors that will receive signals from the brain, so their function will be focused onthe sensation, and as a result, their presence will be more in hands and feet because they are more exposed to surfaces than other parts(McLafferty et al., 2012). Figure 2.2shows the main differences in structure and position between the five layers of the epidermis, in addition to the cells that support the epidermis.

During the formulation of topical pharmaceutical preparation, the most challenging layer among all layers of the epidermis is the stratum corneum,therefore, more

knowledge about the nature and characteristics of this layer should be gained in order to ensure that our preparation is delivered in a proper way.

2.1.1.1.1 Stratum corneum (SC)

As mentioned before, the epidermis is composed of many arranged layers, among these layers, the uppermost layer that is in direct contact with the external environment is the stratum corneum (SC), the SC is formed byan accumulation ofdead, anucleated keratinocytes or corneocytesthat resulted from keratinization process (also named as cornification which gives it its name) from the lowermost layer of the epidermis, the stratum basale. The SC is a very strong, resistant, and organized layer that protects the body from chemical, physical, and microbiological factors that may affect the skin, besides its protection against UV radiation. Water is an essential component of the SC, which prevents cracking of the SC by its plasticizing activity. Therefore, SC has a very important function in the protection of the body from excessive loss of water and electrolytes by acting as a barrier to prevent excess water and electrolytes from getting outside the body, and also prevents chemicals from coming inside the human body. SC maintains the level of transepidermal water loss (TEWL) to the minimum point, therefore, when the SC gets disrupted or damaged, TEWL will increase leading to threatening issues(Anderson, 2012; Benson, 2005). The barrier function of the SCis achieved through surrounding of the corneocytes by the intercellular lipidsthat are released from the lamellar bodies to represent the “brick and mortar” model. This model is divided into two parts, the first one is when the corneocytes filled with keratin filaments and embedded in a lipid matrix, they act like a ``brick`` which gives strong stability to the SC, the other part is the ``mortar`` which gives the barrier function to this layer. In another way to make it easier to be understood, the ``brick`` is the keratin, and the ``mortar`` is the lipids(Glombitza & Müller-Goymann, 2002). The matrix of lipids that gives the SC its ability to act as a strong barrier to the external environment is composed mainly of fatty acids, cholesterol, and ceramides. Ceramides form the majority of the SC lipids, their structure is composed of nonpolar, long hydroxyl or nonhyderoxy groups linked to a polar amino group. Moreover, their structure is saturated (free of double bonds or alkyl branches) which allows the formation of bilayers and compact structure, in opposite to fatty acids and

cholesterol in which they have less compact structure because of the presence of many double bonds. This rigid structure will minimize the amount of water permeation outside the skin(Goldstein & Abramovits, 2003).

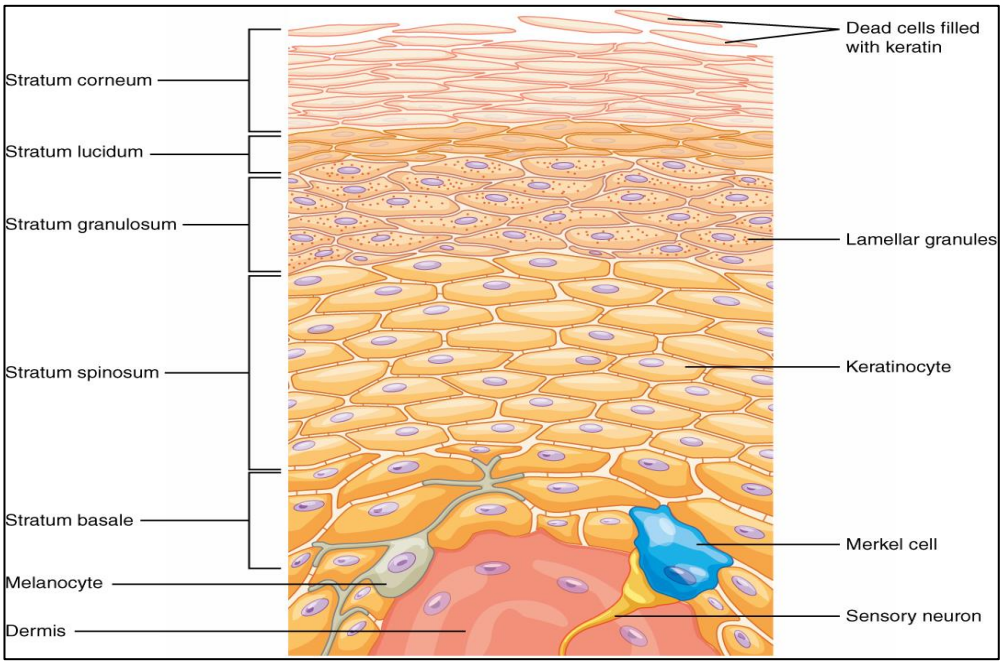


Figure 2.2: Layers of the Epidermis(Betts et al., 2013).

The illustrated figure shows five differentiated layers of the epidermis of thick skin; ascending from down to up, stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. In the case of thin skin, the stratum lucidum layer will not exist.

2.1.1.2 Dermis

The layer that reinforces the epidermis with nutrients and physical support is the dermis, which lies beneath the epidermis and above the hypodermis. In opposite to epidermis, the dermis layer is vascularized (rich in blood vessels) and contains nerve endings, sweat, and sebaceous glands, in addition to hair follicles. This network of the blood vessels existed in the dermis is responsible for the nourishment of the skin and also for thermoregulation, in which it contains plexus that undergoes vasodilation in a warm temperature conditions to help in cooling down and vasoconstriction under cold temperature conditions to help in warming up(Anderson, 2012). There are two layers in the dermis, the papillary layer, and the reticular layerFigure 2.3. The papillary layer is rich in capillaries and nerves that supply the

epidermis, while the reticular layer is composed of strong connective tissue made up of collagen and elastin fibers that are synthesized by fibroblasts. Collagen and elastin support the skin with the required elasticity tostretch without torn and contract to return to its normal position. However, these fibers become less elasticwith increasing in age(McLafferty et al., 2012).

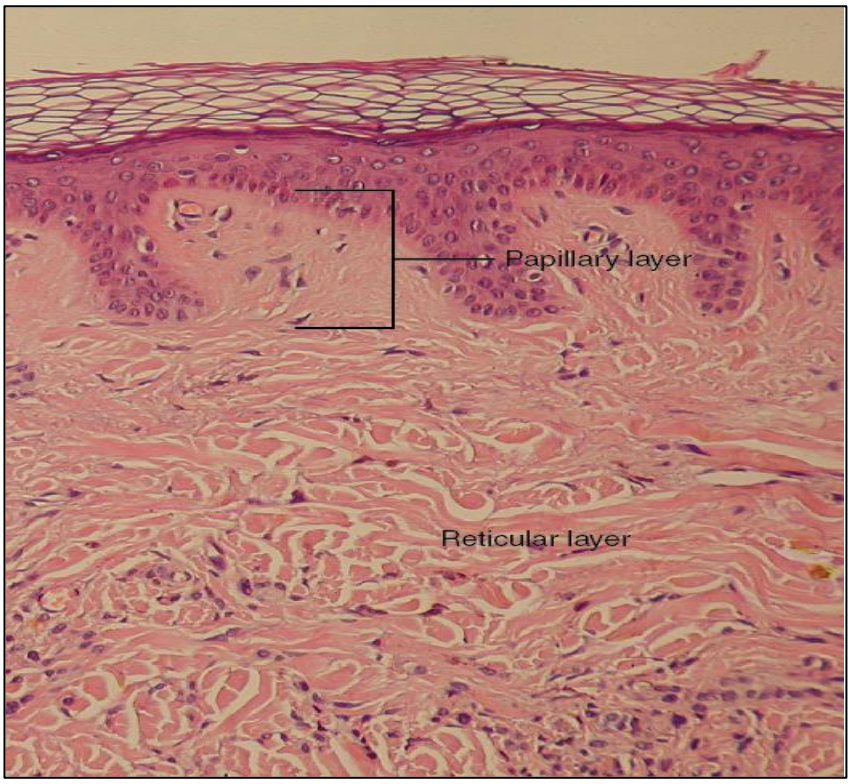


Figure 2.3: layers of the Dermis(Betts et al., 2013).

The stained slide illustrates the two layers of the dermis: the papillary and the reticular layer which give specific reinforcement to the skin.

2.1.1.3 Hypodermis

Hypodermis or subcutaneous layer is the third and innermost layer of the skin and it positioned directly under the dermis, the line between the dermis and hypodermis cannotbe easily distinguished, therefore, they are seen as one layer. The hypodermis is vascularized and composed mainly of adipocytes, which their function is representedby storing fat, providing energy, and insulation of the skin from the external environment(Betts et al., 2013).

2.1.2 Functions of the skin

Skin is the largest organ of the human body; in which it constitutes approximately 15% of the total body weight of an adult. The skin serves the body by many functions that give it the integrity and play an important role in keeping it in a healthy state, these functions include protection against external chemical, physical, and biological factors, regulation of the body temperature, as well as maintaining homeostasis resulting in the prevention of excess water loss out of the body(Kolarsick et al., 2009). Now, after the idea has been built about the structure of the skin, let us figure out the main functions of the skin that support this structure,these functionscan be summarized as follow:

2.1.2.1 Protection

Protection is the main and the most important function of the skin, in which the skin is considered as the first defensive line against external environment from physical, chemical, and microbiological threats, it also protects the internal organs from dehydration by preventing excess water and electrolytes loss out of the body, so providing the skin with the elasticity and the balance of the body's fluids and electrolytes(McLafferty et al., 2012). The acidic excretion secreted by the skin in the way of sweating from sweat glands of the dermis is playing an important role in the prevention of microorganisms from over-colonization on the skin surface by synthesizing dermcidin, which has antimicrobial activities(Betts et al., 2013).

2.1.2.2 Sensory function

Have we ever thought how do we feel when something very small walks on the surface of our skin like a small spider or ant? This is the sensory function of the skin, the hair follicles, which they are the root of the hairs that exposed to the external environment are surrounded by sensory nerves that will sense anychanges in the external environment, then, according to this stimuli, these sensory nerves will send a signal to the CNS and the CNS will translate these signals intoactions, and as a result, we will look at this spider or ant and take it away.Another example can be

noticed is that when we feel cold, we will notice the hairs on our arms are rising or undergoing piloerection. As mentioned before, the epidermis layer of the skin has a type of cells called Merkel cells, contain a neuron in their structure, the nerve receptors of this neuron will respond to pain and the changes in the temperature, and because of the fingertips are more usedfor touching, these receptors will be more condensed(Betts et al., 2013).

2.1.2.3 Thermoregulation

Another function of the skin is thermoregulation, which meant by maintaining the body temperature at a degree of 37℃. Thermoregulation of the body occurs mainly by three mechanisms: insulation, controlling of blood flow, and sweating. Insulation is done by the adipose tissue of the subcutaneous layer of the skin, which is filled with adipocytes. The skin is supplied with plenty of blood supply that assists in thermoregulation by controlling the blood flow within the dermis layer. When the temperature of the body arises, conduction and convection increase the blood flow throughout the body leading to heat loss. On the other hand, when the body feels too cold, increasing body temperature occurs through decreasing of blood flow to the extremities by vasoconstriction. The third mechanism of regulating body temperature is by sweating, which is very common in decreasing the body temperature. Sweat is produced by the sweat glands in the dermis layer. When the temperature becomes above 37℃, heat is lost by the evaporation of the sweat resulting in cooling down the body temperature (Abdo et al., 2020; McLafferty et al., 2012).

2.1.2.4 Vitamin D synthesis

One of the crucial functions of the skin is vitamin D synthesis, which follows exposure to sunlight, and as a consequence, calcium and phosphate homeostasis will be achieved. Exposing the skin to sunlight will lead to immediate production process of vitamin D. In order to synthesize the active form of vit.D, which has important functions in immune defenses, wound healing, as well as regulation of skin differentiation; the light gained from sunlight should undergo some interactions(Anderson, 2012). Themechanism of this production is started by the interaction of UV radiation from sunlight with keratinocytes of the epidermis to convert the provitamin D3 into previtamin D3 and eventually converted to the active

form of vitamin D. Vitamin D is very important in the growing up of bones and teeth and also for supporting them with the required strength, whereas deficiency of vitamin D will result in the formation of weak bones as in Rickets(Anderson, 2012). Overproduction of vitamin D will result in some abnormalities like hypercalcemia and other disorders, so this overproduction is prevented by a feedback tells that the required production of vitaminD is reached, and then previtamin D is converted to the inactive photoproducts; lumistrol and tachysterol(Abdo et al., 2020).Figure 2.4 explains the mechanism of vitamin D synthesis.

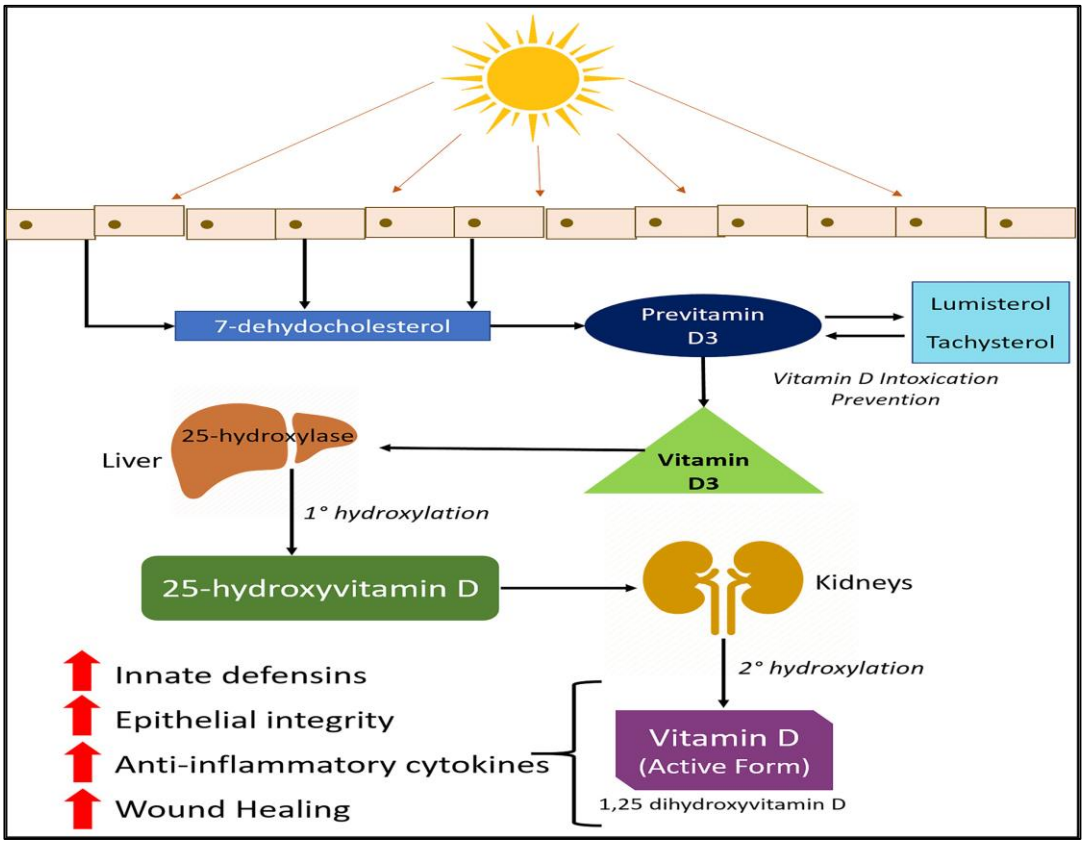


Figure 2.4: Mechanism of vitamin D synthesis(Abdo et al., 2020).

This mechanism shows the production of the active form of vitamin D which have various functions in the human body. Besides, Synthesis of vitamin D will increase innate immunity, resulting in increasing in epithelial cells and anti-inflammatory cytokines, which then affect positively in the acceleration of wound healing process.

2.1.3 Pathways for skin penetration

Pharmaceutically speaking, the overall idea of gaining knowledge about the pathways for skin permeation is to ensure delivering the drug through the skin at a maximum permeation rate.The rigid structure of the skin, especially, the stratum corneum and its selective permeability, creates some challenges during the formulation of topical pharmaceutical preparation, whereby this selectivity will diffuse the drug through the skin at different rates, leading to variation in drug delivery which is undesired. The permeation rate of the drug is expressed as the flux(Ng & Lau, 2015). The penetration pathways through the skin (mainly the SC) are shown in Figure 2.5. Penetration pathwaysthrough the SC can take place by diffusion through one of the following routes:

1) **Transcellular or intracellular route:** In this route, the penetration will occur by passing through the corneocytes existing in the SC that filled with highly hydrated protein, the keratin. This penetration pathway will offer an aqueous environment for the molecules passing through. Therefore, it is the predominant pathway for hydrophilic drugs(Das & Ahmed, 2017).

2) **Intercellular route:** Here, the molecules cross the SC between the cells (corneocytes) without traversing them. Passing through this route means that the molecules will pass through the extracellular lipid matrix which provides a hydrophobic media, and therefore, it is the preferred route of penetration for lipophilic drugs(Shaker et al., 2019).

3) **Transappendageal route:** Transappendageal permeation meant bythe penetration through hairfollicles or sweat ducts, in which this rout accounts for only 0.1% of the surface area of the skin, as a result, this tiny percent limited the availability of the drug to be applied through this route. Despite this limitation, it is an important route for delivering large polar molecules and ions that may be challenging to pass through the other routes. For many hydrophilic drugs, the aqueous pathway of this route is desirable for such drugs, but because of the movement of the sweat against the diffusion of the permeant; the penetration of the permeant may be limited. Likewise, the sebaceous glands that are connected to hair follicles, which filled with lipid-

rich sebum will also act as a barrier for permeation of the hydrophilic drugs(Das & Ahmed, 2017).

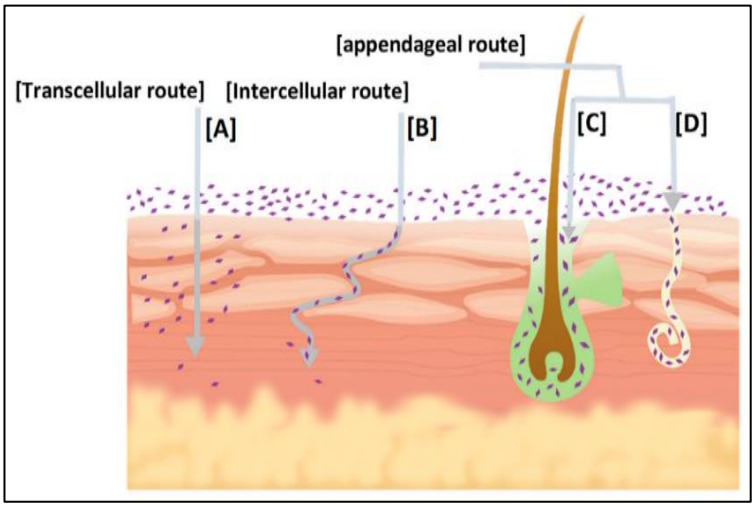


Figure 2.5: Penetration pathways through the skin (SC)(Shaker et al., 2019).

(A) crossing through corneocytes which filled with keratin. (B) passing between corneocytes. and (C) through hair follicle with associated sebaceous gland. (D) via sweat ducts.

2.1.4 Methods for skin penetration enhancement

Briefly speaking, the limitations mentioned about drug permeation motivate the dermatologists with the help of the pharmacists to think in different ways to enhance the permeation of the drug across the SC. Therefore, they found that the enhancement of drug permeationthrough the skin can be achieved by one or more of the following mechanisms(Patel et al. 2011):

1) Modification or disruption of the well-defended structure of the stratum corneum.
2) Interaction with the intercellular protein, the keratin.
3) Partition improvement of the drug, co-enhancer, or solvent into the stratum corneum.

In general, skin penetration enhancers are either chemical or physical enhancers(Mathur et al., 2010). However, more information will be discussed about penetration enhancement in another section in this chapter.

2.2 Woundsof The Human Skin

Skin is the main barrier that protects the whole surface of the body from physical, chemical, and microbiological factors that disseminated in the external environment. It is composed mainly of three layers with their components that give the skin its integrity. Daily exposure of the skin to the external factors may lead sometimes to lose the functionality of the skin with damage in the external surface of the skin, this damage and loss of integrity are generally referred to as wound.

2.2.1 Definition of the wounds

According to Velnar et al., a wound is defined as disruption or damage of the main structure and functions of the skin that may be superficial in the epidermis or deeper reaching the dermis and sometimes hypodermis which may damage other structures like muscles, vessels, tendons, or even bones. Most of the wounds reach the dermis layer of the skin, in exception to the wounds caused by operations, which may reach the subcutaneous layer and deeper(Velnar et al., 2009).

2.2.2 Wound healing and the stages of healing

After the skin got injured, it announces for an urgent situation, and as a result, many factors work together by forming a complex network to respond to this urgent situation and to return the skin to its normal shape, this mechanism defines the wound healing. Successfully healed wounds can be defined as the returning of functions, anatomical structure, and tissue integrity and appearance within a reasonable period(Velnar et al., 2009).Wound healing is a complicated process that occupies a series of interactions from different types of cells and tissue structures(Anderson, 2012). Healing of the wounds is achieved mainly by four overlapped phases as shown in

Figure 2.6, for each of these phases a cell type plays an important role in perfecting this phase. Briefly, these phases are:

2.2.2.1 Hemostasis phase

Instantly, after the skin got injured, hemostasis phase begins with vasoconstriction followed by activation and aggregation of platelets at the site of injury to start the

coagulation process. The platelets will release the components of their alpha granules leading to the formation of a fibrin clot that results from the conversion of fibrinogen into fibrin. As platelets aggregate, clotting factors (fibrinogen, prothrombin, etc) will be released resulting in the formation of a clot, and this clot will then be mixed with the fibrin clot forming a solid clot covers the wound(Schultz et al., 2011). Platelets are also playing a crucial role in the release of growth factors and proteases, which help in the formation of a granulation tissuethat provides a suitable surface for the formation of new epithelial cells, and the most common growth factor is platelets derived growth factor (PDGF)(Anderson, 2012).

2.2.2.2 Inflammatory phase

The inflammatory or defensive phase is the next step after clot formation by hemostasis. This phase is critical due to the possibility of bacterial infections that may alter the healing processes of the wounds. Inflammatory phase starts within 24 hours after skin injury and may be extended to 2 weeks in normal healing. This phase is driven by leukocytes; which the defense is their main function. Neutrophils, monocytes, and macrophages of leukocytes are the key cells that control the inflammatory phase of wound healing, and also they release soluble mediators such as growth factors (have an important role in the activation of epithelial cells and fibroblast), cytokines (important in the regulation of inflammation), and chemokines (which organize the population of leukocytes and activate the cells involved in these leukocytes)(Schultz et al., 2011). Neutrophils have the ability to produce free radicals, which kill a large number of bacteria,and after 2 to 3 days, these neutrophils are subsequently replaced by the tissue of monocytes, which then in wounds are activated to macrophages. Macrophages is very important for proper wound healing, they will secrete cytokines and growth factors which promote the proliferation of keratinocytes, fibroblast, and endothelial cells, resulting in the promotion of ECM synthesis and wound contraction(Larouche et al., 2018).Macrophages will then scavenge the wound from neutrophils and the remaining bacterial debris, whereby the regulation of wound healing cannot proceed without the existence of macrophages.Another important role of macrophages that they produce nitrous oxide, which has the capability to kill bacteria and decrease viral replication(Anderson, 2012).

2.2.2.3 Proliferative phase

After the wound closure achieved by the hemostasis phase and prevention of wound infection is done by the inflammatory phase, the phase of re-epithelialization or proliferation starts to process. Proliferation or re-epithelialization or re-construction phase all give the same meaning which is the formation of new tissue. Usually, this phase begins on the third day of the injury and extends to 2 weeks(Alberti et al., 2017).Proliferation phase is carried out by the activation of fibroblasts, which then produce and secrete collagen that will then replace the extracellular matrix (ECM). Re-epithelialization process is accomplished by the migration of epithelial cells (keratinocytes) from the borders of the wound to the center to form a granulation tissue, which is characterized by angiogenesis (the formation of new blood vessels). The stimulation and migration of keratinocytes is promoted by PDGF(Anderson, 2012).

2.2.2.4 Re-modeling phase

The final stage of wound healing is maturation or remodeling which initiates during the proliferative stage. During this phase, the density of fibroblasts and capillaries decreases with a gradually decreasing in the cellularity of the granulation tissue leading to the formation of an initial scar, which then is replaced by the ECM that has more similarity to the normal skin(Alberti et al., 2017). However, in severe wounds, the scar formed may result in a brittle, less elastic skin, and loss of skin appendages. Remodeling of the wounds may persist for weeks, months, or even for years in case of severe wounds(Schultz et al., 2011).

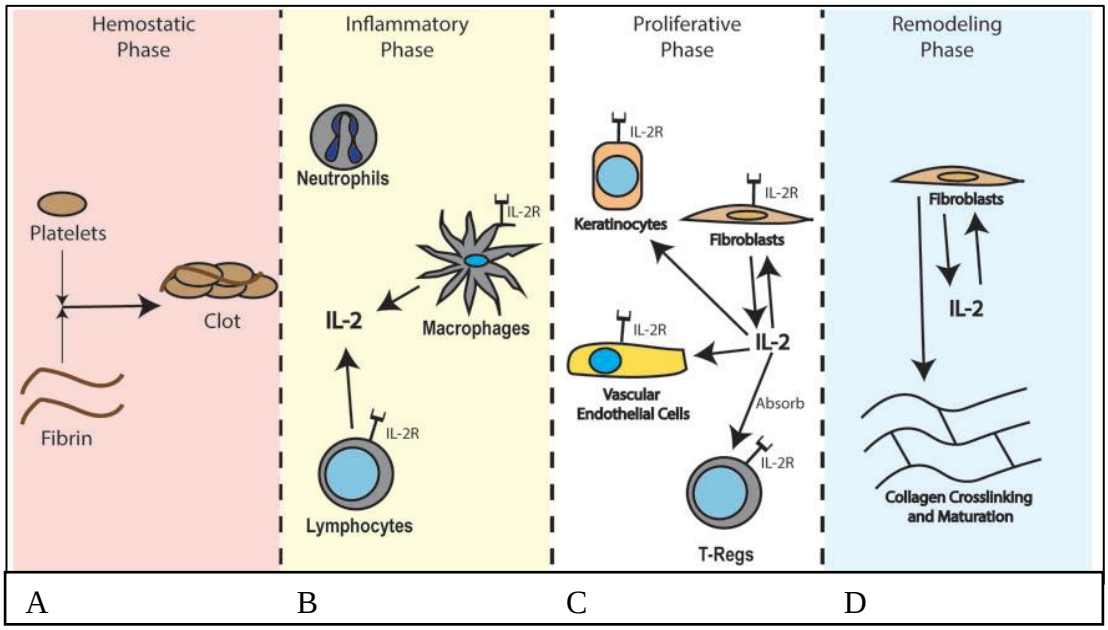


Figure 2.6: Stages of wound healing(Doersch et al., 2017).

(A: Hemostasis) Aggregation of fibrin and platelets directly after the skin got injured to stop the bleeding by forming a solid colt, (B: Inflammation) The immune response driven by lymphocytes is stimulated to prevent the infections by secreting neutrophils and macrophages, (C: Proliferation) Migration of keratinocytes and fibroblasts to surface leading to contraction of the wound, and (D: Re-modeling) Replacement of the fibroblasts by collagen in order to restore the normal shape of the skin.

2.2.3 Classification of wounds

In general, woundscan be categorized by many methods like the location of the injury, the severity of the injury, or the time required for healing. According to following of the wounds to the stages of healing and the time frame for this healing, wounds are classified into healing (acute) and non-healing (chronic). Table 2.1 summarized the main differences between acute and chronic wounds.

2.2.3.1 Acute

When the wounds repair themselves by the normal repairing and proceed with the orderly healing pathways, and ends by functional and anatomical restoration, they considered as acute wounds(Velnar et al., 2009).Examples of acute wounds include surgical wounds, traumatic wounds, and burns(Fletcher, 2008).

2.2.3.2 Chronic

In contrast, when the wounds fail to progress and follow the normal stages of healing, they considered as chronic wounds. In chronic wounds, the process of healing is incomplete and interrupted by many factors like bacterial infections, tissue necrosis, fibrosis, and others that prolong one or more stages of the healing(Velnar et al., 2009). Chronic wounds may sometimes depend on the patient's age or some diseases like diabetes and wound dryness(Negut et al., 2018).Examples of chronic wounds include diabetic foot ulcers, pressure ulcers, and fungating wounds(Fletcher, 2008).

Table 2.1: The differences between acute and chronic wounds (Martin, 2013).

Acute	Chronic
Controlled inflammatory response	Prolonged inflammatory response
Normal levels of inflammatory cytokines	Increased levels of pro-inflammatory cytokines
Levels of neutrophils, elastase and MMPs within normal limits	Elevated levels of neutrophils, elastase and activated MMPs
Controlled bioburden	Elevated bioburden
Growth factors freely available	Limited availability of growth factors
Wound fluid supports cell proliferation	Wound fluid inhibits cell proliferation
Fibronectin intact	Fibronectin degraded
Normal remodeling of extracellular matrix	Defective remodeling of extracellular matrix
Wound fluid does not damage peri-wound skin	Wound fluid causes peri-wound skin irritation and excoriation
Heal with minimal complications and no recurrence	Defective healing, complications common and frequently recurrence

2.2.4 Bacterial infections associated with wounds

A successfully healed wound is achieved when all the phases of wound healing progress properly. Impairment of one or more stages of wound healing will lead to a serious consequence that alter the proper healing which may lead to severe damage

to the skin like loss of skin integrity and functions, harmful sensation, and initiation of bacterial infections. Infection of the wound is very common and unavoidable during wound healing, especially, in case of chronic wounds, in which the wound is exposed to the external environment for a prolonged period of time.Although bacteria are a part of our skin microbiota and are existed in wound healing; butthe dangerous issue is that when they imped the processing of healing by the formation of colonies and microbial biofilm. Among all kinds of bacteria, the most commonly occurring speciesfound in the patient's infected wound are *Staphylococcus aureus* and *Pseudomonas aeruginosa*(Negut et al., 2018). However, prevention of bacterial infection can be achieved by the application of topical antimicrobial agents like silver sulfadiazine, or wound dressings, or by incorporation of some essential oils in the formulation.

2.2.5 Wound dressings

Exposure of the wound for a prolonged time to the external environment, or delay of healing of the wounds (such as chronic wounds), will eventually result in wound infections. For such an issue, the wound should be covered with a material that capable to prevent this infection and accelerate the healing process, these materials are called wound dressings. Wound dressings are applied on the wound area to protect it from the external environment that filled with microorganisms. Unlike the normal dressings that don't have any active ingredients (e.g. bandages which made only from cotton and tape), advanced wound dressings can be designed with the incorporation of ingredients that play a role in wound recovery and prevention or treating the infected wound. Many families of antibiotics can be functionalized in advanced wound dressings such as tetracyclines, cephalosporins, quinolones, etc.(Negut et al., 2018). Examples of wound dressings include foams, alginates, gauze, hydrogel, transparent films, hydrocolloids, and composites dressings. Each of them has its own design and characteristics(Lei et al., 2019).

2.3 Essential Oils and Their Contribution to Human Health

During our daily life, we become in contact with many things likeperfumes, cosmetics, foods, and many others that contain different types of oils in their compositions. Have we ever thought about what kind of these oils are used? Or what is the main source of these oils and how they were extracted? Do they have any benefits to our health? All these questions can be figured out by introducing the Essential oils (EOs). The most common and known example about essential oils is that when we peel off a piece of orange or lemon, sprayed particles in the air will be noticed, in which these volatile particles referred toessential oils.

Essential oils (EOs), or *Quinta essentia* are mixtures of volatile aromatic compounds that are naturally occurred and derived from aromatic plants as secondary metabolites, which can be extracted mainly by distillation from many parts of these plants (like the flower part, leaves, rhizomes, seeds, fruit, and the wood part). The word ``essence`` refers to flammability which is one of the essential oils' characteristics. Because of their hydrophobicity, they are insoluble in water but soluble in alcohol, fixed oils, and ether. EOs are generally liquid and colorless compounds with a distinct odor. The usual pleasant fragrance of essential oils offered them a high priority to be used in wide fields such as cosmetics, perfumes, medicines, inhalation, bath, and even more, in aromatherapy like massage(Dhifi et al., 2016).

2.3.1 History of plant essential oils

Essential oils have been used in different centuries by many cultures for different purposes. For example, in 4500 BC, ancient Egyptian civilization used EO inthe formulation of ointments and various cosmeceutical preparations, furthermore, they used to formulate a mixture of different herbal sources like onion, aniseed, myrrh, cedar, and grapes to prepareperfumes or even medicine (whereby in that time, there wasn't any other source to formulate medications). Going to Chinese and Indian civilization between 3000 and 2000 BC, the use of aromatic oils in their traditional medicines was first recorded. Even more, between 500 and 400 BC, the Greeks also participated in the use of aromatic plants, where the first use of different essential oils was documented by them, such as saffron, cumin, thyme, and

peppermint.Regardless all these uses of the aromatic plants, but the word “essential oils” was first used by Paracelsusvon Hohenheim, who gave the name of the effective components of the drug ``*Quinta essentia*``(Elshafie & Camele, 2017). Recently, in the century of 18^{th} and 19^{th}, as life evolves and with the invention of developed instruments, the chemists became able to determine the active constituents of the medicinal plant with the identification of various substances from this plant (such as morphine, caffeine, and others). This development helped the pharmacists to extract the pharmacological activity of some EO like oregano, rosemary, lavender, peppermint, clove, and many others that may in the future be as an alternative to the use of synthetic medicaments(Edris, 2007).

2.3.2 Chemical constituents of essential oils

Essential oils are composed of many different chemical constituents that belong to many chemical classes, these classes are alcohol, aldehydes, ketones, esters, ethers, amine, amide, phenols, heterocycles, and mainly of terpenes. The majority of EO contents are from the terpene family, and even more, this family has been identified in many other functionalized groups’ derivatives like alcohol (geraniol, α-bisabolol), ketones (menthone, *p*-vitevone), aldehydes (citronellal, sinensal), and phenols (thymol)(Dhifi et al., 2016). Terpenes are made by a combination of isoprene units and they are classified according to the number of these isoprene units. For example, the compounds formed by one isoprene unit are called hemiterpenes, by two isoprene units are monoterpenes, by three isoprene units are sesquiterpenes, and by four isoprene units are called diterpene. However, the volatility of the EOs decreases as the number of the isoprene units increases, in which EOs that made from isoprene units up to three have the highest volatility (De Matos et al., 2019).

2.3.3 Pharmaceutical and therapeutic potentials of essential oils

As mentioned before, the use of aromatic plants for different purposes was since ancient times, in which they can be used in foods, cosmetics, preservation, and im medicinal treatments. In particular, most of the essential oils have pharmacological activities against many diseases, which attracted the attention of the pharmacists to formulate a preparations containing these EOs in their ingredients. These pharmacological activities of EOs can be summarized as follow:

2.3.3.1 Anticarcinogenic activity

Anticarcinogenic property is the ability of specific molecules to suppress or inhibit the activity of carcinogens. The traditional chemotherapeutic agents used in the treatment of cancer showed development in drug resistance to these agents, as a result, the doses of these agents will be increased and this increase will result in severe adverse effects that will mainly affect the liver. These limitations made the researchers to move toward using alternatives and essential oils showed up and they were a good choice to be chosen.However, among the classes of terpenes;monoterpenes have been shown the best chemoprevention activity against tumor cells.Some EOs showedpotential anticarcinogenic activity against different human cancercells like*oregano* EO with its main constituents (carvacrol, thymol, limonene, citral).Hepatoprotective activityagainst liver damage caused by various chemical molecules was provided by myristicin, the main constituent of *nutmeg*. Citral, which is found in manyEOs and compromises 70-85% of *lemongrass* EO induces the hepatic detoxifying enzyme, Glutathione-S-transferase (GST) and increases its secretion(Elshafie & Camele, 2017). The EO of *garlic* is a rich source of oregano sulfur components (OSCs) that exert potential cancer chemopreventive activity, the majority of these OSCs in garlic EO are diallyl sulfide, diallyl disulfide, and diallyl trisulfide. Other examples for essential oils that exhibit chemopreventive activity due to their monoterpenes or phenols content include *orange* EO (d-limonene), *black cumin* EO (thymoquinone), and *sweet fennel* EO (d-limonene and β-myrcene). Eucalyptol, the high concentrated components of *eucalyptus* (60-90%) and *cardamom* 59%, demonstrates suppression activity toward cancer(Edris, 2007).

2.3.3.2 Prevention of atherosclerosis

Atherosclerosis refers to the build-up of a plaque of cholesterol and other substances in the artery walls (which carry oxygen-rich blood to the heart and other parts) that result in restriction of blood flow throughout the body and eventually, clogging of the arteries. This disease is mainly caused by increased levels of oxidative low-density lipoprotein (LDL) in cholesterol. Therefore, atherosclerosis can be inhibited by preventing the oxidation of LDL, which can be achieved by daily intake of antioxidants. In this case, EOs were a very strong candidate to treat or prevent such

disease, because of the strong antioxidant activity for some of them(Edris, 2007). The monoterpene hydrocarbon, *terpinolene*, exhibits an effective inhibition of LDL oxidation. Essential oils that rich in phenolic compounds such as thymol and eugenolprovide the highest antioxidant activity toward LDL oxidation. It has been noticed that when eugenol is the main component of the EO (e.g. clove); the oxidation of LDL is inhibited by 50-100%, on the other hand, the inhibition was only 10-50% when the EO contains a moderate amount of the phenolics;cuminol, thymol, or carvacrol. Some essential oils with their constituents have the ability to lower the levels of cholesterol and triglycerides in blood plasma that may contribute to the formation of the plaque which results in atherosclerosis. One of these oils is *Black cumin oil*which showed decreased levels of cholesterol and triglycerides in blood plasma. α-Curcumene, the main component of *Javanese turmeric*, provided lowering activity against triglycerides in serum as well as triglycerides of the liver. *Garlic* EO decreases the serum cholesterol and triglycerides levels significantly with the advantage of increasing the levels of high-density lipoprotein (HDL). Combinations of cinnamon, oregano, cumin,and other EOs decrease systolic blood pressure if administered orally. Intravenous administration of *basil* EO exerts significant and simultaneous hypotension and bradycardia (slowing down of the heartrate) due to the vasodilation activity of this essential oil(Edris, 2007).

2.3.3.3 Anti-inflammatory activity

Inflammation is a protective immune response stimulated by tissue injury or infection which usually associated with pain, swelling, and redness, leading to functionality weakness. This response to fight the foreign bodies will induce and increase the permeability of the fighting cells to the site of injury. These defenders are the endothelial cell antibodies, macrophages of leukocytes, cytokines, and the stimulation of arachidonic acid activity, as well assome enzymes like (oxygenase, nitric oxide synthase). For many decades, essential oils have been used a lot in this field to relieve the pain caused by inflammation diseases like arthritis, rheumatism, and others. In fact, it was noticed that EOs provided an effective activity of pain-relieving more than synthetic pharmaceutical analgesics did(Dhifi et al., 2016). *Chamomile*oil was documentedto have more flavonoids with anti-inflammatory activity than other species, in which these compounds penetrate easily to the skin and

relieve inflammation. The anti-inflammatory activity of *tea tree* oil (TTO) with its main constituent (α-terpineol) is achieved by inhibition of the release of histamine or by reducing inflammatory mediators' production by activating monocytes. Another anti-inflammatory effect was reported for *Japanese nutmeg*, where they are considered as COX-2 inhibitors. Therefore, EOs represent a good option in the treatment of inflammatory disease(Elshafie & Camele, 2017).

2.3.3.4 Antioxidant activity

Oxidation is a process that will result in the formation of radicals, and by mean, antioxidants are molecules that have the ability to react with these radicals or prevent oxidative stress of the radicals by their oxidation reducing activity(Elshafie & Camele, 2017). Free radicals may contribute to cellular damage that may result in many diseases like cancer, immune system suppression, in addition to brain and heart dysfunctions. It was demonstrated that some EOs have very strong antioxidant activity, and therefore, they are capable to scavenge the free radicals, keeping in consideration that this activity is associated mainly with their compositions. Among all the constituents of EOs, phenolic compounds were the best to show a strong antioxidant activity because of their redox properties that can neutralize the free radicals and decompose peroxides. However, essential oils like *cinnamon*, *oregano*, *thyme,clove,basil*, and *nutmeg* are characterized by having the best antioxidant activity, in which phenols (thymol and carvacrol) are the main constituents(Dhifi et al., 2016).

2.3.3.5 Antiviral activity

Synthetic antiviral drugs are used to treat the viral infections caused mainly by Herpes Simplex virus (HSV) (type I and II), some of these antivirals weren't effective enough, especially in genital herpes infections, besides, a developed resistance toward one of acyclovir family was documented. Some essential oils were a good alternative to traditional synthetic antiviral drugs by offering a potential virucidal activity against both type I and type II. Such oils are *lemongrass*, which possesses the most potent antiviral activity against HSV-I, it offered a complete inhibition of viral replication. *Peppermint*essential oil was demonstrated with strong

virucidal activity toward both HSV-I, HSV-II, and also against the acyclovir-resistant strain of HSV-I(Edris, 2007).

Under the circumstances we are living now around the whole world to fight the new pandemic type of viruses, the Coronavirus 19 (COVID-19), hopefully, the promising effects of essential oils against viral infections offer us a perfect treatment for this virus, as well as HIV.

2.3.3.6 Antibacterial activity

Bacterial infection is one of the most common issues that occurs to the human body. It is defined as the proliferation of harmful strains of bacteria internal the body or externally. They have three main shapes: spherical (cocci), rod (bacilli), and helical (spirilla). Bacterial strains are either Gram+ve (e.g. *Staphylococcus aureus*, *Streptococcus pneumonia*, etc) or Gram-ve bacteria (e.g. *Pseudomonas aeruginosa*, *Escherichia coli, etc)*,in which the main difference is that Gram+ve bacteria have athicker wall than Gram-ve. From ancient times, the aromatic plants, in particular, the essential oils, were used in the preservation of foods due to their strong constituents that prevent any bacteria to colonize, and because of their pleasant smell, they were also used in cosmetics, in addition to their absolutely crucial role in the medical field to combat bacteria and to prevent decay and toothache. EOs have the ability to prevent or inhibitthe bacterial growth through their components that have a strong bactericidal or bacteriostatic activity against a wide spectrum of harmful bacteria strains. Due to the lipophilicity of the EOs, their mechanism of fightingis achieved by disturbing the cell structure of the bacteria through partitioning into their lipid bilayer, which makesthe bacteria more permeable and hence, results in leaking of the bacterial ions and molecules leading to lysis of the bacterial structure(Dhifi et al., 2016).A comprehensive study was performed by Puškárováet al. on six EOs toward different bacterial strains (Gram +ve and Gram – ve) to figure out their antibacterial activity against these strains, these oils were*oregano*, *thyme*, *clove*, *arborvitae*, *lavender*, and *clarysage*, and the chloramphenicol was the control. The results showed that among the selected six EOs, oregano and thyme EOs were strongly effective against all tested bacteria than other EOs. The reason behind this strong activity is their constituents that contain

high concentrations of phenolic compounds, mainly, thymol and carvacrol. Generally, it was reported that EOs that characterized by high concentrations of phenolic compounds such as thymol, eugenol, and carvacrol, exhibit the highest antibacterial activity(Puškárová et al., 2017). A formulation containing *tea tree* oil (TTO) provides short killing time against multidrug-resistant organisms like methicillin-resistant *staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *glycopeptide-resistant Enterococci*. This activity of TTO was attributed to its high concentration of α-terpineol(Dhifi et al., 2016). Peppermint and spearmint EOs also have inhibition activity against methicillin-resistant *staphylococcus aureus* (MRSA). However, EOs showed antimicrobial activity against Gram+ve more than Gram-ve(Edris, 2007).

2.3.3.7 Other uses for essential oils

In addition to the all beneficial uses of essential oils, they can be used as repellent and insecticides. In this field, *wintergreen* and *eucalyptus* EOs exert very toxic effects against insects. Due to the pleasant fragrance of essential oils, besides their powerful activities, they have been used as inhalants and as marvelous oils for massage therapy (Dhifi et al., 2016). Inhalation of *peppermint*, *lavender*, *rosemary*, and *clary-sage* EOs decrease anxiety and stress. On the other hand, *lavender* and *geranium* EOs are good choices for massage therapy due to their ability inthe relieving of inflammatory symptoms associated with allergy and arthritis(Edris, 2007).

2.3.4 The role of essential oils in wounds healing

As explained before about wounds and wound healing, a wound is a rupture or any cut in the skin that leads to loss of skin’s functionality,and this wound by normal should be followed by the healing process, in order to prevent any complications. Wound healing is a complex process achieved by four overlapped phases work together to give back the skin its normal integrity. The most important phase in wound healing is the inflammatory phase, in which if this phase was well-controlled, the whole process will progress normally. Therefore, essential oils played a vital role in controlling this phase along with the other phases through their essential constituents that possess different activities. Phenols and aldehydes exhibit a critical

role in the prevention of the oxidation that may lead to prolong the healing process. Further details will be discussed in the following examples:

A study achieved by Seyed Ahmadi et al. showed that the application of *cinnamon* EO with its main constituents (cinnamaldehyde and eugenol) accelerates the wound healing by its antibacterial and antioxidant activity that will protect the inflammatory stage from any oxidation, and as a consequence, accelerates the transition from inflammatory into proliferative phase, and assists the granulation tissue to be formed faster by increasing keratin biosynthesis(Seyed Ahmadi et al., 2019).

Another results extracted by Dai et al. and Hajialyani et al. about *Curcumin*, another EO with potent antioxidant activity.Curcumin is one of the most important phenol compounds that has the ability to interact with free radicals, and therefore, reduction in the inflammatory responses will be achieved. Curcumin has a crucial role in the formation of new blood vessels (angiogenesis), in which it facilitates capillary blood formation. Another critical function of curcumin which employed by its capability to enhance fibroblast and collagen migration and deposition in the granulation tissue; an essential step in the re-modeling phase(Dai et al., 2017; Hajialyani et al., 2018).

*Achillea biebersteinii*EOis an oil extracted from a plant found mainly in Turkey under the name (Sarıçiçek)was documented by Akkol et al. to has antimicrobial and antioxidant activity and was able to reduce edema of the granulation tissue that contains fibroblasts and collagen fibers. Furthermore, Sarıçiçek increased the contraction of the wound area resulting in the acceleration of the wound healing process. The activity of *Achillea biebersteinii* was noticed to play an important role in the proliferative phase rather than other phases. The main constituents identified in this plantare camphor, eucalyptol, α-terpinene, borneol. Synergistic interaction between these components may promote and improve the wound healing process(Akkol et al., 2011).

One of the most powerful antibacterial and antioxidant EOs is *rosemary* essential oil (REO) with its main constituent (1,8 cineol). A study was carried outbyKhezri et al. showed that the activity of REO was by decreasing the inflammatory cytokines (that mainly associated with inflammation of the tissue) and promoting the wound healing process in chronic wounds (e.g. diabetic wounds). Furthermore, prevention of

lipoperoxidation of the lipids existing in the skin due to its high contents of oxygenated monoterpenes. Considering antibacterial activity, rosemary EO exhibits the most antibacterial activity against Gram+ve bacteria like *S.aureus, S.epidermidis*, and *L.monocytogenes*. Another critical function of rosemary EO was reported for its improvement of angiogenesis which gives the positivity in the formulation of granulation tissue and acceleration of the healing process(Khezri et al., 2019).

Epithelialization of the wound was noticed by Kadhim and Amerto be improved and accelerated by the application of *eucalyptus* essential oil throughmaintaining the moisture of the wound and thickening the epidermis by increasing the proliferation of keratinocytes(Kadhim & Amer, 2018).

Multi-drug resistance of many bacteria has become challenging, especially in the case of methicillin-resistant Staphylococcus aureus (MRSA). *Oregano* EO, was studied by Lu et al. and it offered an absolutely fantastic solution to overcome this obstacle by its outstanding bacteriostatic andbactericidal activity through the prevention ofbacteria from the formation of biofilms(Lu et al., 2018).

Other essential oils like *Clove* EO (Alam et al., 2017), *peppermint* EO(Ghodrati et al., 2019),*Tea tree*EO (Lee et al., 2014),*garlic* EO (Ejaz et al., 2009),*Hypericumperforatum*(Süntar et al., 2011),*Thyme*and *Laurusnobilis* EOs(Costa et al., 2019)have been documented with their ability to accelerate the process of wound healing by antibacterial and antioxidant activity or by assisting in re-epithelialization of the wound to restore the integrity and functionality of the skin.

2.3.5 Toxicity of the essential oils

Essential oils are Generally Recognized As Safe (GRAS) which provided by the United States Food and Drug Administration (USFDA), but because of their chemical constituents, misusing essential oils or administering them in high doses can cause serious adverse effects like irritation, skin allergy, phototoxicity, and others.

2.4 Dermaland Transdermal Drug Delivery System(TDDS)

Administration of the pharmaceutical dosage forms can be achieved through various routes like oral, buccal, nasal, topical, parenteral, rectal, vaginal, and others. Each of these routes has its own characteristics that may offer superiorities over the other routes.

Among these routes, delivering the formulation by topical\dermal or transdermal route has attracted the researchers to formulate a dosage form through this route, because of the advantages that can be offered by it, especially, in case of the oral route, by which the transdermal route might be the best alternative for such route. When formulating a dosage form that intended to be used through the skin; it is crucial to have an idea about the differences between topical and transdermal use. The terms topical/dermal and transdermal are not fully understood, and sometimes are confusing whether this formulation is considered as topical/dermal or transdermal. Generally, all medications that applied on the superficial area like skin, eyes, vagina and exert a local effect are usually referred to as site-specific or ``topical`` preparations, whereas the medications that exhibit a systemic effect and penetrate through the skin into deeper layers (by the assistance of permeation enhancers to cross the stratum corneum) are usually considered as ``transdermal`` preparations(Wilbur, 2017). In another way, topical (dermal) preparations should be used when targeting a pathological site within the skin to exert a local effect (like when treating dermatological conditions such as psoriasis, eczema, acne, and microbial infections) with ensuring only minimal systemic absorption, whilst the transdermal delivery is only restricted to the preparations that diffuse through the skin layers and reach the bloodstream to exert systemic effect and the required therapeutic effect (such as using nicotine patches in the treatment of withdrawal symptoms of narcotics) as illustrated in Figure 2.7. However, to make the things simple, dermal or topical preparations such as creams, ointments, and sunscreens, etc. are usually used for the conventional release (for short time release) and exert a local effect, whereas transdermal preparations like some patches that intended for different purposes are used for controlled release (releasing of the drug gradually for a long sequence of time) and exert a systemic effect(Brown et al., 2006).

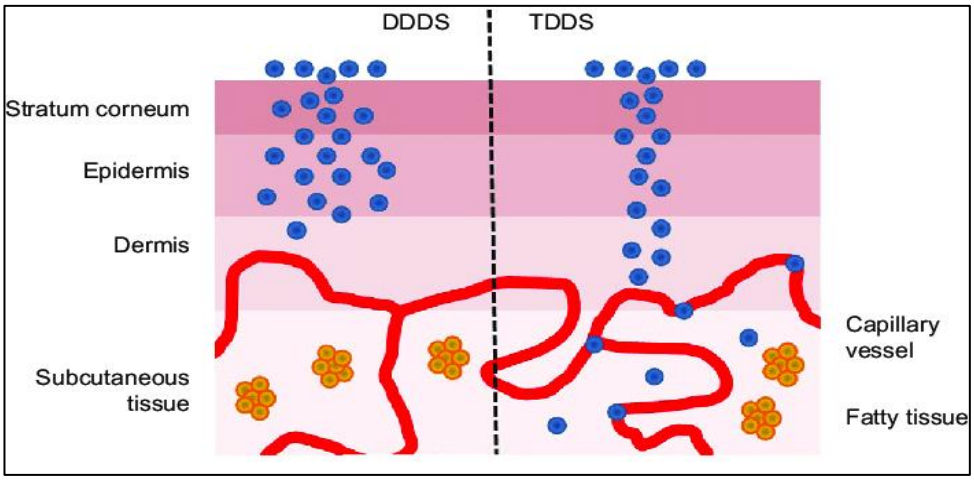


Figure 2.7: Difference between DDDS and TDDS(Muzzalupo & Tavano, 2015).

Simple illustration for the difference between dermal DDS and transdermal DDS, the drug in TDDS reaches the blood vessels in the dermis layer and then diffuses to the blood circulation to provide systemic effect, while in DDDS the drug remains in the dermis and upper layers to exert only local effect (a very minimal quantity may diffuse to blood stream but without exerting systemic effect).

2.4.1 Purposes of dermal and transdermal preparations

In order to acquire the desired effects of the preparationsand to formulate an efficient topical formulation;the intended usage and the purposes of the preparation along with the site of action should be figured out. Topical preparations might be used to exert(Bhowmik et al., 2012):

- Superficial effects: protection purposes (prevention of dehydration, protection from sunlight), cosmetics (for enhancement of the appearance), or for cleansing purposes (removing of germs and microbes).
- Effects on the stratum corneum: keratolytic effect (exfoliation of the skin, which is useful in the treatment of psoriasis), protective effect (moisturizing and protection from harmful sunlight).
- Epidermal and dermal effects: various drug classes may permeate to these layers to exert their local pharmacological activity like (antibacterial, anesthetic), once they reach the dermis layer, they might diffuse to the blood circulation. However, it is difficult to formulate a preparation to exert only

local effect without minimal diffusion to bloodstream, this is because of vascularization of the dermis with the existence of the veins and arteries.

- Appendageal effects: some drug classes exert their pharmacological activity in the appendages (hair follicles and sweat glands), these activitiescan be depilatory, antiperspirant, antimicrobial, or exfoliant activity.
- Systemic effects: usually transdermal preparations are meant to reach the bloodstream and provide their systemic activity.

2.4.2 Advantages and disadvantages of Dermal and TDDS

The topical and transdermal route is usually compared with the oral route, therefore, the main advantage of topical and transdermal administration over the oral route (in case of TTDS) is the avoidance of the first-pass metabolism of the gastrointestinal tract (GIT) and enzymatic degradation which will lead to other advantages like(Tanwar & Sachdeva, 2016):

- Reduction in the dose due to no loss of drug that caused by metabolism.
- Reduction in the undesired side-effects that caused by plasma levels fluctuations.

As summarized by(Rastogi & Yadav, 2012; Tanwar & Sachdeva, 2016; Yadav, 2012), other advantages are:

- Self-administration and ease of use, leading to patient compliance.
- Painless and non-invasive technique compared to parenteral therapy.
- Site-specific targeting will ensure the other areas not get damage.
- Are good choice to be used for the unconscious patients and patients with difficulty in swallowing.
- Transdermal patches are the best choice if the drug substances are broken by the GIT, and these patches permit constant dosing, which exerts a controlled release, and as a result, multi-day therapy with a single application can be achieved, besides, this therapy stops whenever the patch removed.

- The activity of drugs with short half-life can be extended through incorporation of the drug into a reservoir system offered by TDDS.

Usually, everything in life has its advantages and also disadvantages, therefore, the drawbacks of the dermal and transdermal route can be summarized as follow(Rastogi & Yadav, 2012; Yadav, 2012):

- Possibility of skin irritation locally or at the site of application.
- Barrier functions of the SCwill limit the permeability of the applied drug, and this function may vary in different areas of the skin, and from person to person.
- Allergic reactions may progress.
- Drugs with large molecular weight, high doses, and drugs that require a high blood level cannot be administered through this route.
- Transdermal patches may cause inconveniences if they didn't adhere well, and if applied on frequently-used areas.
- Not all drugs are suitable for DDDS and TDDS.

2.4.3 Skin penetration enhancers

Skin is the largest organ that covers all areas of the human body and isolates it from the external environment, besides, it provides many vital functions like protection, heat regulation, prevention of water loss, and more. Skin acts as a barrier from the external factors and this barrier function is maintained mainly by the rigid, well-engineered structure, the stratum corneum (SC). This rigidity of the SC is due to its construction that filled with dead, flattened keratinocytes and surrounded by lipids that composed mainly of ceramides. Therefore, successful penetration of the dermal\transdermal dosage forms is limited and became challenging. This issue was undesired and made the researchers to think about approaches that have the capability to alter this barrier function, which drove them to ``the penetration enhancers`` group.As mentioned earlier, the drug can penetrate through three main routes of the SC which are transcellular, intercellular, and transappendageal. So, penetration enhancement will be achieved through manipulating these three layers.

Penetration enhancers are compounds that improve the permeation of the drug through the SC by several methods like interacting with the lipids existed in this layer, modification of this layer, or with the assistance of mechanical approaches. The ideal permeation enhancers should acquire the following properties(Roy et al., 2017):

- They should be non-irritating, non-allergic, and pharmacologically inert (biocompatible).
- They should be tasteless, odorless, and colorless.
- They must be compatible with the other excipients and with the drug itself.
- They must not have any pharmacological activities and free of side effects.
- They should be unidirectional (deliver the therapeutic agents to the body with the prevention of leakage of the body fluids and endogenous components outside the body).
- In-vitro evaluation should be performed for them.
- They should be stable physically and chemically.
- When their action finished, the barrier functions of the skin should be restored.
- And they should not damage the skin cells.

Enhancement of the drug penetration can be achieved through hydration of the stratum corneum, in which water is the easiest, safest, and the most widely used to enhance the permeation of both lipophilic and hydrophilic compounds. Water constitutes around 15-20% of the stratum corneum, and this percent may change according to the external humidity. The increased amount of water within the SC will lead this layer to swell and to alter the barrier functionality, and as a result, permeation of the permeant will be enhanced. A study was performed on alcohol permeability across hydrated skin, the results showed that the permeation coefficient of alcohol was ten times in hydrated skin more than in dry skin. However, this hydration process can be acquired by the use of occlusive materials like waxes and

paraffin alone or incorporated in the topical preparations. These occlusive compounds will cover the skin by forming a waxy layer that will prevent the evaporation of water and transepidermal water loss (TEWL). This prevention will swell the SC leading to opening of the barrier gates, and eventually, easier permeation of the applied dosage form will be succeeded (H. J. Patel et al., 2011). Other penetration enhancers can be:

- **Physical penetration enhancers:**Enhancement of the drug permeation through physical enhancers can be achieved by the application of mechanical methods like ***iontophoresis*** (application of minimum levels of electrical currents of ~ 0.5 A/cm on the surface of the skin, which ensures the permeation of polar and charged molecules in large amounts. This method was successful for the penetration enhancement of NSAIDs for the treatment of rheumatoid arthritis(Okyar et al., 2012)), ***sonophoresis*** (application of ultrasonic energy (usually between 20 kHz and 16 MHz)which providestemporary improvement of the drug permeation (Rodríguez-Cruz et al., 2016)), ***electroporation*** (application of high electrical pulses that create nano-sized pores which allow the permeation of ions and macromolecules across the skin(Rodríguez-Cruz et al., 2016)), ***microneedles*** (the use of needles with a length of 100-500 μm to deliver the drug molecules, in which each needle acts as a drug reservoir that contains the active substance which will be released after a successful skin penetration(Rodríguez-Cruz et al., 2016)), ***radiofrequency*** (exposing the skin to high-frequency currents (usually between 10 kHzand 900 MHz) which create microchannels that induced by heat in the membrane(Mathur et al., 2010)), ***thermophoresis*** (increase the permeation of the drug by exposing the surface of the skin to elevated temperatures. This elevation will fluidize the lipids existing in the SC leading to weakening of the barrier functions, then, the blood vessels of the hypodermis will undergo vasodilation which eases the permeation of the drug to the blood circulation(Mathur et al., 2010)).

- **Chemical penetration enhancers:**The mechanism of enhancement by chemical agents is achieved through reversibly changing the structure of the SC to increase the efflux of the drug molecules into the skin. These changes

could be by disrupting the structure of the intercellular lipids in the SC, modification or denaturation of the intracellular keratin conformation of the SC, or improving partition of the drug into the SC by a drug reservoir(Okyar et al., 2012). Chemical penetration enhancers are such as ***Sulphoxides and similar chemicals*** (dimethylsulfoxide (DMSO) is one of the most widely used penetration enhancers.Alone, it was applied as a systemic anti-inflammatory agent, but with the time, DMSO became to be usedas an accelerant permeation enhancer and the concentration required for optimum efficacy is 60%. This relatively high concentration resulted in skin erythema, stinging, and burning sensation, and therefore, it became substituted by chemically-similar agents like dimethylacetamide (DMAC), dimethylformamide (DMF), and decylmethylsulfoxide (DCMS)), ***surfactants*** (these materials consist of a hydrophilic head and lipophilic tail which give them the ability to solubilize the lipid compounds in the aqueous media and vice versa. Therefore, they will act by solubilizing the lipid contents in the SC which improves the permeation through this barrier. However, more details will be discussed in another section), other enhancers like azones, pyrrolidones, urea, fatty acids, oxazolidinones, alcohols, fatty alcohols, and glycolshave the capability to enhance the penetration across the SC by different mechanisms(H. J. Patel et al., 2011).

- **Natural permeation enhancers (NPEs):**This class of penetration enhancers offered superiorities over other penetration enhancers like their abundance, low cost, and better safety profile. Because of their lipid nature, their main mechanism of enhancement will be through modifying the diffusivity and disrupting the intercellular lipids of the SC, which will lessen the barrier functionality of the rigid structure of the SC. Due to these distinct characteristics of natural permeation enhancers, they will offer a promising alternative to the synthetic penetration enhancers. These enhancers can be natural plants such as ***piperine*** (which enhances the transdermal permeation of aceclofenac), ***capsaicin*** (which enhances the permeation of naproxen gel), and ***myristica fragrans*** (showed better penetration enhancement of transdermal diclofenac sodium gel when compared to synthetic enhancers), or

can be essential oils like ***eucalyptusoil*** (when combined with isopropyl alcohol, it enhanced the penetration of chlorhexidine into the dermis rather than using isopropyl alcohol alone), ***almond oil*** (enhanced the penetration of transdermal ketoprofen gel and patches), ***rosemary oil*** (penetration activity and absorption were enhanced when using rosemary oil for an applied topical gel of diclofenac sodium), ***menthol*** (which is one of the most potent permeation enhancers and along with limonene, they can be used as a prototype of penetration enhancers), ***eugenol*** (penetration profile of lornoxicam transdermal patches across the skin was enhanced when eugenol oil is used), and many other natural oils(Das & Ahmed, 2017).

2.4.4 Nanotechnology and the classification of novel nanosystems used fordermal andtransdermal drug delivery system

Delivering the pharmaceutical preparations through the skin to target specific areas for the treatment of many dermatological diseases like acne, psoriasis, dermatitis, and other diseases has offered many advantages such as ease of accessibility, patient compliance, etc. but because of the skin is an excellent barrier against passaging of any materials from the external environment into the body, and due to this good barrier functionality that is driven by the stratum corneum; the penetration of the drug into deeper layers is prevented. Therefore, a lot of penetration enhancement methods have been exercised to overcome this rigid barrier. During the last two decades, nanotechnology has grabbed the eyes of the researchers and much efforts have been paid to afford and extend its applications in pharmaceutical sciences. Therefore, the successful enhancement of drug penetration across the SC was achieved by the incorporation of nanosized drug carriers for the treatment of various dermatological diseases. These nanocarriers enhanced the efficacy of the pharmaceutical preparations by providing superiorities such as increased solubilization of the hydrophobic drugs, improvement of the chemical stability, targeting the drug to the site of action, and side effects have been limited by releasing the drug over prolonged periods of time(Kahraman et al., 2017).

The following classification will present an overview of different nanocarriers that enhance and target the transportation of the drug molecule to the desired site of action into the skin or the body:

- **Micro and Nano-emulsion:**An emulsion is a coarse dispersion heterogeneous system consisting of two immiscible liquid phases dispersed into each other. Microemulsion (ME) and Nanoemulsion (NE) are nanometric dispersion systems of two immiscible phases which usually are water and oil and dispersed into each other according to the system type whether O/W or W/O, in which this dispersion is assisted and stabilized by the use of amphiphilic molecules whereby their structure have the ability to solubilize and decrease the interfacial tension between the two immiscible phases, these amphiphilic molecules are the surfactants. ME and NE seem similar in general view which generates some confusion, but there are many significant differences between them led to classify them in these different classifications. The obvious difference between them is in the terminology in which the word ``micro`` refers to the size of 10^{-6}m, while the word ``nano`` refers to 10^{-9}m. Beside the difference in terminology, size of the distribution of the oil droplets in both ME and NE was also confusing to be consideredas micro or nano, therefore, several authors defined the value of the oil droplet size distribution to liein the range between 100-500nm(Nastiti et al., 2017). The main discrimination between ME and NE is the free energy of the colloidal dispersion in relation to that of the two separated phases which influence the preparation and the stability of these two systems. MEs are thermodynamically stable, which means that the reaction or the formation of ME can be achieved spontaneously, by mixing water, oil, and surfactants together. Nevertheless, to overcome the kinetic issues that may delay the formation of ME, external energy throughheating and magnetic stirring is usually applied. In contrast, the separated phases of NEs possess lower free energy with respect to the colloidal system, therefore, an external energy input (will be discussed later) should be applied in order to achieve the successful formation of this system. For long-term stability, this difference in the freeenergy between these two systems is critical. The other and crucial

difference between MEs and NEs is that NEs are capable to load higher amounts of the dispersed phase (our API) with considerably lower amounts of surfactants, in contrary to MEs. This advantage was attractive and it gave the NEs the superiority over MEs, since the lower amounts used of surfactants, the better toxicological/safety profile. Furthermore, NEs can be prepared with divers kinds of surfactants while due to that MEs are prepared by the dependence on a very low interfacial tension; they are specified with only surfactants that provide an ultralow interfacial tension(Pavoni et al., 2020).

The great advantages that offered by NEs gave them the applicability in a wide range of fields like:

- Application in cosmetics:Nanoemulsion has gained importance as a vehicle for controlled delivery of cosmetics in particular skin layers. Moreover, their nano-sized droplets and the offered large surface area permit an effective delivery of the active materials. Another interesting importance of NEs is their ability to reduce transepidermal water loss (TEWL), which keeps the skin hydrated and soft. Another use of nanoemulsion is for hair-care products, in which their prolonged use will provide the hair with shiny, less brittle, and non-glassy features.What makes NEs acceptable in cosmetics is the absence of any chance for physical instability like flocculation, coalescence, creaming, and sedimentation that mightbe observed in MEs (Abolmaali et al., 2011; Chime et al., 2014).

- Antimicrobial activity: The antimicrobial activity of NE is due to the ability of the oil droplets of NE to fuse with lipids existing in the bacterial cell-wall or viral envelopes, resulting in destabilization and disruption of the pathogen(Abolmaali et al., 2011).

- Intranasal drug delivery: NEswere formulated as intranasal dosage form with Tadalafil as an API for the treatment of erectile dysfunction (ED), the results showed an enhanced efficacy of TD-NE in comparison to the oral administration of Tadalafil.These results may

offer a promising platform for the other treatments with different APIs(Elbardisy et al., 2019).

- Intravenous drug delivery: NEs also formulated as an intravenous formulation for delivering Rifampicin for the treatment of tuberculosis. Formulation of Rifampicin as NE showed excellent efficacy with stability profile for more than 19 months(Ahmed et al., 2008).

- Ocular drug delivery: Poor bioavailability and nonproductive absorption of the drugs from ocular dosage forms were observed, which is related to the corneal barrier of the human eyes, blinking of the eyes, as well as tear production. Therefore, incorporation of the drug into NE was a good choice and it effectively enhanced the permeation of the drug across the cornea as in delivering the drug Celecoxib (the selective COX-2 inhibitor from NSAIDs)(Moghimipour et al., 2017).

Many other applications of NEs were formulated like topical application of antiacne agents(Najafi-Taher & Amani, 2017), topical treatment of psoriasis(Khurana et al., 2018), for the improvementof oral delivery of poorly soluble drugs and targeting the drugs to specific sites in cancer therapy(Chime et al., 2014), and others.

- **Polymeric nanocarriers:** These nanocarriers are prepared from biodegradable and biocompatible polymers in which the drug is entrapped in a reservoir system or dissolved or encapsulated in a polymeric matrix. These nanocarriers are ***Nanospheres*** (are homogenous matrix systems in which the drug is entrapped within a polymeric matrix or adsorbed on the surface of this polymer), ***Nanocapsules*** (are a reservoir system in which the core is a hydrophilic drug surrounded by a polymeric shell), ***Lipid-based nanocapsules*** (these are the same as nanocapsules but the core is consisting of a lipophilic drug), ***Micelles*** (polymeric micelles are formed by amphiphilic block copolymers with a hydrophobic core that carries and protects the drug, and hydrophilic shell that stabilizes the hydrophobic core), ***Dendrimers*** (are radially symmetric molecules composed of a homogenous structure made

from three components: a central core, branches as a shell, and outer shell of functional groups(Abbasi et al., 2014)).Figure 2.8summarizes the differences between these nanocarriers in a perfect way.

- **Lipid Nanocarriers:** The third type of novel penetration enhancers is the enhancement by lipid nanocarriers. Lipid nanocarriers are classified into two systems:

  - **Lipid-based nanocarriers:** These nanocarriers are like nanoemulsion but differ in lipid nature. They are based mainly on lipids in their formulation to deliver the drug, in which these lipids could be solid as in ***Solid-lipid nanoparticles(SLNs)*** (they are formed by a matrix of biodegradable lipids that are solid at room and body temperature), or a mixture of solid and liquid lipids like ***Nanostructured-lipid carriers (NLCs)*** (they are formed by mixing the solid lipids with the liquid lipids to form a matrix of lipids that is solid at body temperature. They offer advantages over SLNs like higher drug upload and enhanced penetration due to the liquid nature of the lipids)(Uchechi et al., 2014).

  - **Vesicular nanocarriers:** The second system of lipid nanocarriers is the vesicular system which is composed of ***Liposomes***, ***Niosomes***, ***Transfersomes***, ***Ethosomes***.

The differences between lipid-based and vesicular nanocarriers are illustrated and summarized in Figure 2.9.

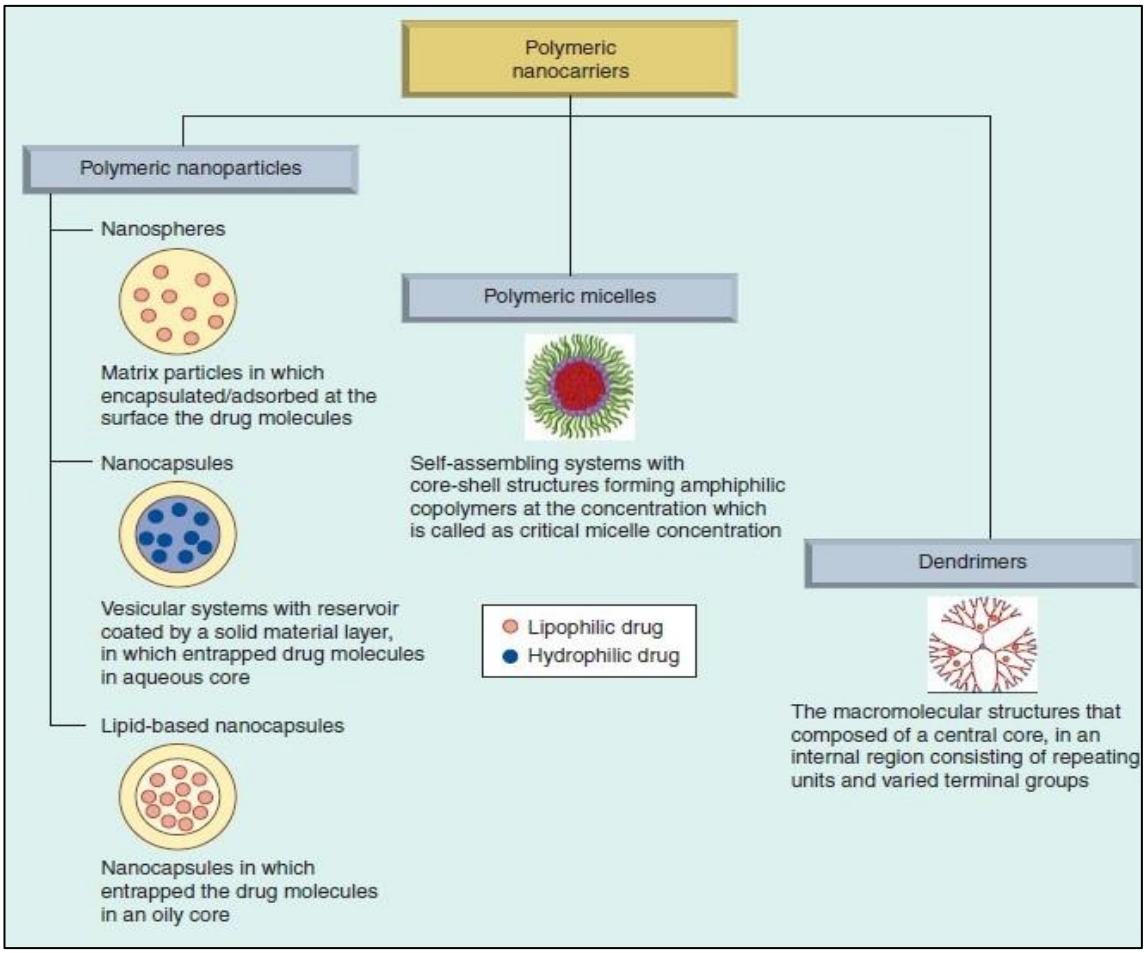


Figure 2.8:Types anddifferences betweenpolymeric nanocarriers(Kahraman et al., 2017).

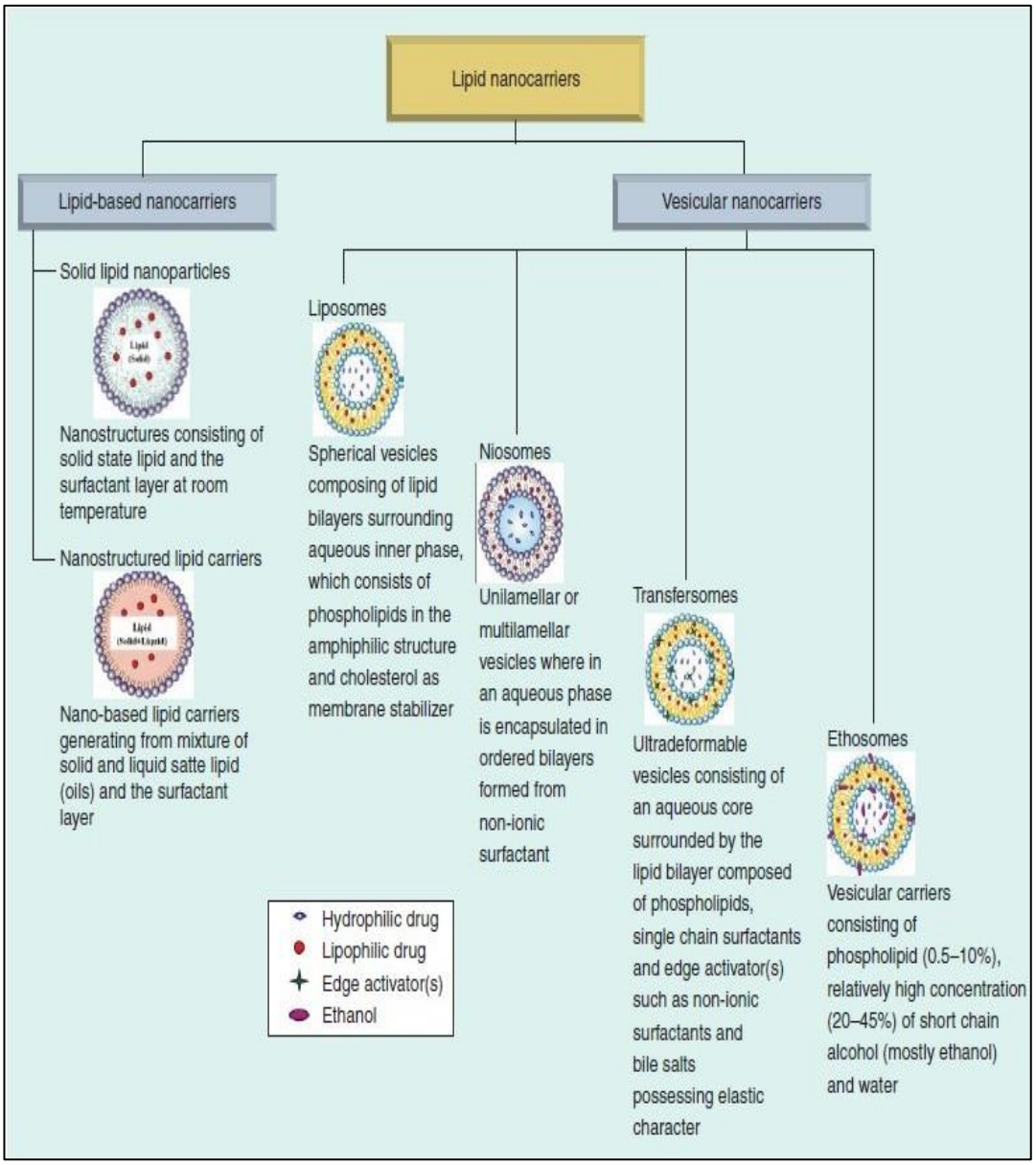


Figure 2.9: Types and differences between lipid nanocarriers(Kahraman et al., 2017).

However, regarding the topical applications, among all these classifications of novel nanocarriers that used to enhance the permeation across the skin and the SC; nanoemulsionswere the best candidate to deliver the drugs topically rather than transdermally due to the enhanced stability characteristics when compared to the other nanocarriers(Uchechi et al., 2014).

2.4.5 Dermatopharmacokinetics

To understand the kinetic of the applied formulations through the skin, or to study the biological effects of these applied formulations toward the skin; dermatopharmacokinetics studies should be performed. Dermatopharmacokinetics describe the pharmacokinetics along with pharmacodynamics processes of the applied drug on the stratum corneum. This examination is used to assess the cutaneous drug concentration at the site of application which can be performed by two smart techniques. The first technique is ***Stratum corneum tape-stripping***, which is used to analyze the penetration of the drug into the SC. This tape-stripping is performed by repeated applications of adhesive tapes on the site where the topical formulation applied, then the drug levels in the SC isdetermined from these collected tapes(Uchechi et al., 2014). The second method to study the pharmacokinetics and pharmacodynamics of the topical and transdermal applied drug is ***Cutaneous microdialysis***. This method is performed by insertion of an ultrathin semipermeable microdialysis probe or perfusate into defined skin layers (epidermis or dermis) directly under the applied formulation, then a physiological solution consists of saline or Ringer's solution is slowly pumped by using a pump. After that, the compounds that existed in the interstitial fluids diffuse into this semipermeable perfusate to be collected then with the dialysate. This dialysate is collected at different time periods and the existence of the drug is analyzed by using high-performance liquid chromatography (HPLC) as illustrated in Figure 2.10(Ruela et al., 2016).

Cutaneous microdialysis offers a good overview of the skin absorption and clearance of the drugs from topical and transdermal applications. However, it isn't suitable with large molecular weight compounds in which they may cutoff the semipermeable membrane(Ruela et al., 2016).

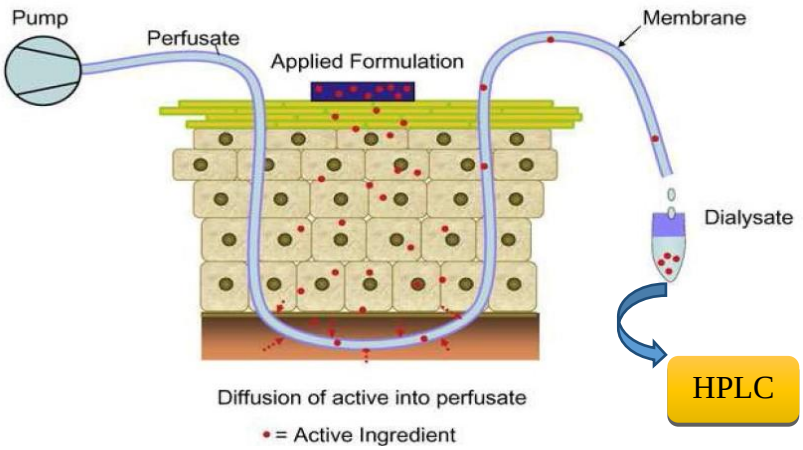


Figure 2.10:The mechanism of cutaneous microdialysis (Uchechi et al., 2014).

After applying the topical formulation, to obtain the level of penetration of the applied formulation, an ultrathin microdialysis semipermeable probe is inserted just directly under the applied formulation, then a physiological solution is pumped through this semipermeable membrane or perfusate to be collected then with the dialysate. After that the sample can be analyzed by using HPLC to obtain the existence and the amount of the applied active ingredient onto the skin.

2.5 The Essential Oils of Oregano and Rosemary for Wound Healing

2.5.1 An overview of oregano essential oil (OEO) androsemary essential oil (REO)

The use of natural herbs was common from ancient times for different purposes in daily products especially in therapies and food flavoring and preservation. Their uses in phytotherapylike an antimicrobial, pain-relieving, antioxidant, hepatoprotective, and many other uses are directly proportional to the active constituents of plants secondary metabolites, the essential oils (EOs). The use of EOs as antioxidants and antimicrobial is absolutely crucial in the prevention of lipoperoxidation and prevention of microbial growth, whether in food or in the human, so these two functions are critical when we talk about wounds and their healing processes, and we expect that EOs will provide us promising results for the improvement of the stages of wound healing. However, these strong activities of EOs may possess a potential alternative to synthetic products that used for same purposes.

Origanum vulgare L., from the family Lamiaceae and the herb where the essential oil of oregano (OEO) is extracted from, is the most common plant that was -and still- used as a spice to enhance the flavor of food, it is also used in the traditional and modern medicine, beside their use in the pharmaceutical industries for variety of purposes. There are four main groups of oregano that have been distinguished, these are; Greek oregano (*Origanum vulgare L. hirtum*), Spanish oregano (*Coridothymus capitatus L.*), Turkish oregano (*Origanum onitesL.*), and the Mexican oregano (*Lippia graveolens*), but *O.vulgare L* was the most common one that has been used for different purposes (Elshafie & Camele, 2017). Generally, Turkey is considered as the largest oregano producer around the world in which approximately over 10,000 ton of dried oregano is harvested in this country, and about 1000 ton is consumed domestically, while some of the harvested oregano is manufactured as essential oil, and the rest of that large produced quantities is exported to the other countries (Can Başer, 2008).

Rosmarinus officinalis L., which is the origin of rosemary essential oil (REO), is a powerful aromatic plant belonging to the family Lamiaceae (Labiatae), and originating in the Mediterranean regionespecially in Turkey, which is affordable in

abundance.It is widely used in folk medicine, food flavoring agent, cosmetics, and many other uses will be discussed later. This plant is also known by many other *Latin* names like alecrim-da-horta, alecrim-de jardim, alecrim-de cheiro, alecrim-rosmarinho roris marino, in *English* it is called rosemary, while in *Spanish* it is known as romero, and in *French* it is romarin, and romarino in *Italian*, whilst rosmarin in *German*. The parts used where the essential oil is extracted are the flowering leaves and summits(Araújo et al., 2017).

2.5.2 Chemical constituents of oregano EO and rosemary EO

In comparison with rosemary EO, oregano EO has less chemical constituents that give this oil its activities. Oregano EO is composed approximately of twenty chemical ingredients and the main compound that constitutes the majority of the oil isCarvacrol from the family phenolic monoterpenes. Other compositions are available in the oregano EO in lower percent like; Thymol, γ-Terpinene, p-Cymene, Myrcene, Linalool, and α-Pinene. The rest components are existing in very low concentration such as; Camphor, Borneol, α-Thujene, Camphene, and others (Lu et al., 2018).

The essential oil distilled from the plant *Rosmarinus officinalis L.*was obtained to be composed of approximately 37-40 chemical constituents. These contents are mainly belonging to the family oxygenated monoterpenes (46.9%) and the family monoterpenes hydrocarbon (46.7%), along with other families that constitute very small percent. The contents of the main two families are as follow(Bozin et al., 2007):

- **Oxygenated monoterpenes (46.9%):** Under this group, the main active constituents are:1,8 cineole (eucalyptol), camphor, linalool oxide, and borneol. Otherconstituents like menthone, α-terpineol, carvacrol, and many others form a little amount of this family(Bozin et al., 2007).
- **Monoterpenes hydrocarbon (46.7%):** The main components of this family are: α-pinene, limonene, and camphene. Other contents such as γ-terpinene, sabinene, o-cymene, and more that constitute the remaining of this group(Bozin et al., 2007).

2.5.3 The uses oforegano EO androsemary EO

2.5.3.1 Oregano EO

Oregano essential oil has been used in different fields due to its attractive effectivity and improved functionality, beside its affordability to be administered orally, by inhalation, or even to be applied topically. Oregano EO has a crucial activity against atherosclerosis (the accumulation of fats and cholesterol in the blood vessels which restricts the blood flow leading to blockage of the arteries and eventually serious health risks like coronary disease and others), therefore, oregano EO possesses a very strong antioxidant activity that inhibits the free radical chain reaction which is directly proportional to the LDL oxidation. For this field, as reported by Kulišić et al, the aqueous tea infusion contains oregano inhibits completely the oxidation of LDL even at small doses(Kulišić et al., 2007). For the oral administration, oregano EO has very effective activity toward gastrointestinal illnesses and abdominal pain, besides, the oregano water showed hypertensive activity, while the oregano oil exhibited hypotensive activity. Furthermore, carvacrol (the main constituent of OEO) demonstrated a successful inhibition of the platelet aggregation and it was much more potent when compared with aspirin(Can Başer, 2008). Oregano EO with its main constituent carvacrol that is responsible for the oil's biological activities has shown activities in other different fields such as; antimicrobial activity, antifungal, antiparasitic, analgesic, anti-inflammatory, antimutagenic, antitumor, insecticidal, hepatopretective, and other applications in multiple areas(Béjaoui et al., 2013).

Talking about wounds and their healing processes, oregano EO plays an absolutely vital role in improving the stages of the wound healing especially for the second phase; the inflammatory phase, in which the antimicrobial and antioxidant activities of the oregano EO prevent the proliferation of the microorganisms inside the wounds, which provides the advantage of speeding-up this stage. Many studies proved that carvacrol induces the synthesis of growth factors and cytokines, and as a consequence, the inflammatory and proliferation phasesare promoted and speeded-up. Moreover, carvacrol was demonstrated to have the ability to modulate the

fibroblast migration and collagen synthesis, resulting in the formulation of the granulation tissue (which is the last step of wound healing) in a faster way (Costa et al., 2019). Other studies showed that carvacrol destroyed the outer membrane of *P. aeruginosa* along with the lipopolysaccharide barrier of this bacterium, besides, carvacrol led to leakage of the phosphate ions of *S. aureus* and *P. aeruginosa* resulting in the overall weakness of the microbes, providing us better enhancement of the wound healing processes. Carvacrol also prevented the formation of biofilm of two species of bacteria;*S. aureus* and *Salmonella Typhimurium*(Ravishankar et al., 2010). Lu et al. results proved that oregano EO can destroy the biofilm of the bacteria faster than the synthetic antibiotics, furthermore, the results showed that the topical application of OEO reduced the burden of the bacteria in burn wounds and it inhibits the growth of the Gram -ve*P. aeruginosa* and the Gram +ve methicillin-resistant *S. aureus* (MRSA) without showing any adverse effects on the human keratinocytes, along with the absence of any cytotoxicity or genotoxicity of the skin(Lu et al., 2018). A study was performed by Béjaoui et al. on five types of bacteria to figure out the antibacterial activity of oregano oil against these bacteria, the tested bacteria were *E. coli* Gr -ve, *S. typhimurium* Gr -ve, Ampicillin-resistant *P. aeruginosa* Gr -ve, *S. aureus* Gr +ve, and *B. subtilis* Gr +ve. The results showed that the EOs existed in oregano inhibited the bacterial activity of the all five tested bacteria. Among the five tested bacteria, the highest antibacterial activity was against *E. coli* with the largest inhibition zones(Béjaoui et al., 2013). Another study was carried out by Amini et al. on *Acinetobacter baumannii* by using oregano EO as a growth inhibitor, the results showed that the antibacterial activity of oregano EOcan cure the wounds and shorten their healing duration, besides, the activity was against both gram (+) and gram (-) bacteria and in particular *Acinetobacter baumannii*(Amini et al., 2019).

2.5.3.2 Rosemary EO

The oil obtained from the species *Rosmarinus officinalis L.* is well-known since ancient times for its uses in many fields, mainly in food and therapeutics. In folk medicine, rosemary is used as antiseptic, antibacterial, general stimulant, antihypertensive, carminative, anti-inflammatory, and other uses (Araújo et al., 2017). Rosemary EO is also used to treat headaches, epilepsy, insufficient

circulation, and as a mild analgesic (Labib et al., 2019). REO has also been used to treat fever, colds, asthma, cough, sinusitis, and rheumatism (Sienkiewicz et al., 2013). Besides, rosemary EO showed activity in memory enhancing; in which by lowering the anxiety, the ability of the brain to concentrate increases (Heerema, 2020). Regarding antibacterial activity of REO, the results of an experiment performed by Sienkiewicz et al. on rosemary EO along with basil EO showed strong activity against multi-drug resistant strains of *E. coli*, it's found that all clinical strains of *E. coli* were sensitive to rosemary and basil EO, and therefore, they can be widely used to eradicate the strains of *E. coli* that diagnosed in different clinical conditions. It was also reported that extended-spectrum β-lactamase (ESBL) that is produced by the clinical strains of *E. coli* were sensitive to REO(Sienkiewicz et al., 2013). Rosemary, along with sage, possesses the best antioxidant activity among various types of herbs. Furthermore, rosemary is the only herb that is commercially available as an antioxidant ready to use in the United States and Europe, and it is marketed in different formulations like oil-soluble, dry powder, water-miscible, and water-dispersible forms. The antioxidant activity of REO was demonstrated as both free-radical scavenging capacity (RSC) and strong protection against lipoperoxidation (LP) (Bozin et al., 2007).

Rosemary EO plays an important role in wound healing, thanks to its antimicrobial and antioxidant properties, in which the decreased inflammation levels, contraction enhancement of the wounds that lead to reduced wound's area, angiogenesis, and improvement of collagen deposition which speeds-up regeneration of the granulation tissue, all were observed after treatment of the wounds with the topical application of rosemary essential oil (Araújo et al., 2017). Rosemary EO, with its antibacterial activity that is achieved by the main constituents; 1,8 cineole, has the ability to inhibit the synthesis of pro-inflammatory cytokines that directly proportional to tissue inflammation (Khezri et al., 2019). It was demonstrated by Labib et al. that the topical application of REO on the wound of diabetic mice possesses noticeable effects in the different stages of wound healing, in addition to wound contraction and enhancement of the oxidative stress status, which offers a reduction in the lipid peroxidation and elevation of glutathione (GSH) levels. The high quantity of oxygenated monoterpenes in REO plays a crucial role in the antioxidant activity and

acceleration of the wound healing process (Labib et al., 2019). Another study was performed by Nejati et al. to elucidate the antifungal activity of REO against *Candida albicans*in wounds; the rosemary oil was formulated as an ointment for topical preparation The results illustrated that REO reduced the inflammatory agents (*C. albicans* in this study) which resulted in shortening the inflammatory phase, moreover, the proliferative phase and the contraction of the wound were speeded-up, and as a consequence, the overall processes of the wound healing were enhanced (Nejati et al., 2015).

2.6 Topical Nanoemulsions: Compositions, Methods of Preparation, and Characterizations

As mentioned earlier, the stratum corneum (SC) is the uppermost layer of the epidermis that acts as a strong barrier against the applied formulations. This issue made the permeation of the prepared formulations across the skin challenging, therefore, many strategies of micro and nanosystems were studied and generated to overcome this obstacle. One of the most feasible strategies was the incorporation of the active ingredient(s) into a carrier of nanoemulsion, so what is a nanoemulsion? Nanoemulsion (NE) is an emulsion with nanometer-sized droplets that ranged between 20-200nm. It is a colloidal system made up of two immiscible phases, an oily phase and aqueous phase in which when the oily phase is dispersed into the aqueous phase; the system is considered as oil-in-water (O/W) NE, while when the aqueous phase is dispersed into the oily phase; the system is referred to as water-in-oil (W/O) NE. Both systems are stabilized by amphiphilic molecules that decrease the surface tension of the system and enhance the solubility of the two phases into the NE system, these molecules are the surfactants. Sometimes co-surfactants/co-solvents are added to the NE system to aid solubilization and stabilization of the system by decreasing the interfacial tension between the two phases. Figure 2.11 represents the main structure of NEs.

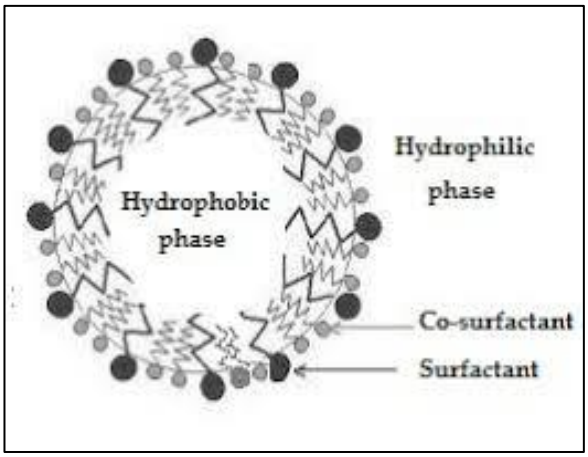


Figure 2.11: Structure of NEs (Kale & Deore, 2017).

Nanoemulsion is a transparent or translucent liquid-in-liquid dispersion system that is kinetically stable and thermodynamically metastable which offers long-term physical stabilities with no coalescence or flocculation in the systemFigure 2.12. Therefore, NEs provide superiorities over the conventional emulsions such as the encapsulation capacity of the drug into the system is larger, besides, dispersibility of the system is much higher due to the nanometer-sized droplets; which prevent flocculation, coalescence, and phase-separation, hence, long-term stability enhancement is achieved(Shaker et al., 2019).

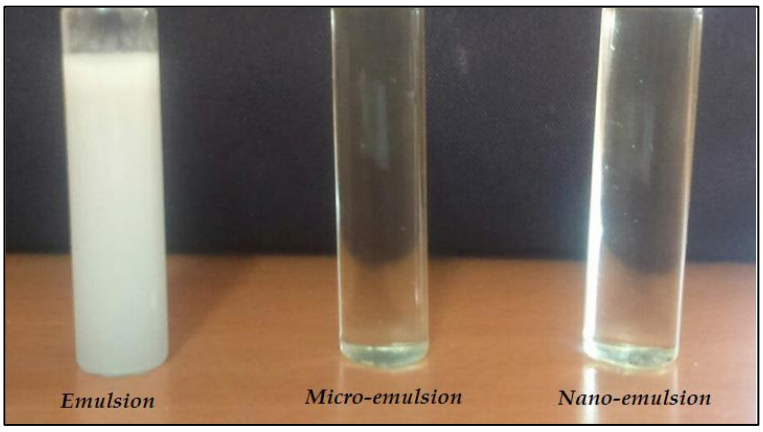


Figure 2.12: Emulsion, Microemulsion, and Nanoemulsion (Kale & Deore, 2017).

The figure illustrates the differences in visual appearance between emulsion, micro and nano-emulsion, in which the coarse emulsion has turbid and milky appearance, while micro and nano-emulsion are transparent but microemulsion is less in transparency when compared to nanoemulsion.

Topical nanoemulsions are novel dermatological nanocarriers that because of their nano-sized;they ease the permeation of the incorporated drug across the SC and improve occlusion, hence the release profile of the drug is improved, which as a consequence, the enhancement of the bioavailability of the applied formulation is achieved(Dal Mas et al., 2016). There are many advantages offered by using nanoemulsion as a carrier for delivering the topical dosage forms, these advantages are; the drug permeation through the SC is increased, the ability of incorporation of both hydrophilic and lipophilic drugs, a high interfacial area is provided which increases the solubility of the drug, protection of the drug from oxidation and hydrolysis, and lower usage of the surfactants when compared to microemulsion, which led to lower toxicity and irritation profile, and as a result, it will reflect the compliance of the patients(Dal Mas et al., 2016).Figure 2.13 illustrates the difference of drug permeation between conventional emulsion formulation and nanoemulsion formulation through the skin and the effect of droplet size on the drug permeation. The figure shows that the topical application of nanoemulsion offers penetration into deeper layers; which enhances the bioavailability of the delivered drug.

A study was performed by Dal Mas et al. to obtain the anti-inflammatory activity of the topical use of *Rapanea ferruginea* extract. The extract was incorporated in conventional cream and in nanoemulsion system to compare the activity differences in the inhibition of the inflammatory inducers, Interleukins (IL) and Tumor Necrosis Factor (TNF). The results showed that the topical application of *R. ferruginea* extract incorporated nanoemulsion was 160% more efficient than the applied cream of that extract(Dal Mas et al., 2016).

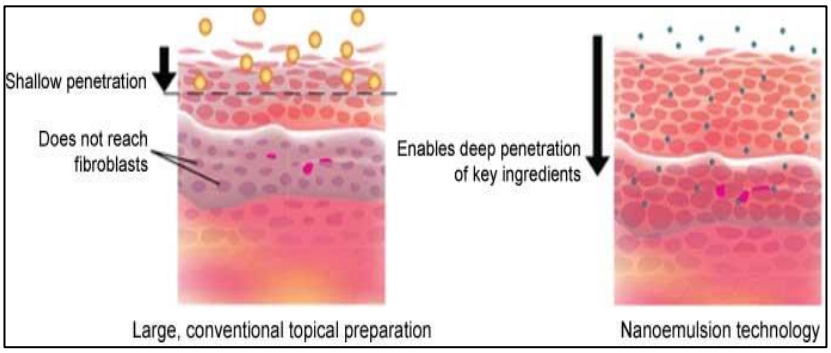


Figure 2.13:Comparison between drug penetration of conventional emulsion formulation and nanoemulsion formulation through the skin (Sutradhar & Amin, 2013).

As obtained from the figure above, nanoemulsions – because of their small size – have the ability to penetrate into deeper layers which offers better bioavailability, on contrary to the conventional emulsions which are not capable to reach deeper layers.

Topical nanoemulsions alone may possess antimicrobial activity against bacteria, viral envelope, and fungi by fusing with the lipid bilayer of the microbial cell membrane and destabilizing those lipids thet existing in the cell membrane, leading to the antimicrobial activity, so by incorporation of essential oilsinto the nanoemulsion system, we expect a promising and synergistic antimicrobial activity as shown and proved by(Al-Sowayigh et al., 2019) against *S. aureus*, MRSA, *E. coli*, and *P. aeruginosa*.

2.6.1 Composition of NEs

The selection of the nanoemulsions' components is very critical, because the stability of the system is strongly dependent and affected by the correct choice of the appropriate components. NEs are composed mainly of oily phase and aqueous phase, surfactants, and/or co-surfactants/co-solvents.

2.6.1.1 Oil

The oil is the most important component in nanoemulsions; because of that the desired activities are achieved through it. Therefore, it may act as a carrier to dissolve the lipophilic API, or can be itself as an active ingredient (as in our study, the EO). The stability of the colloidal system is strongly influenced by the characteristics of the selected oil, like *viscosity* (oils with low viscosity require a short time to be disrupted by the applied external energy, hence the formation of the smaller droplets are achieved faster), *interfacial tension* (low interfacial tension of the oil eases the size reduction process, which leads to reduction in the energy required to decrease the droplet size), and *polarity* (low polarity or highly hydrophobic oils harden the formation of NEs). Essential oils offer low viscosity, low interfacial tension, and high polarity, and therefore, they might be the optimal candidates to achieve a proper NE(Pavoni et al., 2020). Ostwald ripening, a form of the physical instabilities of NEs that meant by the formation of large droplets in the system. This phenomenon is prevented or slowed down by the addition of a group

called ``ripening inhibitors`` like medium or long-chain triglycerides (MCTs/LCTs) or some vegetable oils such as corn oil and sesame oil. These oils act as a kinetic barrier and they have the ability to influence their partitioning between the aqueous phase and the oil droplets, beside their activity to make EOs less water-soluble. For these reasons, mixing of EOs (as an active ingredient) with the ripening inhibitors will assure the long-term stability of the system (Pavoni et al., 2020). A stable antimicrobial NE formulation and prevented Ostwald ripening phenomenon were achieved by Liang et al. when they added MCTs (as a ripening inhibitor) to the peppermint EO (as an active ingredient)(Liang et al., 2012). Asensio et al. added MCTs to retard the formation of Ostwald ripening in an oregano EO-based NE(Asensio et al., 2019). Vegetable oils (the edible oils) are effective as ripening inhibitors in the edible systems (e.g. foods and beverages), they showed a stable NE system when corn oil was added to thyme EO to formulate an antimicrobial NE formulation for food and beverages products(Ziani et al., 2011).Moreover, it was reported that the addition of MCTs as a carrier onto EOs to form the oily phase offered many advantages like alteration of the absorption rate of the EOs (offering effectivity for long time) and prevention of the EOs evaporation. Since MCTs are made of fatty molecules, they will prevent the EOs evaporation, which provides efficacy for longer time (Pavoni et al., 2020).Synthetic MCTs (the hydrolyzed form of regular MCTs) showed to have surfactant-like activities, therefore, they may replace the regular MCTs due to their dual activity(V. P. Patel et al., 2010). In general, regarding the oily phase, MCTs are preferred on LCTs because of the chemical structure of MCTs makes them easier to nanoemulsify than LCTs(Date et al., 2010). Some oils like oleic acid, isopropyl myristate, and capryol 90 have penetration enhancement activity through the SC layer(Shaker et al., 2019). In the penetration enhancement field, EOs like rosemary, eucalyptus, cardamom, menthol, and many other EOs play an absolutely vital role due to their strong capability of enhancing the permeation of the applied formulation across the SC(Das & Ahmed, 2017).

Some examples of the most commonly used oils in the formation of the oily phase of NEsare Oleic acid, Captex® (200,300,355,500), Miglyol® (810, 812, 840), Labrafac®, Capryol® 90,Brij®, and others(Date et al., 2010).

2.6.1.2 Surfactants

The second component of the NEs is the surfactants. Surfactants are amphiphilic molecules that have the ability to interact with both; water (by their hydrophilic head) and oil (by the hydrophobic tail) and disperse the two phases together. Surfactants are not less important than the oil component during the formulation of the NEs, in which the whole system is stabilized and solublized through these molecules by decreasing the interfacial tension between the aqueous and the oily phase in addition to the assurance of the proper dispersion of the system's components by merging the two phases together.

Surfactants are classified into four groups; anionic, cationic, non-ionic, and zwitterionic surfactants. Among these four types, the most frequently used group is the non-ionic surfactants due to their low toxicity and irritation profile when compared to the ionic ones, and they are less affected by the changes in pH, moreover, they are well-known by their good biological acceptance(Salim et al., 2016).Non-ionic surfactants also have the ability to fluidize and solubulize the lipids of the SC, hence, they enhance the permeation and absorption of the applied formulation on the skin (Shaker et al., 2019). Non-ionic surfactants are classified according to a scale invented in 1954 by William C. Griffin, this scale is the Hydrophilic-Lipophilic Balance (HLB), it is a specific empirical expression of the non-ionic surfactants that describes the relationship between the hydrophilic portion of the surfactant to the hydrophobic portion. The HLB scale ranges from 0-20, where the surfactants with HLB 3-6 are lipophilic (e.g. SPANS) and used in the preparation of W/O NEs, and the surfactants having HLB 8-18 are hydrophilic (e.g. TWEENS) and used in O/W NEs, while surfactants having HLB more than 20 are used as co-surfactants(Zheng et al., 2015). The addition of the surfactants is not random; hence they are added gradually till reaching a specific point when the surface becomes saturated with the surfactants and the micelles start to form, this point is called critical micelle concentration (CMC).Therefore, the addition of more surfactants after reaching CMC will no longer affect the surface tension as illustrated in Figure 2.14. However, the surface tension can be measured by using tensiometer (Zheng et al., 2015). It was claimed that a mixture of surfactants may improve the stability of the NE system(Vilasau et al., 2011). Moreover, a mixture containing hydrophilic and

lipophilic surfactants showed higher stability formulation instead of using one surfactant alone, besides, the chemical similarity between the hydrophilic and lipophilic surfactants offers higher stability of the system rather than using surfactants from different chemical families. For example, the combination between SPAN 80/TWEEN 80 from the same family (oleates) provides higher NE stability along with smaller droplets size when compared to using a mixturecontaining SPAN 80/TWEEN 20 from different families (oleate and laurate) (Salim et al., 2016).

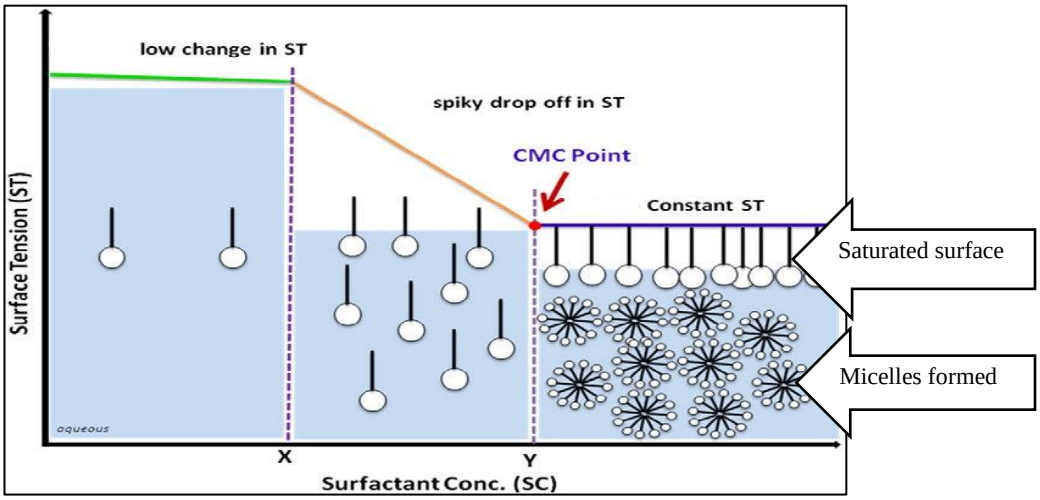


Figure 2.14: The rule of surfactants addition(Singh et al., 2014).

Surfactants will be added till reaching the CMC point in which the surface will be saturated with surfactants and the micelles start to form and the addition of more surfactants will no longer have efficacy.

Regarding the incorporation of the EOs into the oily phase during the formulation of EOs-based NEs, the best choice of choosing surfactants is to select surfactants with intermediate-high HLB value (11-16), it is more suitable with respect to selecting surfactants with too-low or too-high HLB value. Considering EOs-based NE, the most two used surfactants are TWEEN 80 and TWEEN 20, since they possess stability of the NE system without the assistance of co-surfactants(Pavoni et al., 2020).

Examples of the most commonly used surfactants during formulation of the topical NE formulations are TWEENs®, Cremophor®, Plurol Oleique®, Plurol Isostearique®, Labrasol®, and others(Nastiti et al., 2017).

2.6.1.3 Co-surfactants/co-solvents

Co-surfactants are surface-active molecules that assist the action of the surfactants synergistically by reducing the interfacial tension of the system, increasing the fluidity of the liquid-liquid interface, and allowing a higher penetration of the oil between the surfactant tails, which ensure the stability of the system. Their addition may decrease the amount of surfactants used, but also they can't stabilize the NE system alone due to the smaller size of the polar head when compared to the surfactant molecules(Pavoni et al., 2020). Generally, the most frequently used co-surfactant in the NE formulations is Transcutol®, on the other hand, the most commonly used co-solventis alcohol. The effect of the co-solvents on the stabilization of the NEs is depending on the length of alcohol chain. For example, the use of long-chain alcohol such as heptanol or octanol will result in the formation of a less organized micelle system, while using short to medium-chain alcohol provides stable oil droplets and less sign of phase separation. The use of co-surfactants enhance the solubility of the drug-loaded into the system, and by using multiple co-surfactants, the flux of the drug may increase without the need to incorporate the permeation enhancers (Shaker et al., 2019).

Regarding the EOs-based NE, the most commonly used co-solvent is ethanol due to its ability to improve the formation of a system that able to be diluted into the aqueous phase(Pavoni et al., 2020). Co-surfactants/co-solvents such as ethanol, polyethylene glycol, and glycerol when mixed with thesurfactant, Cremophor®, the stability of the NE system was increased, moreover, the antimicrobial activity of the EO also seen to be enhanced(Pavoni et al., 2020). In general, along with Transcutol® and ethanol, there are many other co-surfactants/co-solvents used frequently during formulation of the NEs, these are glycerol, ethylene glycol, propylene glycol (PG), polyethylene glycol (PEG), propanol, isopropanol, and others(Chime et al., 2014).

2.6.1.4 Other additives

During the formulation of nanoemulsions, other components rather than the oils, surfactants, and co-surfactants along with water might be added, generally for stability purposes. Thickening agents or viscosity enhancing agents are rheology modifiers that could be added to the system to provide more stable NE, they reduce

the fluidity or increase the viscosity of the system and form gel-like nanoemulsion or referred to as nanoemulgel. Examples of thickening agents that could be added to the NE systeminclude Carbopol®, Aerosil®, hydrocolloids such as cellulose derivatives, gelling hydrocolloids like gelatin and alginate. Penetration enhancers also can be found in the NE system to improve the permeation and assure the delivery of the applied drug across the SC(Nastiti et al., 2017). Since nanoemulsion is a water-based system, the addition of preservatives is required and important in order to prevent the existence of microorganisms and their proliferation in the system.

In the case of EOs-based NE, the addition of preservatives seems to be unnecessary since EOs have antimicrobial and antioxidant activity. However, oregano, rosemary, and thyme EOs were found to be enhancers of the overall system's antimicrobial activity(Pavoni et al., 2020).

In the end, the whole compositions of the emulsion system should be compatible to each other, otherwise many forms of the instability of the system that lead to phase separation like creaming or sedimentation, flocculation, coalescence, and Ostwald ripening will be appeared(Pavoni et al., 2020).

2.6.2 Preparation methods of NEs

As mentioned earlier, the formulation of nanoemulsions requires external energy input in order to achieve the formation of the nano-sized droplets, this applied energy could be low or high-energy. The differences between these two energies and the types of each will be explained briefly in the following sections:

2.6.2.1 Low-energy emulsification (LEE) methods

Low-energy methods, as it is obvious from the name, require low input energy to fabricate the nanoemulsion. These methods depend mainly on the modulation of the phase transition and physicochemical properties of the system's components(Chime et al., 2014). LEE methods can be performed though:

2.6.2.1.1 Spontaneous emulsification

This method is easy and simple technique to formulate the emulsion with nano-sized droplets; in which oil, surfactants, and/or co-surfactants/co-solvents are mixed

together along with the aqueous phase by using a magnetic stirrer. Spontaneous emulsification is prepared by first mixing the oil(s) with the lipophilic surfactant(s) to form the oily phase, then mixing water with the hydrophilic surfactant(s)to form the aqueous phase, after that the oily phase is poured into the aqueous phase under magnetic stirring to form the NE. This method is extremely dependent on the physicochemical characteristics of the system's components and it's influenced by the chemical compatibility between the emulsion ingredients(Bouchemal et al., 2004). Stirring speed is an important factor that influence the formation of an ideal or an emulsion with nano-sized droplets; in which the particle size decreases with increasing the stirring speed(Komaiko & Mcclements, 2016). Nanoemulsions prepared by this method can be also formulated by another technique through diluting the microemulsion using alcohol as co-solvent, in which alcohol will diffuse from the oil phase into the aqueous phase causing the system to be no longer thermodynamically stable (which is a property of ME), and therefore the system will be converted to NE(Salim et al., 2016).Figure 2.15 shows the method of NE formulation by spontaneous emulsification.

There is another method to form NEs spontaneously which is the phase inversion emulsion (PIE). This method is opposite to the spontaneous emulsification; in which here the aqueous phase is titrated onto a container that contains the oil phase and the surfactant(s). when the water is titrated onto the organic phase, a W/O emulsion will be formed and with the continuous addition of water; a multiple O/W/O emulsion will be generated forming a bicontinuous microemulsion, and eventually this microemulsion breaks down to form O/W emulsion with droplets in nano-sized. During the continuous addition of the water to the organic phase, a liquid crystalline phase might be formed, and this formed liquid has relatively high viscosity which is undesired property in the formation of an ideal NE, therefore, to overcome this obstacle; the system can be heated to decrease the viscosity of this liquid crystalline phase (Komaiko & Mcclements, 2016). Figure 2.16 provides an identification of the formulation of NEs by phase inversion emulsion.

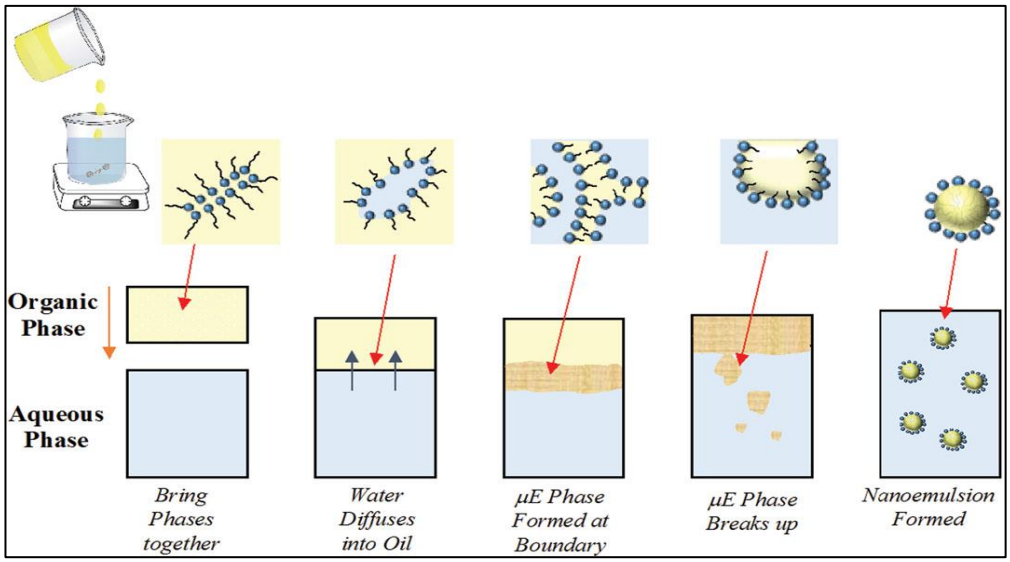


Figure 2.15:Schematicdiagramillustrates the formation of the NE by spontaneous emulsification (Komaiko & Mcclements, 2016).

The oily phase that composed of oil and surfactant(s) is titrated into a container that contains the aqueous phase with a magnetic stirring. After adding the oil phase to the aqueous phase, a bicontiuous microemulsion will be formed at the boundary where the two phases are in contact. By the continuous stirring, the formed bicontinuous microemulsion will be broken forming very fine droplets, and as the speed of the stirrer increased, the formation of a small droplets will be facilitated forming nanoemulsion.

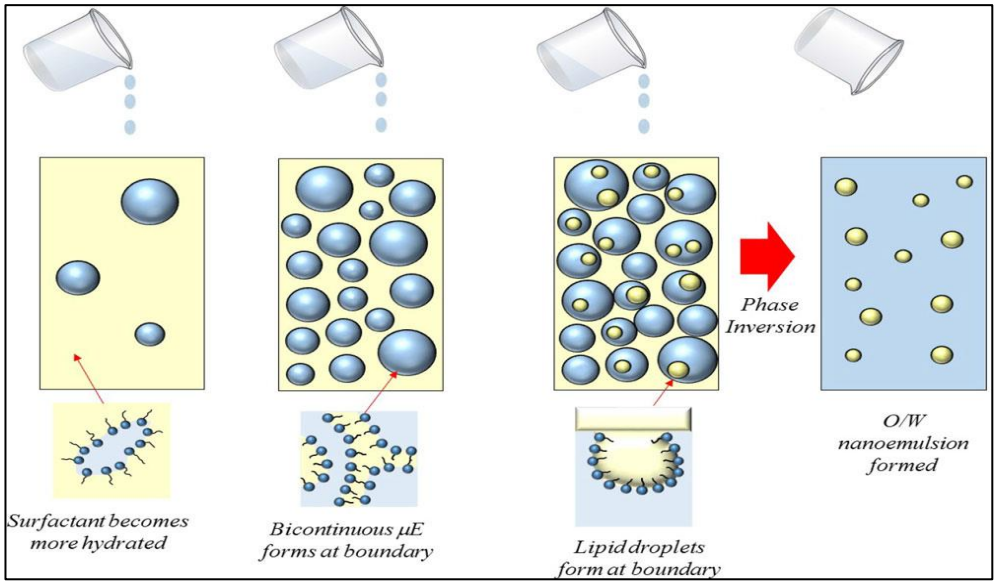


Figure 2.16: Schematic diagram illustrates the formation of the NE by phase inversion emulsion(Komaiko & Mcclements, 2016).

As illustrated in the figure, after titrating the aqueous phase onto the organic phase; a bi-continuous W/O microemulsion will be formed, and by continuous water addition with stirring, a multiple O/W/O emulsion will be generated. After the addition of the required amount of water with continuous stirring, the formed droplets will then be broken down facilitating the system to be inverted O/W emulsion system with nano-sized droplets.

2.6.2.1.2 Phase-inversion temperature (PIT)

PIT emulsification method is based mainly on the transformation of the primary W/O emulsion into an O/W emulsion with nano-sized droplets. This inversion of the phase is achieved by modifying the temperature in order to reach the inversion point where the fine droplets are formed at very low interfacial tension. This method is based on various non-ionic surfactant solubility, in which at room temperature that is below the PIT, these surfactants are almost soluble in the aqueous phase forming O/W emulsion, at a certain temperature (the PIT which is reported to be around 90℃)the surfactants have similar solubility in both the aqueous and oily phase, while raising the temperature above the PIT will weaken the interaction between the water and the hydrophilic heads of the surfactants, hence their solubility in the oil phase increases which reverses the emulsion into W/O system. Regarding the high temperature applied in this method, the heat-labile oils are not suitable to be used in this technique(Pavoni et al., 2020). In general, NE prepared by this method is achieved through three steps (1) mixing surfactants, oil, and water together at room temperature to form O/W coarse emulsion. (2) the mixture is gradually heated till reaching the PIT (90℃) resulting in W/O emulsion. (3) the heated emulsion is then rapidly cooled or diluted into cold water with continuous stirring leading to the formation of O/W nanoemulsion as illustrated in Figure 2.17. The critical step that should be monitored is to rapidly cool down the system from above the PIT to below the PIT, whereby cooling the system slowly will result in the formation of emulsion with large droplets(Komaiko & Mcclements, 2016).

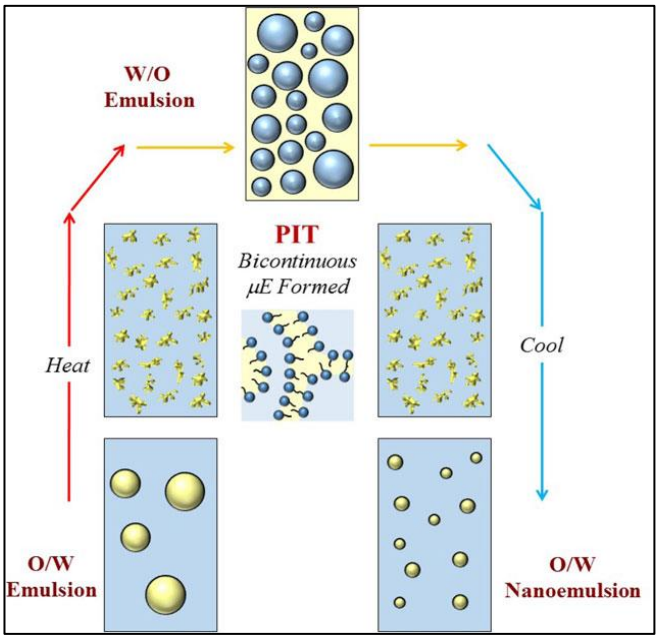


Figure 2.17: Formation of NE by PIT method(Komaiko & Mcclements, 2016).

The figure above shows the steps of the formation of NE by PIT method which can be summarized by three steps; (1) an O/W emulsion is formed at room temperature by mixing oil, surfactant, and the water together, (2) then the formed emulsion will be heated till reaching the PIT ~ 90℃ leading to converting the system into W/O emulsion, (3) and eventually the system is directly cooled down to achieve the production of O/W emulsion with droplets in nano-sized.

2.6.2.1.3 Solvent displacement method

The formulation of NE using this method is achieved through dissolving the organic phase into water-miscible organic solvent e.g. ethanol, acetone, or ethyl methyl ketone. The resulting mixture is then added to the aqueous phase that contains surfactants and/or co-surfactants to yield spontaneous formation of NE by prompt diffusion of the organic solvent, in which this organic solvent is then removed from the system by a suitable method such as vacuum evaporation. This method doesn't require energy as it accomplished instantaneously at room temperature(Chime et al., 2014).

2.6.2.2 High-energy emulsification (HEE) methods

HEE methods are meant by the formation of emulsion with nano-sized droplets through using instruments that apply very high mechanical energy to the injected

system. These methods are sophisticated and consume large energy, therefore, theyare expensive but offer the advantage of controlling the size of the droplets, hence ensuring more uniform nanoemulsion droplets. Due to the high energy applied, this method is not suitable for thermo-labile components such as retinoid, enzymes, proteins, and nucleic acids(Jasmina et al., 2017). The formulation of NE through HEE methods is achieved generally by two steps, the first one is the preparation of the coarse emulsion which is formed by mechanical stirring of all components together, second, the prepared emulsion system is then converted into nanoemulsion through large disruptive forces that applied by mechanical instruments which break up the large droplets of the system into very small droplets forming the desired nanoemulsion system. However, these HEE methods are high-pressure homogenization, micro fluidization, and ultrasonication(Pavoni et al., 2020).

2.6.2.2.1 High-pressure homogenization

This method is the most commonly used method to formulate nanoemulsions, in which the prepared coarse emulsion is pumped into the chamber of the instrument and then forced under high pressure (usually 500-5000 psi) to pass through a very small orifice, which produces disruptive forces that able to smash the large droplets of the systeminto smaller ones. This method proved to be effective in the production of very small droplets(Pavoni et al., 2020). The applied high pressure is achieved by the assistance of several forces which are hydraulic shear, intense turbulence, and cavitation, whereby when they act together, extremely small droplets are produced, hence nanoemulsion is fabricated. Smaller nanoemulsion droplets can be produced through repeatedly subjecting the resultant to the high-pressure homogenizer until the desired droplet size is obtained(Chime et al., 2014). This method is only for the preparation of O/W nanoemulsions with oil contents less than 20%, whereby using a large oil content will reduce the productivity of the method, leading to undesired results(Jasmina et al., 2017).

2.6.2.2.2 Microfluidization

Microfluidization method produces NEs by the use of a high-pressure displacement pump that applies a very high pressure (500-20,000 psi) on the injected emulsion. After the formation and injection of the coarse emulsion into the instrument, this

pump forces the phases of the system to pass through an interaction chamber that consists of micro-channels which reduces the size of the droplets. Repeated passing of the coarse emulsion to these micro-channels will result in the production of very fine droplets, hence nanoemulsion system is achieved. In order to assure the uniformity of the droplets of the NE produced, the bulk emulsion is filtered through using a filter under a nitrogenic atmosphere to remove the large droplets (if existed) from the system(Salim et al., 2016).

2.6.2.2.3 Ultrasonication

The ultrasonic homogenization method that has been used to prepare NEs is meant by exposing the coarse emulsion (that is prepared before) to high-intensity ultrasonic waves (generally 20-24 kHz) by the use of a sonicator probe, whereby sudden transformation of the turbid coarse emulsion into transparent nanoemulsion occurs. Figure 2.19 shows nanoemulsion prepared by ultrasonication method. This sonicator creates disruptive forces that have the ability to exert cavitational effects on the droplets causing these droplets to break-up which offers the transformation of a coarse emulsion into nanoemulsion(Kentish et al., 2008). The mechanism of this production is shown in Figure 2.18The particle and droplet size of the system decrease as increasing the duration of exposure of the system to the ultrasonic waves, along with increasing the intensity of these waves, with considering the types and the amounts of the surfactants used in the system(Jasmina et al., 2017). It is important to optimize the intensity of the applied ultrasonic waves because it could damage the structure of the components existing in the nanoemulsion system(Pavoni et al., 2020).

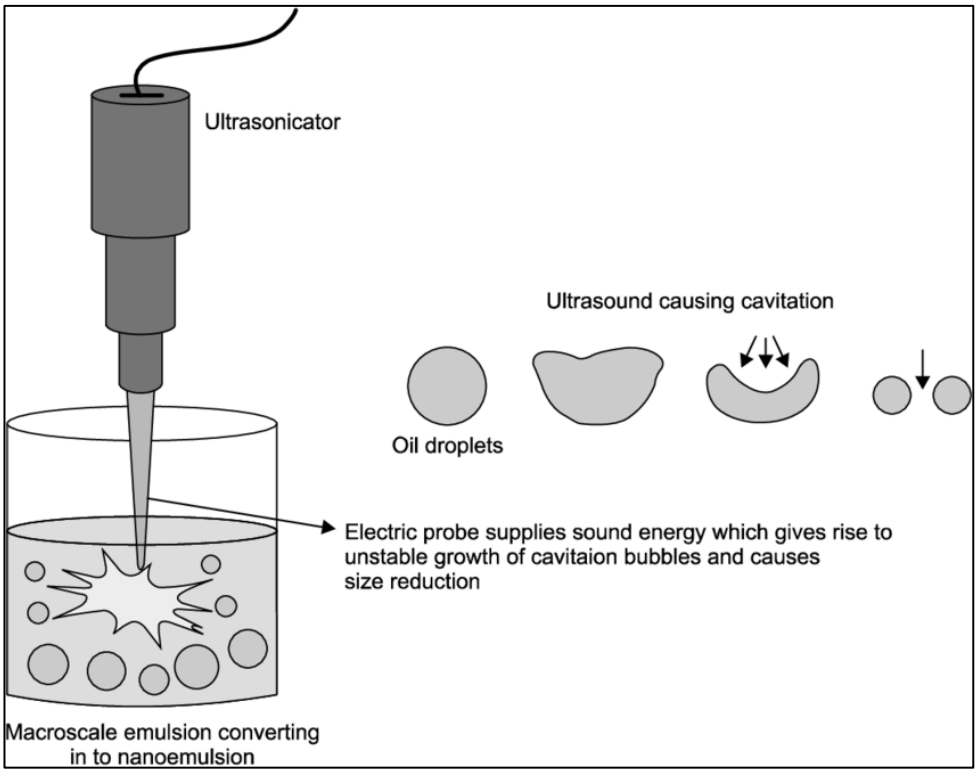


Figure 2.18: The production of nanoemulsion by Ultrasonication method (Kumar et al., 2019).

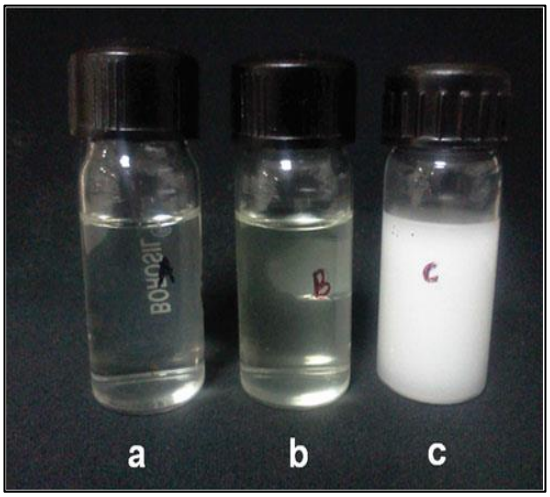


Figure 2.19: The effect of ultrasonication on the visual appearance on the NE at differenr time intervals (Sugumar et al., 2014).

As shown in Figure 2.19, the sample (C) is the coarse emulsion with turbid and milky appearance, after exposing this coarse emulsion to the ultrasonic waves, the sample (B) which is microemulsion will be produced and by the continuous exposure of the sample to these ultrasonic waves, the sample (A) will be resulted which contains an emulsion in nano-sized droplets with transparent appearance.

However, among these three methods, high-pressure homogenization is the most frequently used method to fabricate nanoemulsions and can be used in both, laboratory and industrial-scale, while microfluidization and ultrasonication; in addition to their expensive cost, they are only used in laboratory-scale, hence they cannot be used in the production of nanoemulsions in large amounts(Jasmina et al., 2017).

2.6.3 Characterizations of NEs

The achievement of an ideal nanoemulsion cannot be done by chance or randomly, ratherit is achieved according to specific criteria and tests performed on the sample after getting the final formulation of this sample. These tests are performed in order to assure the stability and the perfection of this finished product, and also to ensurethe stability of this formulation for long term which then reflects the acceptability of the consumer. These characterizations can be summarized as follow:

2.6.3.1 Visual assessments

Visual evaluation by the naked eye is the initial part of evaluating the NE finished product. After centrifugation of the sample for 30 minutes, the absence of phase separation and creaming or any physical instabilities, along with the production of transparency or translucency sample refer – initially – to an excellent formulated NE(Dal Mas et al., 2016). Different visual assessments of the emulsion formed are elucidated in Table 2.2.

Table 2.2: Visual assessment of NE (Suresh & Sharma, 2011).

NE grade	Emulsification time	Observation	Visual appearance
Grade A	Less than 1 minute	Rapidly forming of clear and transparent NE with high dispersibility	Clear or slightly bluish
Grade B	Within 1 min	Rapid NE formation but with less transparency, and less clear	Less clear and slightly white bluish
Grade C	Within 2 min	The formation of NE is less rapid and turbid in nature	Slightly milky white
Grade D	Within 3 min	NE devoid of minimal emulsification or take a long time to emulsify, the oil droplets are non-uniform distributed	Fade, grayish white emulsion with slightly oily appearance
Grade E	Longer than 3 min	Longer emulsification time is required with poor NE properties	Large oil globules present on the surface

2.6.3.2 Viscosity and electrical conductivity

Viscosity and electrical conductivity measurements are useful to predict the emulsion type and to detect the phase inversion phenomena. The viscosity of the NE formulations is a crucial factor whereby it influences the stability of the formulation and the drug release from that formulation. The viscosity is a function of surfactants, water, and oil composition along with their concentrations, and this viscosity is measured by a viscometer. Generally, the viscosity of the emulsion is decreased with increasing the water content, while decreasing the surfactant and co-surfactant content will result in a reduction of the interfacial tension between the oil phase and the water phase, leading to increased viscosity. However, highly viscous NE formulation will retard the penetration and the release of that applied formulation, therefore, O/W NE has faster drug release and penetration than W/O NE,

furthermore, they are less greasy and easily washable(Shaker et al., 2019). Electrical conductivity measurement is simple and inexpensive method; in which it is performed by inserting conduct o meter electrodes into the NE sample. In general, an aqueous continuous phase ((O/W) nanoemulsion) conducts high electricity, whilst an oil continuous phase ((W/O) nanoemulsion) has low or no electrical conductivity(Nastiti et al., 2017).

2.6.3.3 Morphology of the NE droplets

This evaluation is crucial to verify the consistency in shape, size, and distribution of the emulsion's droplets to be in the nano-range.The most commonly used instruments to measure the nanoemulsions' droplets properties are transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In TEM, higher resolution image of the dispersed phase can be obtained, while SEM gives 3D image of the globules. The determination is achieved through negatively staining of the NE sample with a 1% aqueous solution of phosphotungstic acid or by dropping 2% solution of uranyl acetate and then the sample applied on a 200 μm mesh size copper or carbon-coated grid. The used accelerating voltage is usually 20 kV, and then the results are figured out by using the appropriate magnifications and software. Other sophisticated techniques such asatomic force microscopy, X-ray, and cryo-electron microscopy can be used to obtain the behavior and morphology of the formed nanoemulsion(Chime et al., 2014).

2.6.3.4 Droplet size, zeta potential (ZP) and polydispersity index (PDI)

The stability of the NE system is strongly affected by these three factors; in which they might be considered almost the most important characterization to evaluate the ideal NE formulation. As mentioned earlier, the normal range of the nanoemulsion droplets is in between 20-200nm. The NE system is composed of many particles, each of these particles has a surface charge (usually negative(Carpenter et al., 2019)), the surface charge and the repulsive forces between these particles are called zeta potential (ZP), while the broadness of the size distribution and the quality or homogeneity of the dispersion, or in another definition, the degree of the agglomeration or aggregation of the particles in the sample, both definitions are indicated by polydispersity index (PDI)(Nastiti et al., 2017). Therefore, the higher

the ZP values of the NE sample (in positive or negative as demonstrated in Table 2.3), and the lower the PDI values (<0.2), will provide us excellent stability of the NE system(Shaker et al., 2019). The stability of the colloidal system is a balance between the vender der Waals' attractive forces and the electrical repulsion between the particles due to the net surface charge, therefore, if the zeta potential values fall below a certain level, the attractive forces will lead the system particles to agglomerate and instability of the system will be resulted. On the other hand, if the zeta potential values were high (whether in positive or negative), generally more than 30 mV, the overall stability of the system will be maintained and an ideal NE formulation will be produced(Abolmaali et al., 2011). However, droplet size, zeta potential, and polydispersity index can be measured by using an instrument called Zetasizer (Mohamed Salama & Ahmad Mustafa, 2013). Zetasizer is based on the measurement by dynamic light scattering (DLS) which is also called photon correlation spectroscopy (PCS), this DLS is used to analyze the fluctuations by using laser light which passes through the droplets/particles that are subjected to Brownian motion (the random and uncontrolled movement of the particles that are suspended in the fluid)(Nastiti et al., 2017).

Table 2.3: Zeta potential values and their effects on the stability of the colloidal system (Arulprakasajothi et al., 2018).

Zeta potential value (mV)	Stability behavior of the system
0 to ± 5	Rapid aggregation/agglomeration
± 10 to ± 30	Initial instability
± 30 to ± 40	Moderate stability
± 40 to ± 60	Good stability
$> \pm$ 61	Excellent stability

2.6.3.5 Thermodynamic stability studies

These studies are performed in order to ensure the stability of the formulated NE for the long-term when being storaged by subjecting the formulation to three different conditions; heating-cooling cycle, centrifugation, and freeze-thaw cycle.

These three studies are explained briefly by(Galvão et al., 2018)as follows:

- **Heating-cooling cycle:**This is the first step in studying the stability of the formulated NE. The sample is subjected to six cycles between cooling at(4℃) for 24 h and heating at (45℃) for 24 h, if the sample passed without any form of instabilities, then it will be transferred to the next step.
- **Centrifugation:** The next step of evaluating the stability of the NE formulation is centrifugation, where the sample is centrifuged at 5000 rpm for 30 min. If the centrifugation of the sample is performed without separation of the two phases, then we will move to the next cycle.
- **Freeze-thaw cycle:** This step is meant by exposing the sample to an accelerated environment. The sample is subjected to three freeze-thaw cycles in which it is incubated in the freezer at -20 ℃ for 22 h, then followed by thawing in the water bath at 30 ℃ for 2 h. If the three cycles performed without showing any signs of instability, then the formulated NE is considered to have good stability.

After performing these tests, the sample is analyzed by HPLC to obtain the validity of the drug loaded into the NE sample along with the other compositions.

2.6.3.6 In-vitro skin permeation for the topical applied NE formulation

Skin permeation and drug release of the applied formulation can be assessed by using Franz Diffusion Cell for in-vitro evaluation. Franz Cells offer many advantages over other techniques like; (1) simple method with few handling of tissues, (2) continuous sample collecting is not required, and (3) the amount of drug required for the analysis is very low(Salamanca et al., 2018). Determination of the drug release and permeation can be achieved by dispersing a little amount of the formulated NE on the donor chamber of the Franz cell that has a membrane acts as a barrier, then the permeation of the encapsulated drug is monitored in the receptor chamber which contains phosphate buffer saline (PBS, pH 7.4) and a magnetic stirrer at 100 rpm at 37±1 ℃(Chime et al., 2014).

The mechanism of in-vitro assessment of the drug permeation by Franz diffusion cell is simplified in Figure 2.20.

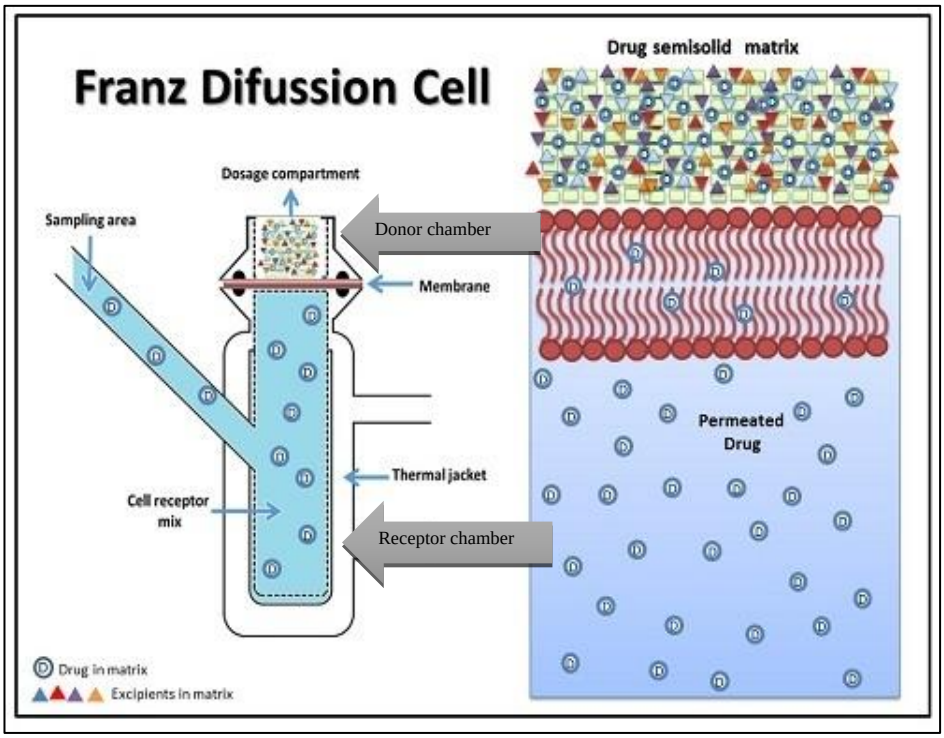


Figure 2.20: Franz Diffusion Cell (Salamanca et al., 2018).

The figure above illustrates the Franz Cell that provides in-vitro evaluation of the topical formulations. This Franz Cell consists of upper donor chamber that contains the formulation wanted to be tested, and lower receptor chamber that contains phosphate buffer saline solution with a magnetic stirrer. The two chambers are separated by a cellulose membrane that acts as a barrier to test the permeability of the applied formulation. To obtain whether the drug permeated or not, a sample can be taken from the receptor chamber through the sample port, then the collected sample is analyzed using HPLC to check the existence of the active ingredients.

2.6.3.7 Other characterization

Other studies can be applied to the formulation to assure the achievement of an ideal NE formulation. However, these studies are pH, skin irritation, rheology (by rheometer in the case of nano emulgel), antimicrobial activity against the microbial strains (like in our study), and other evaluations according to the type and aim of the study.

CHAPTER THREE

3. MATERIAL AND METHOD

3.1 Materials

Oregano (*Origanum vulgare L*) and Rosemary (*Rosmarinus officinalis L*) essential oils were gifted from (Bezmialem Vakıf Üniversitesi, Istanbul, Turkey)to form the oil phase, the non-ionic surfactants used were Kolliphor RH® 40 (HLB= 13)and Kolliphor PS® 80 (HLB= 15)and they were gifted from (BASF company, Germany), and purified water was used as an aqueous phase.

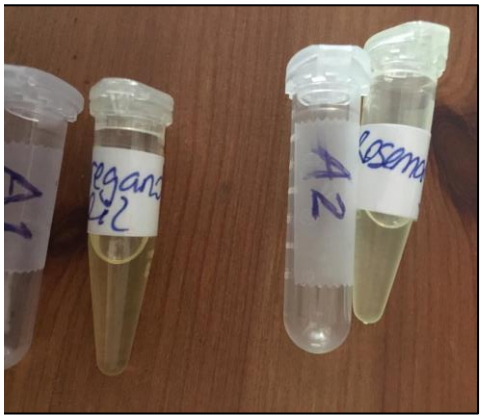


Figure 3.1: Oregano (A1) and Rosemary (A2) essential oils.

Figure 3.2: Digital balance (METTLER TOLEDO).

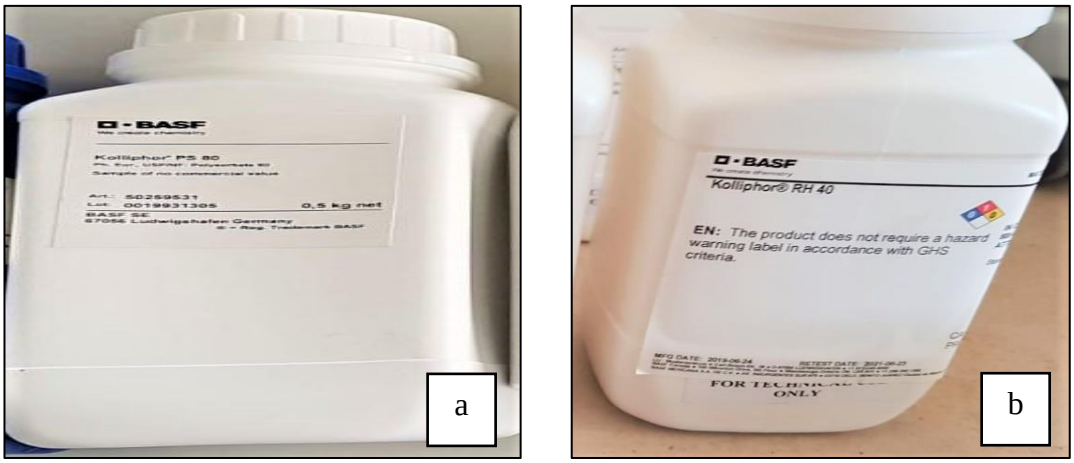


Figure 3.3: Kolliphor PS 80 (a) and Kolliphor RH 40 (b).

3.2 Methods

3.2.1 Gas chromatography-mass spectroscopy (GC-MS)

GC-MS test was provided from (Bezmialem Vakıf Üniversitesi Fitoterapi Eğitim Uygulama ve Araştırma Merkezi, Istanbul, Turkey)(Agilent Technologies, US) and it was used to obtain the constituents of Oregano and Rosemary EOs and to elucidate the active substance(s) of the oils.

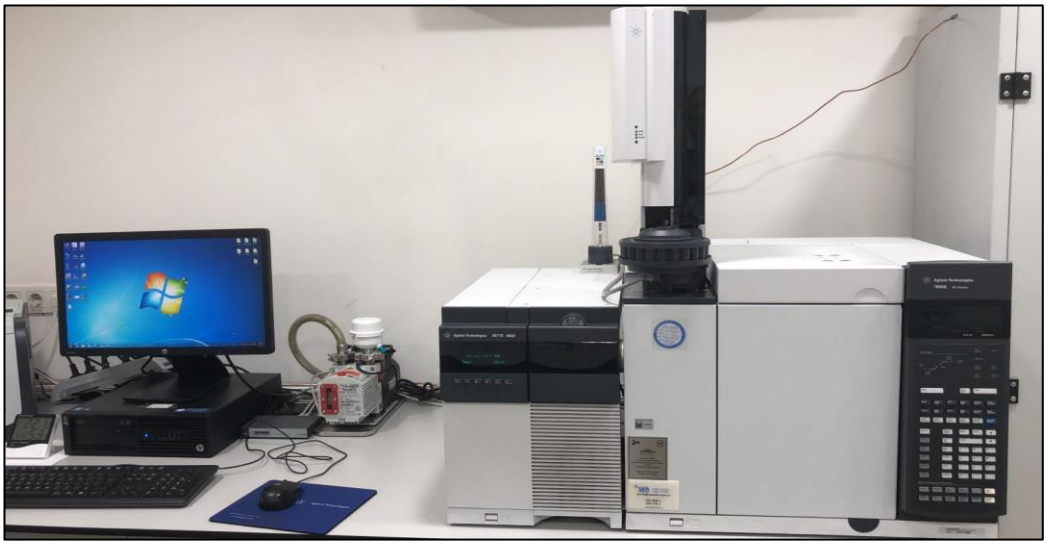


Figure 3.4: Gas Chromatography-Mass spectroscopy (GC-MS) instrument.

3.2.2 Antibacterial activity of oregano and rosemary EOs

3.2.2.1 Bacterial inhibition zone

We took **Oregano** (A1) and **Rosemary** (A2) EOs to compare the antibacterial activity between these two oils against *Staphylococcus aureus* bacterium which is the usual associated bacterium with wounds. The method used to identify the inhibition zone was Disc Diffusion Method.

The bacterium was incubated in a medium at specific temperature for specific time. A suspension of McFarland standard was prepared from the isolated colonies. Then the essential oils impregnated blank discs were placed on the plates. Petri plates were incubated at specific temperature for specific time. At the end of the period, the zone diameters were measured. The levofloxacin disc was used as a control. The test was provided by (Ankara University, department of pharmaceutical microbiology).

3.2.2.2 Bacterial minimum inhibitory concentration (MIC)

The MIC of the selected oils was performed using Broth microdilution method.
The bacterium was incubated on in a medium at specific temperature for specific time. From the isolated colonies, a suspension McFarland standard was prepared and diluted with the medium to give the final concentration. A1 oil was first diluted this stock was used in broth microdilution plates. Samples were added to the first wells and serial dilutions were made. Afterwards, bacterial suspensions were added to the wells and after specific time incubation at determined temperature, Ciprofloxacin was used as a standard antibiotic.

3.2.3 Nanoemulsion preparation

3.2.3.1 The preparation of NE formulations with different oil and surfactant percentages

The preparation of nanoemulsion will be performed after selecting the suitable oil with better antimicrobial activity. So according to the results that will be shown and discussed later, oregano oil was chosen to have better antimicrobial profile. Eight formulations (O_1-O_8) were prepared with different ratios of the oil and the two selected surfactants along with purified water as shown in Table 3.1. The NE preparation method was Ultrasonication homogenization. The preparation method started by first preparing the coarse O/W emulsion with specific ratios in a beaker with a magnetic stirrer. The selected two surfactants were dispersed in 5 ml purified

water. Magnetic stirring was applied at 10 rpm (IKA Werke RT 15 Power) for 30 minutes. The specific amounts of Oregano oil were added to water-Kolliphor PS® 80 and water-Kolliphor RH®40 dispersant in approximately 30 seconds when homogenization was applied at 2000 rpm (IKA T25 Digital Ultra Turrax). The homogenization process was maintained specific time and rpm. After the homogenization process achieved, the samples were taken to probe sonicator (Sonics VibraCell). Ultrasonic processor was adjusted at 130 watts 20 kHz and maintained for 10 minutes. The samples were kept at 8 ^{o}C.

Table 3.1: The eight different formulations of NE with different oil and surfactants percentages.

<table>
<tr><th>Formulation code</th><th>Oregano oil (%)</th><th>Kolliphor RH® 40: oregano oil</th><th>Kolliphor PS® 80: oregano oil</th><th>Purified water</th></tr>
<tr><td>O1</td><td>50 µl</td><td>-</td><td rowspan="4">Starting from 1:1</td><td>5 ml</td></tr>
<tr><td>O2</td><td>50 µl</td><td>-</td><td>5 ml</td></tr>
<tr><td>O3</td><td>50 µl</td><td>-</td><td>5 ml</td></tr>
<tr><td>O4</td><td>50 µl</td><td>-</td><td>5 ml</td></tr>
<tr><td>O5</td><td>50 µl</td><td rowspan="4">Starting from 1:1</td><td>-</td><td>5 ml</td></tr>
<tr><td>O6</td><td>50 µl</td><td>-</td><td>5 ml</td></tr>
<tr><td>O7</td><td>50 µl</td><td>-</td><td>5 ml</td></tr>
<tr><td>O8</td><td>50 µl</td><td>-</td><td>5 ml</td></tr>
</table>

These eight NE formulations were prepared to figure out which samples will give the best results that will be taken for further studies to reach the optimum results of a prepared NE formulation. Therefore, after selecting the optimum results among the prepared eight NE samples, the preparation methods that will be mentioned in sections3.2.3.2 and 3.2.3.3 were run.

3.2.3.2 The preparation of NE formulations with different sonication amplitude percentages

The amplitude percentage study had similar preparation process as the beginning preparation. As distinct from all, this process was prepared as 10 second for the addition time of oil, 13800 rpm as homogenization rate, and 1 minute as

homogenization time. In amplitude study with the selected surfactant concentration, the amplitude percentage was manipulated as 35, 55, 75 and 95%, while the sonication process took a fixed time as **3 minutes as standard parameter**. The test was run after diluting the selected optimum samples with 1% dilution.

3.2.3.3 The preparation of NE formulations with different sonication times

The time of sonication study had the same preparation process as the amplitude percentage study. The process had 10 seconds as adding time of oil, 13800 rpm as homogenization rate and 1 minute as homogenization time. In the time of sonication study with the selected surfactant concentration, now sonication time was manipulated as 3, 5, 7, and 10 minutes, while the sonication amplitude was kept fixed at **%35 as standard parameter.** The test was run after diluting the selected optimum samples with 1% dilution.

After finding out the best NE samples' results, these samples were taken and specific parameters were run to carry out the final optimum NE formulations that correspond the criteria of the ideal NE characteristics.

3.2.4 Characterization of NE

The following evaluations will be performed on the prepared eight NE formulations to obtain the ideal and most stable one.

3.2.4.1 Visual assessment

The prepared NE formulations are assessed by noticing the transparency of the samples through visual evaluation.

3.2.4.2 Droplets size, zeta potential, and PDI

These three parameters are measured by Zetasizer Nano ZS (Malvern Instruments 1000 HS®, Worcestershire, UK) after diluting the NE samples with a ratio of1:100 with purified water.

3.2.4.3 pH

The pH of the NE formulations was measured by pH meter (HANNA instruments, Woonsocket, Rhode Island, US) to obtain the most suitable pH that is compatible with the NE components and the skin. The results are then recorded.

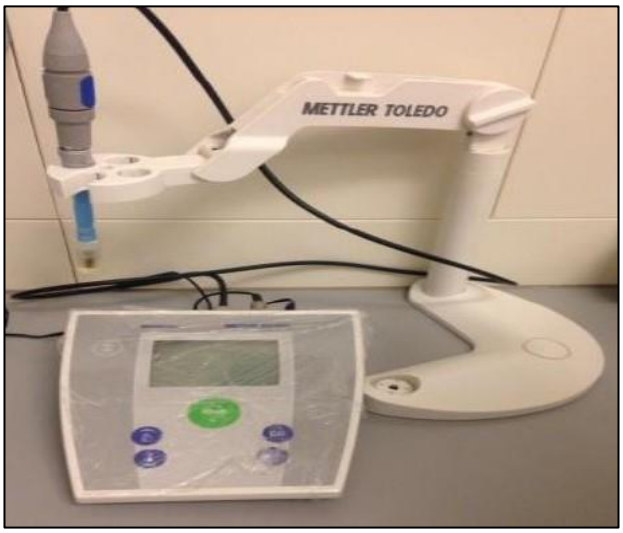


Figure 3.5: pH meter (METTLER TOLEDO)

3.2.4.4 Viscosity

The viscosity of the optimum NE sample was measured by RheoStress RS1 rheometer(HAAKE^{TM}, US).

CHAPTER FOUR

4. FINDINGS

4.1 Gas Chromatography-Mass Spectroscopy (GC-MS)

The main active substance of **Oregano** oil that is responsible for the oil's antibacterial activity was found to be carvacrol with a concentration of (50.61%). The chromatogram of Oregano oil's constituents and their abundance in the oil are illustrated in Figure 4.1.

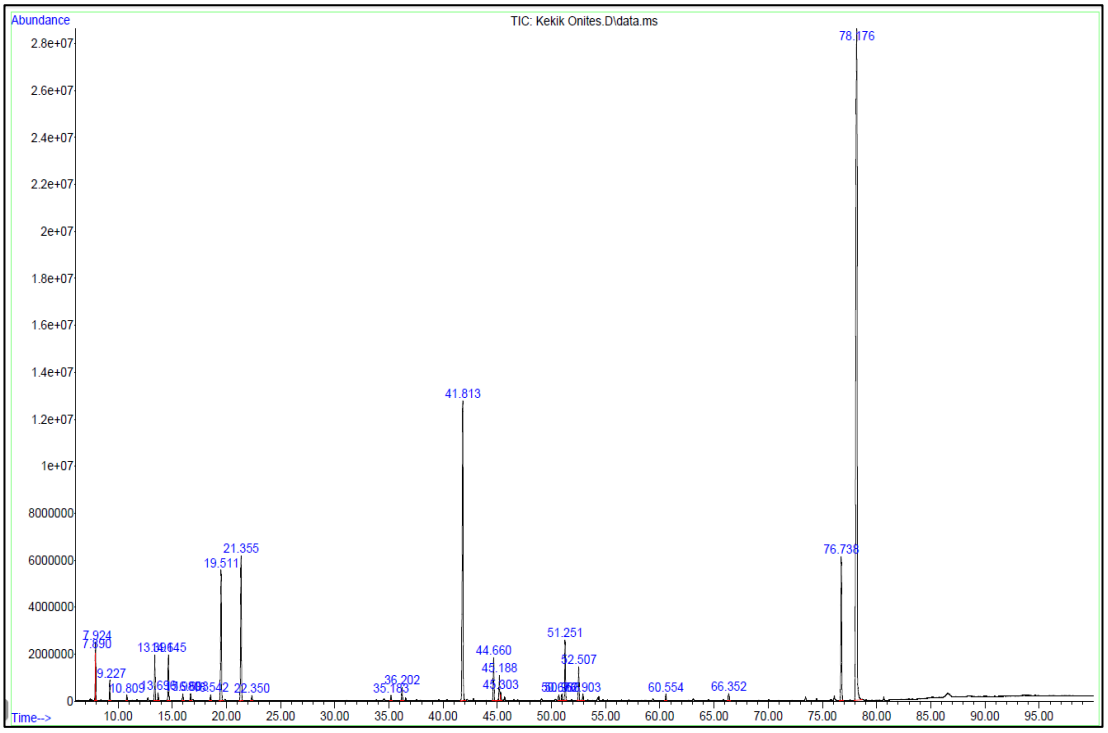


Figure 4.1:Gas chromatography-Mass spectroscopy (GC-MS) chromatogram of Oregano oil constituents.

While for **Rosemary** oil, the main active substance that is responsible for the antibacterial activity of the oil was shown to be 1,8-Cineole with a concentration of (39.35%). The chromatogram of Rosemary oil's constituents and their abundance in the oil are illustrated in Figure 4.2.

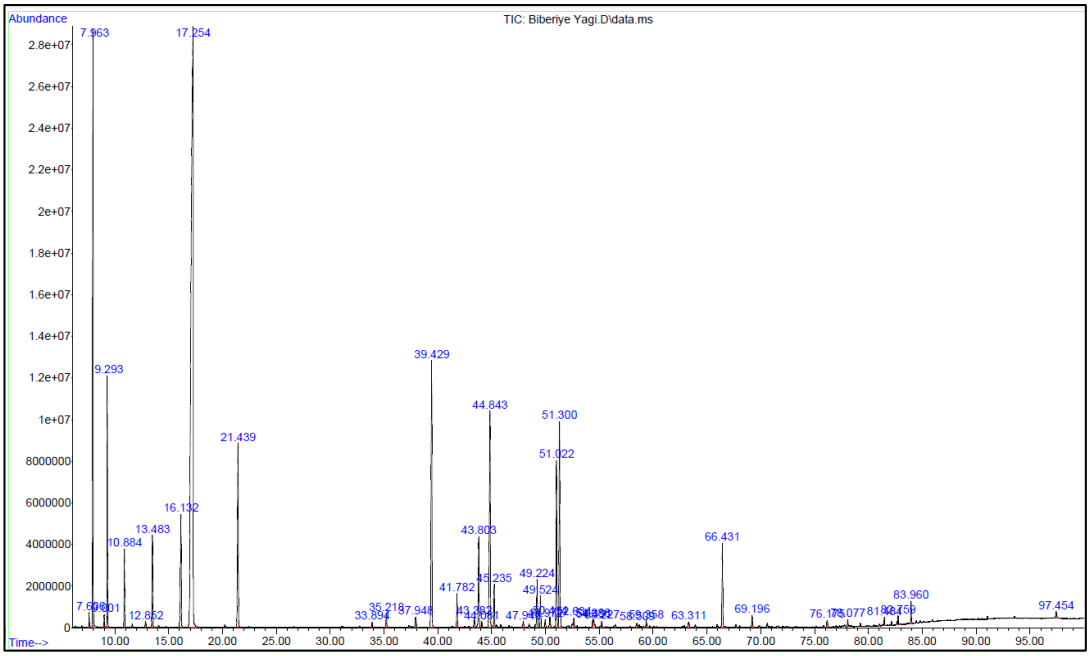


Figure 4.2:Gas chromatography-Mass spectroscopy (GC-MS) chromatogram of Rosemary oil constituents.

4.2 Antibacterial Activity of Oregano and Rosemary EOs

4.2.1 Bacterial inhibition zone

Regarding the bacterial inhibition zone, the tests on *Staphylococcus aureus* bacterium was carried out. The results showed that **A1** oil (Oregano) has inhibition zone much wider than **A2** oil (Rosemary) which informs us that **A1** oil is more effective against the selected bacterium than **A2** oil. The inhibition zone diameter in the disc diffusion is illustrated in Figure 4.3.

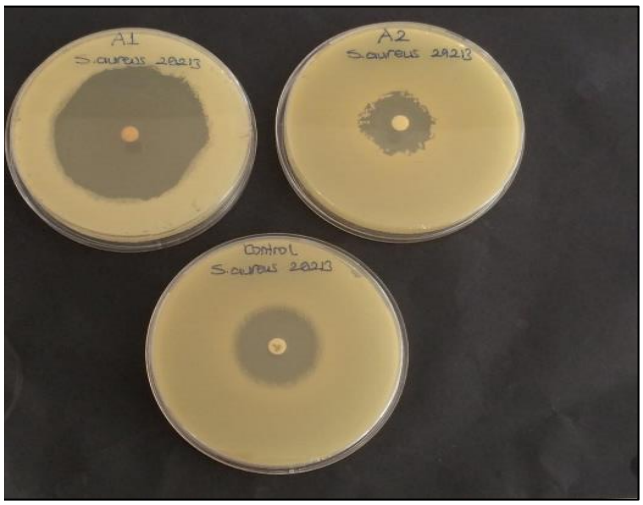


Figure 4.3: Disc diffusion inhibition zone resultson *Staphylococcus aureus* bacterium.

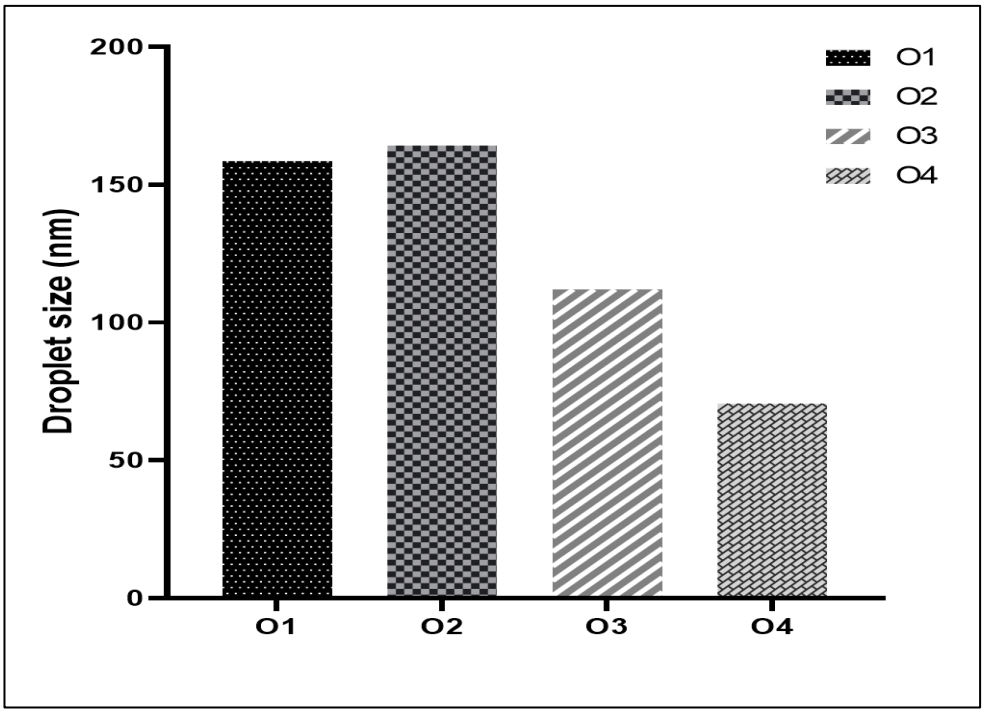
4.2.2 Bacterial minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of **A1**that required to inhibit the growth of the *Staphylococcus aureus* bacteriumwas 0.024 μg/ml and it wasmuch smaller than that required from**A2** oil which is 0.48 μg/ml and the synthetic antibiotic Ciprofloxacin that is 0.5 μg/ml.

4.3 Nanoemulsion Preparation

4.3.1 The preparation of NE formulations with different oil and surfactant percentages

After comparing Oregano oil with Rosemary oil for the best antibacterial activity as shown earlier, Oregano oil was taken to be our model for the study due to its outstanding results against the selected bacterium. So, the components and quantities of the prepared eight NE formulations with Oregano oil were given in **Error! Reference source not found.**3.1 and the droplet size and PDI results of these prepared NE formulations are illustrated in



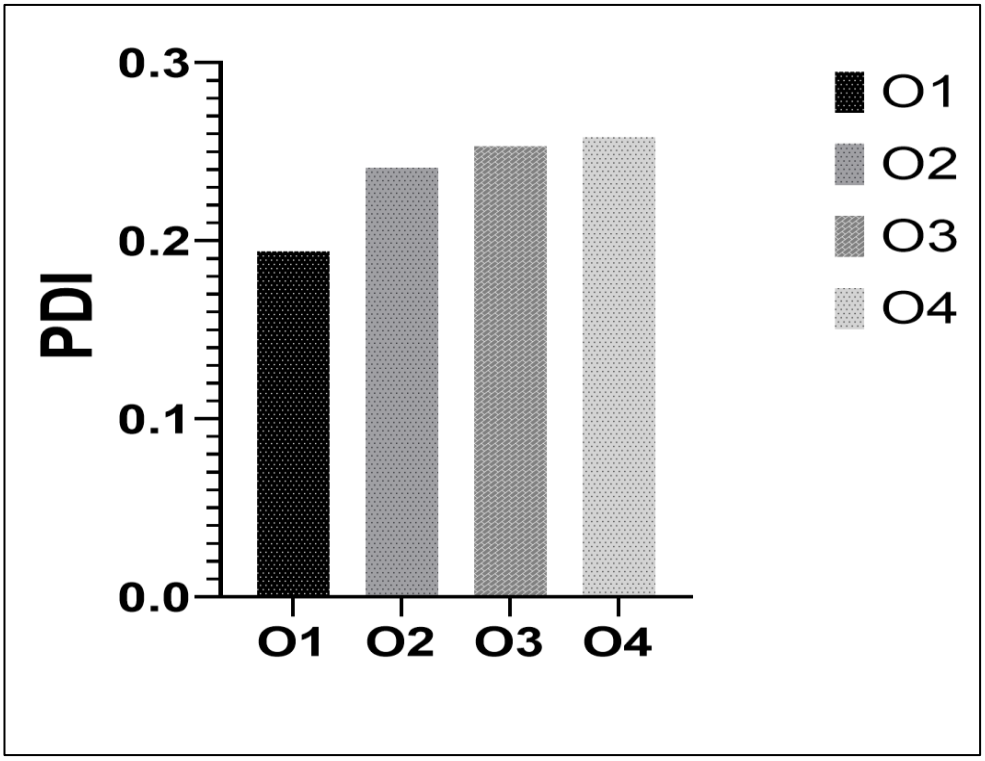
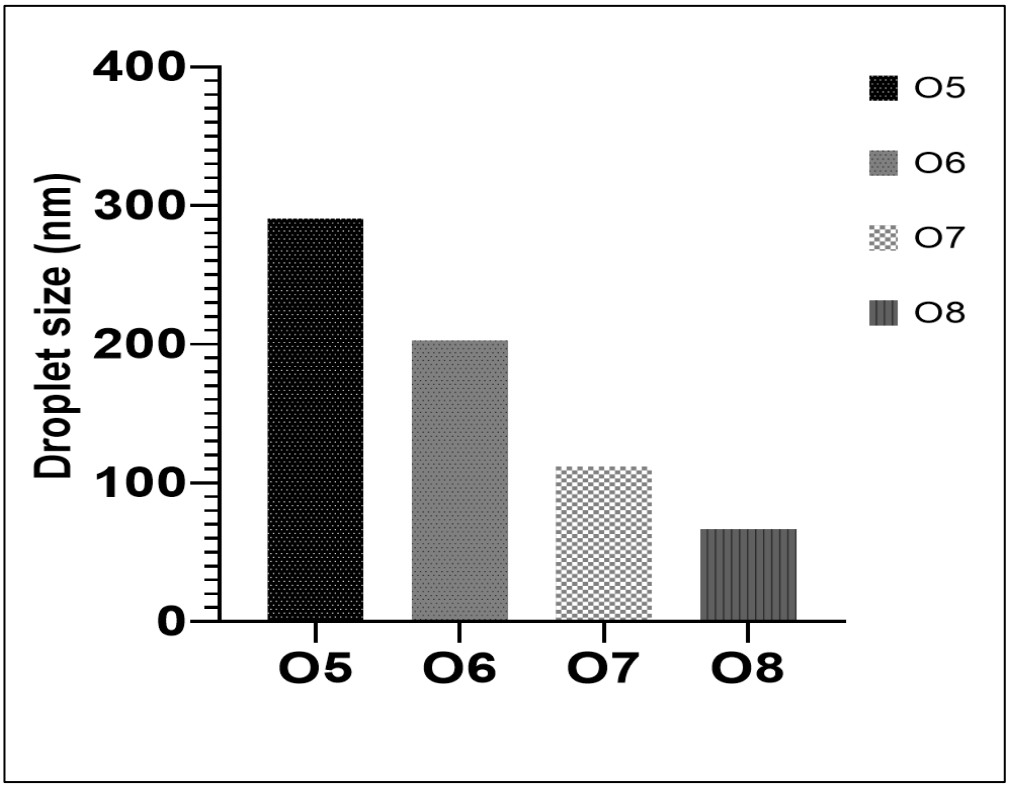


Figure 4.4 and


Droplet size (nm)
400
300
200
100
0
O5
O6
O7
O8
O5
O6
O7
O8

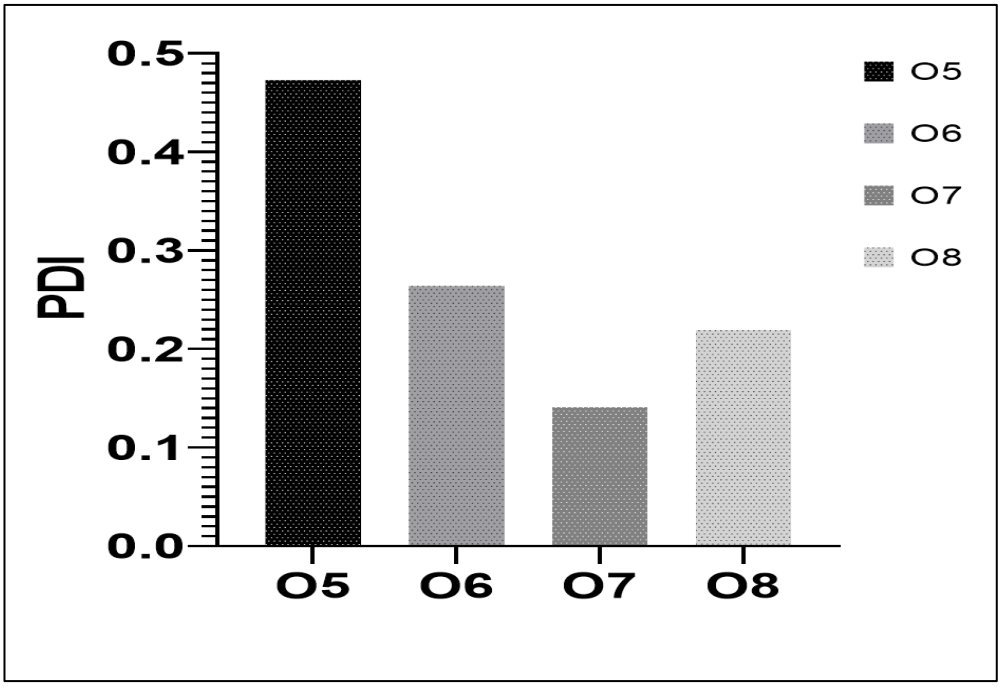

PDI
0.5
0.4
0.3
0.2
0.1
0.0
O5
O6
O7
O8
O5
O6
O7
O8

Figure 4.5.

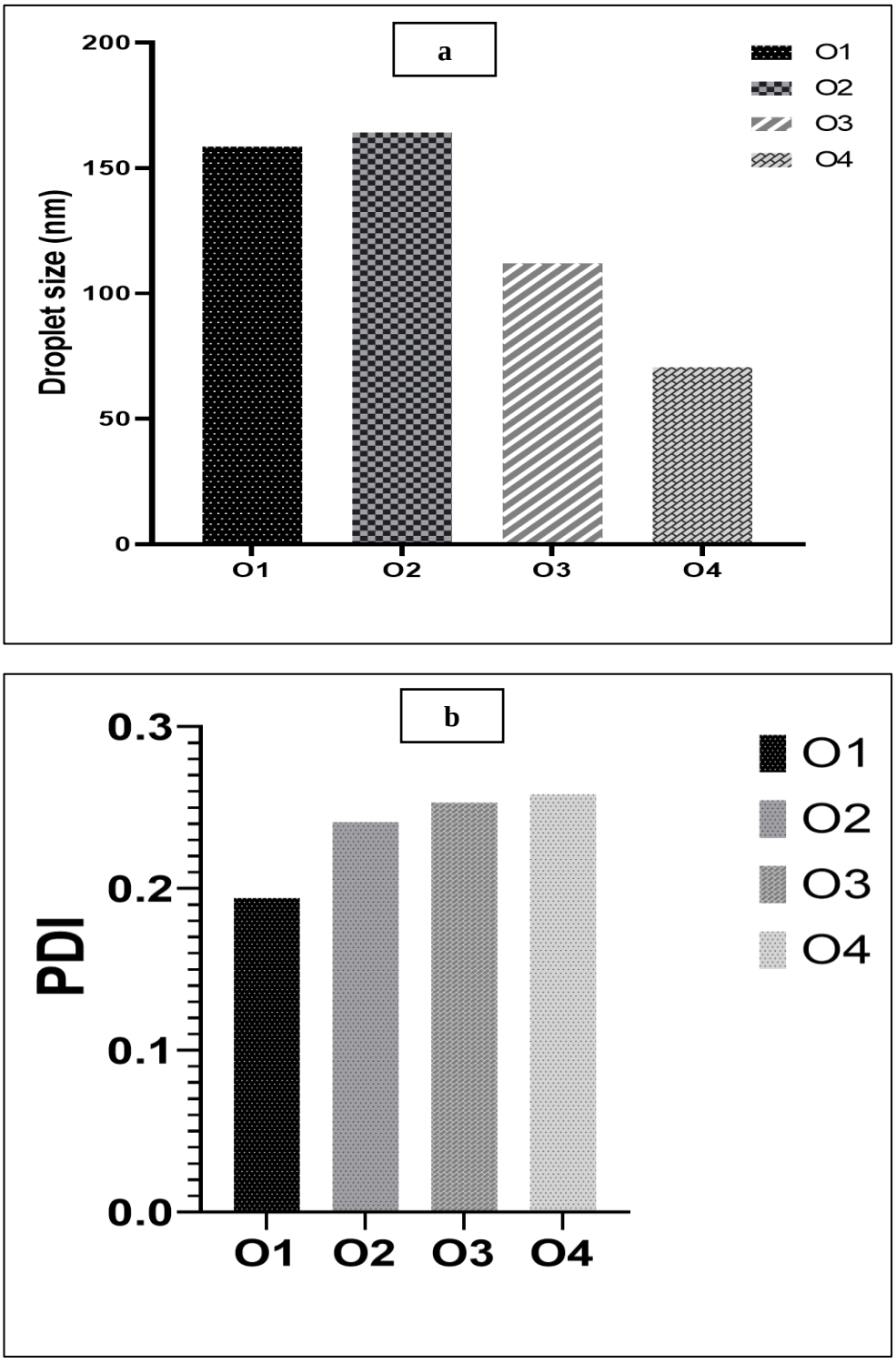
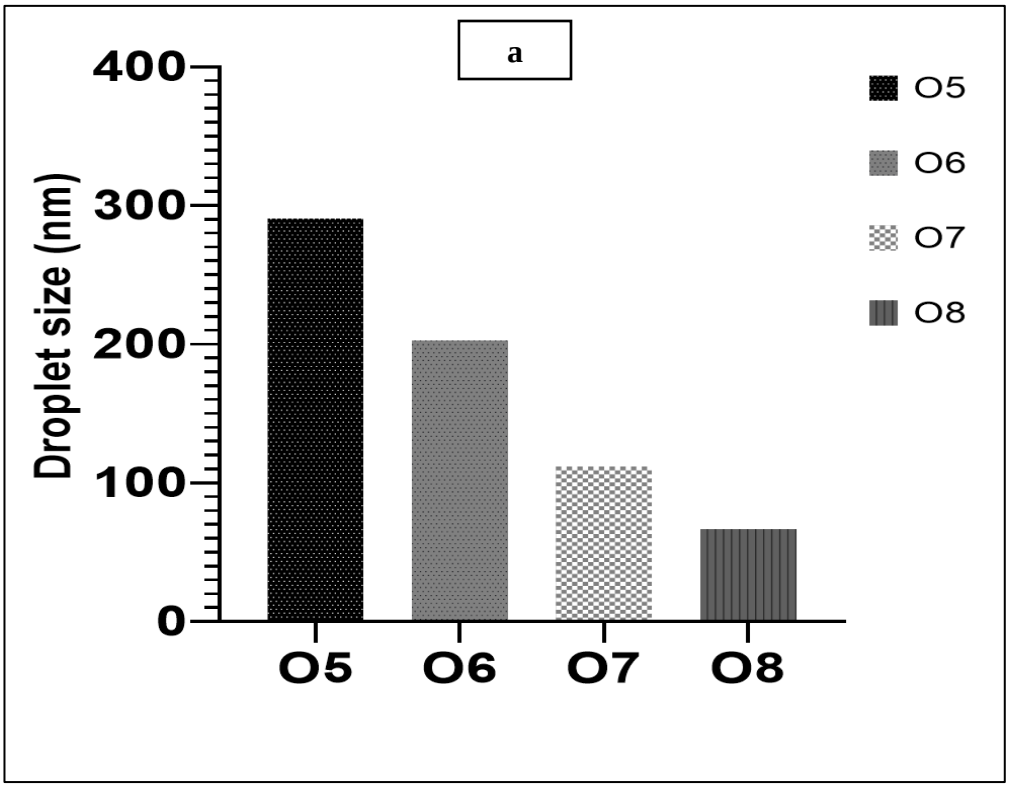


Figure 4.4: The droplet size (a) and PDI (b) results of NE formulations with Kolliphor PS® 80 (O_{1-4}).



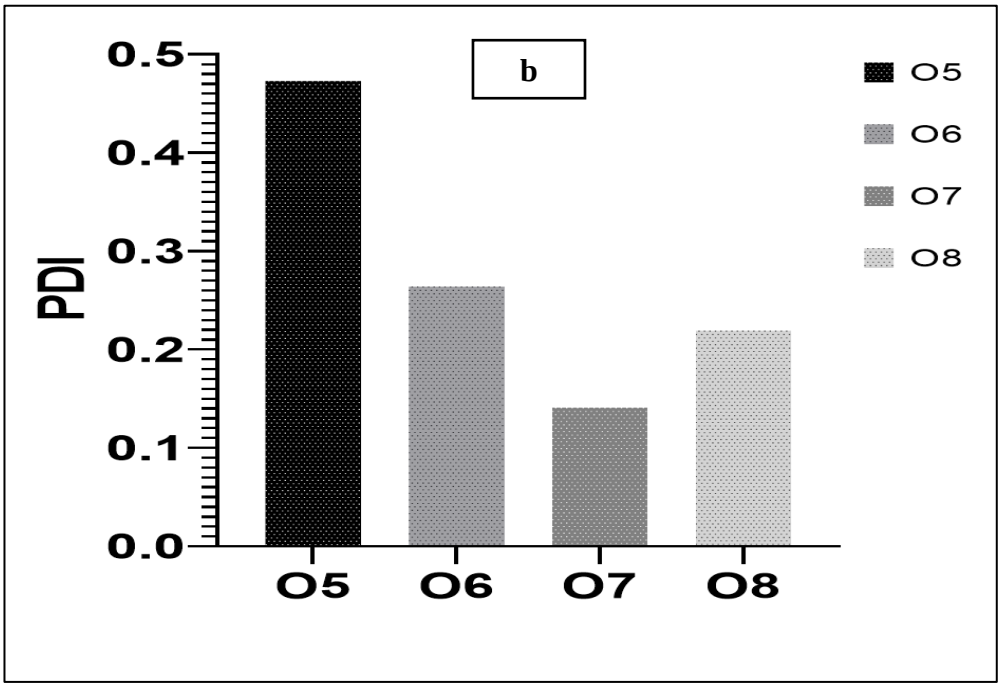


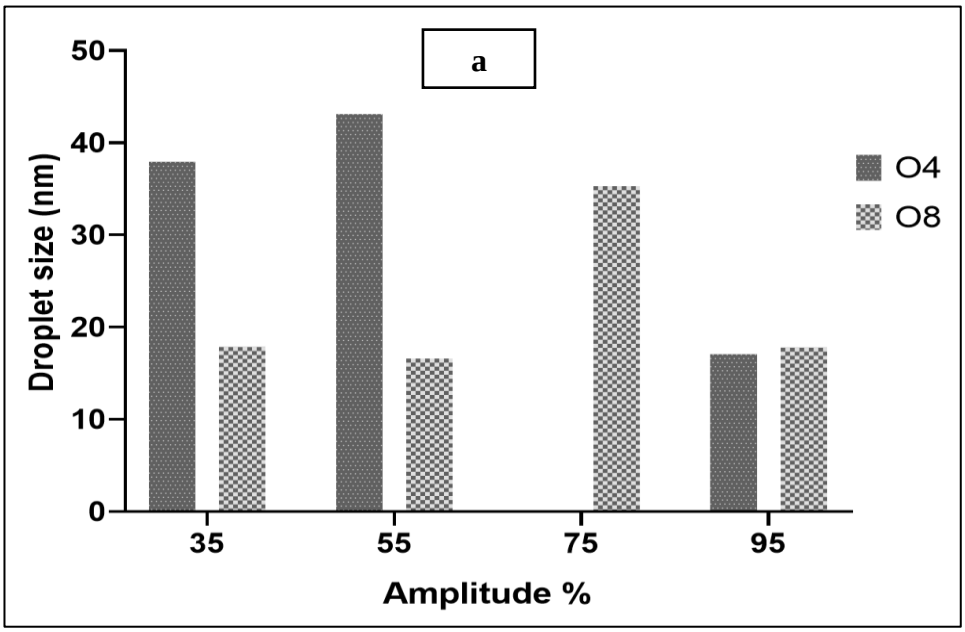
Figure 4.5: The droplet (a) size and PDI (b)results of NE formulations with Kolliphor RH® 40 (O_{5-8}).

Therefore, when we look at the droplets size and PDI values of the prepared eight NE formulations; **O4** (with Kolliphor PS® 80) and **O8** (with Kolliphor RH® 40) were noticed to have the optimum results among the other NE formulations. These two formulations were taken for further studies and prepared by a minute homogenization in 13200 rpm and 3 minutes of sonication in 130 watts 20 kHz. They were kept at 8 ^{o}C. The oil was applied in 10 seconds at 2000 rpm in homogenization.

After running the previous preparation method, the selected two formulations (**O4** and **O8**) were prepared under different conditions to carry out the optimum formulation. These conditions were at specific time of sonication and specific sonication amplitude percentage.

4.3.2 The preparation of NE formulations with different sonication amplitude percentages

The first study was performed on **O4** and **O8** samples by fixing the time of sonication at 3 minutes with changing the sonication amplitude as mentioned earlier in section 3.2.3.23.2.3.2. The droplet size and PDI results of this study are illustrated in Figure 4.6.



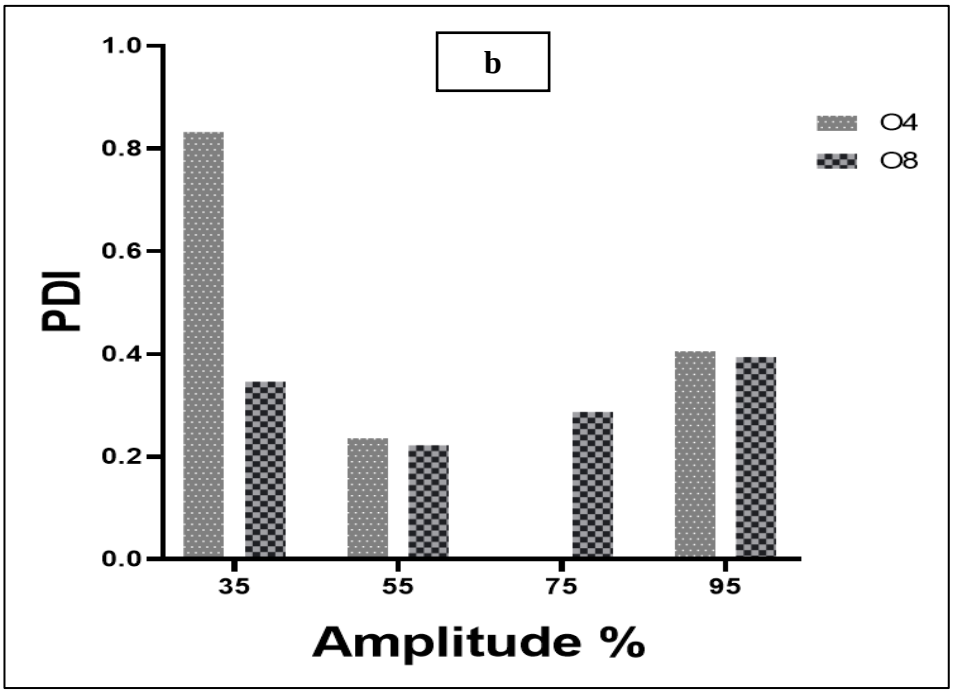
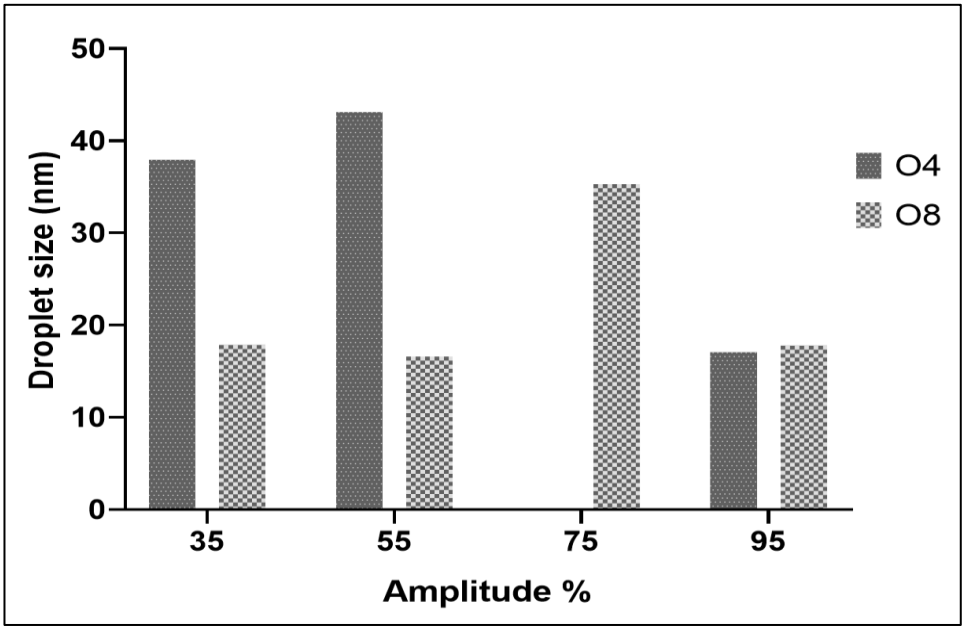


Figure 4.6: The results of droplets size (a) and PDI (b) for O4 and O8 at different amplitudes with standard time at 3 min.

As shown from the results that are given



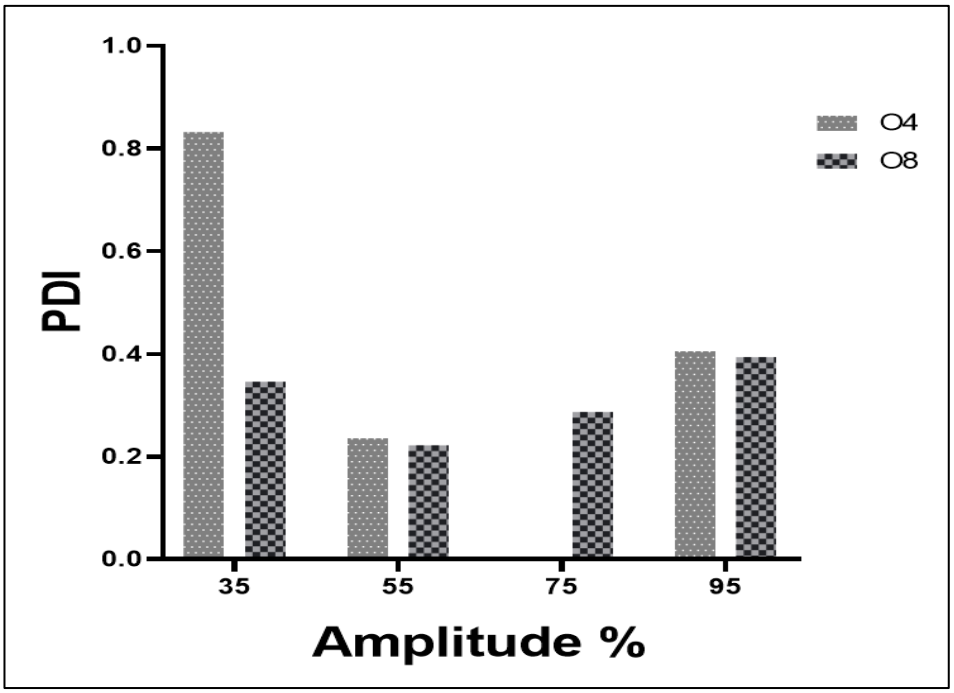


Figure 4.6, for **O4** formulations, 95 A4 sample has acceptable droplet size results among others, but the PDI value was relatively high. On the other hand, for **O8** formulations, 55 A2 sample has optimum results of PDI and droplet size among the other formulations.

4.3.3 The preparation of NE formulations with different sonication times

The next study was performed by fixing the sonication amplitude at 35% with manipulating the time of sonication by a preparation method explained before in section 3.2.3.3. Again, the aim is to carry out the optimum results among the tested NE samples. The droplet size and PDI results for **O4** and **O8** are shown in Figure 4.7.

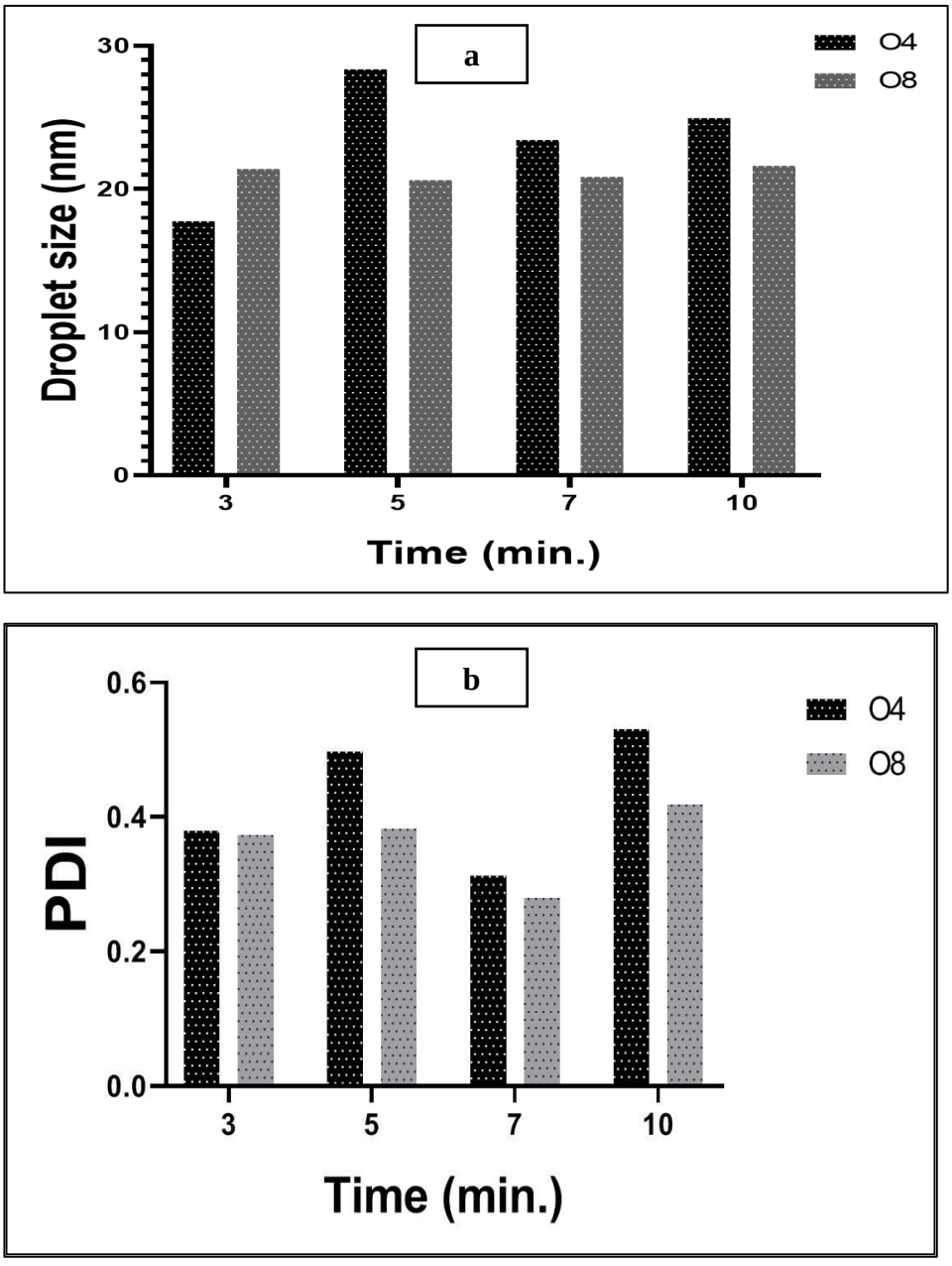


Figure 4.7: The results of droplets size (a) and PDI (b) for O4 and O8 at different time of sonication with standard amplitude at 35%.

From Figure 4.7 results, we can notice that for **O4** formulations, 3 T1 sample has an only acceptable result for droplet size, but for PDI values; all four formulations were not as needed. In contrast, for **O8** formulations, 7 T3 sample got the optimum values of PDI and droplets size over the other formulations.

4.4 Characterization of The Prepared NEs

4.4.1 Visual assessment

Figure 4.8 shows the differences in visual appearance between **O4** and **O8** NE formulations at different amplitudes with standard time at 3 min. By assessing the samples, the results are relatively close to each other in transparency but the formulations of **O8** samples seem to be more transparent especially for the sample **O8(***55 A2***)** due to the given values of droplet size and PDI given for this sample.

Figure 4.8: Visual appearance of O4 (a) and O8 (b) at different amplitude percentages with standard time at 3 min.

Figure 4.9 shows the differences in visual appearance between **O4** and **O8** NE formulations at different time of sonication with standard amplitude at 35%.. By assessing the samples, this time the differences in transparency between **O4** and **O8** samples are obvious, in which **O8** NE samples were more transparent than **O4** samples, especially for the sample **O8(***7 T3***)** due to the provided values of droplet size and PDI given for this sample.

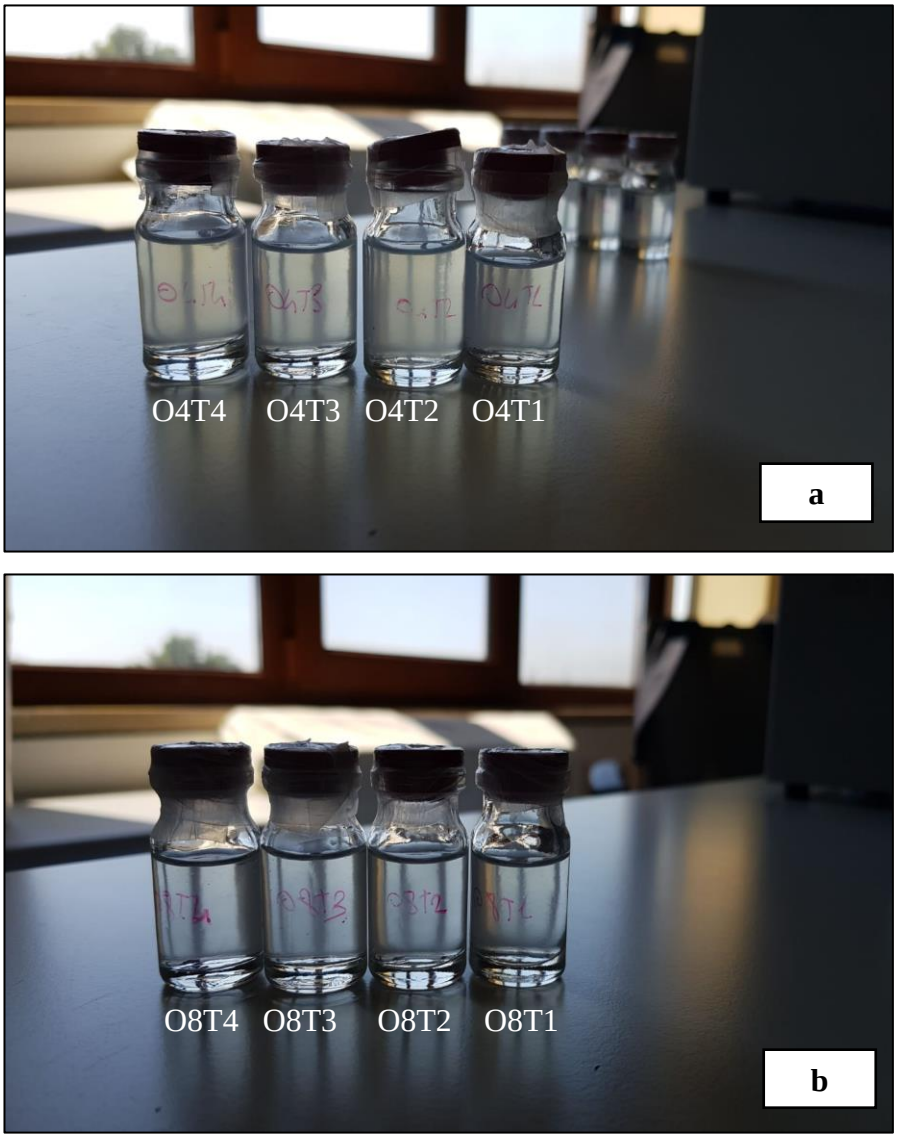


Figure 4.9: Visual appearance of O4 (a) and O8 (b) at different times of sonication with standard amplitude at 35%.

4.4.2 Droplets size, zeta potential, and PDI

After the optimum NE samples were found (**O8 A2 T3**), these samples were taken as one sample by the selected parameters (O8 at 55% amplitude (A2) and for 7 minutes sonication time (T3)) and the rest of the NE characterization will be run on this NE sample. After running the analysis on the sample, the droplet size, the ZP, and the PDI values of the sample were optimized.

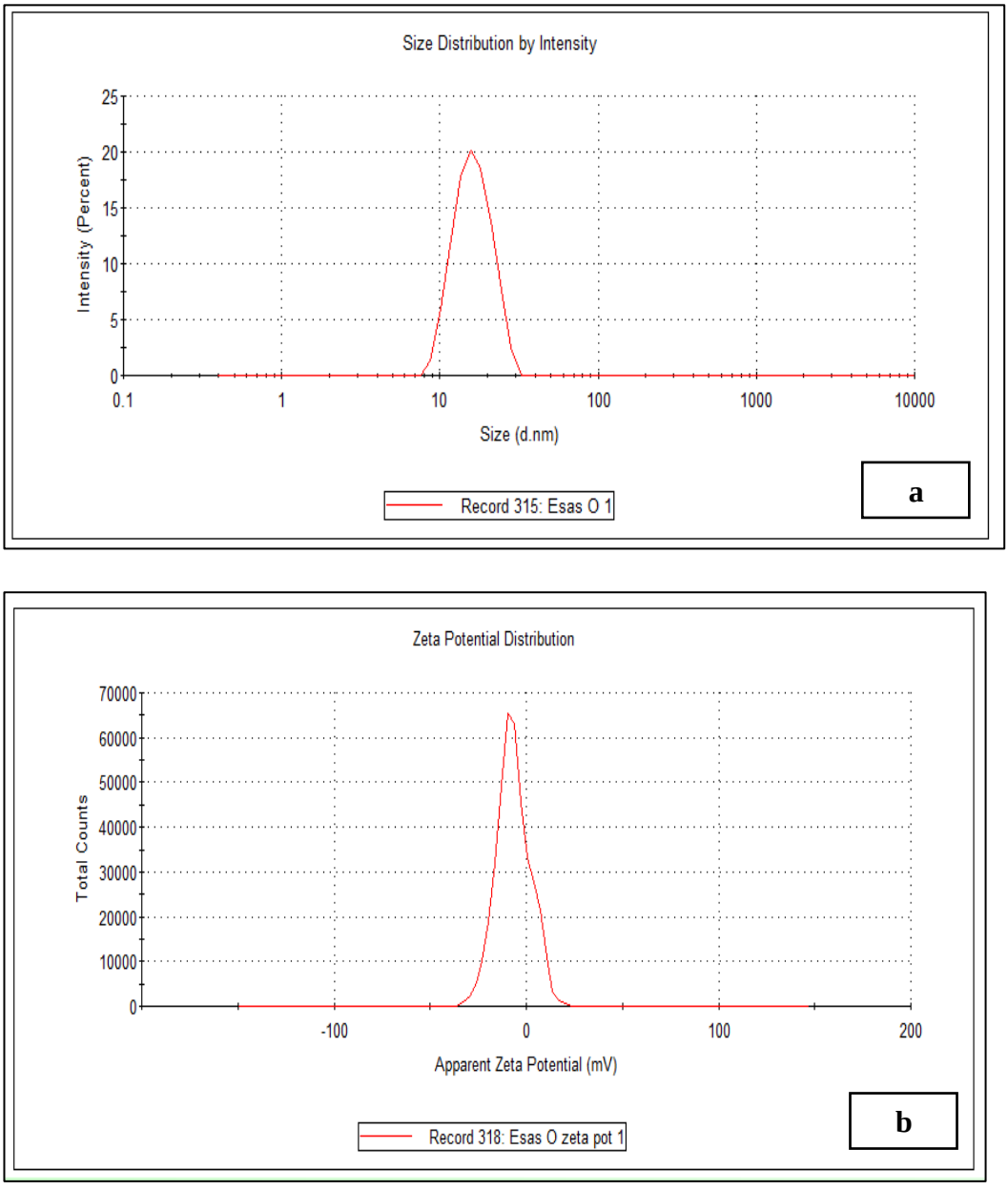


Figure 4.10:The droplet size (a) and ZP(b) values of O8A2T3 NE sample.

4.4.3 pH

The pH of **O8A2T3** NE sample was 5.76 which is suitable with skin's pH (4.5 - 6).

4.4.4 Viscosity

The measured viscosity of **O8A2T3** NE sample was 0.887 cP which refers to a great result obtained from this sample.

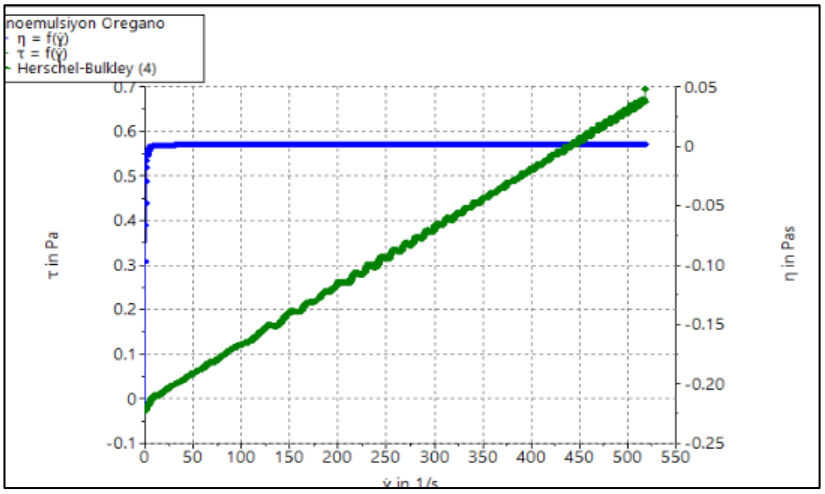


Figure 4.11: The measured viscosity of O8A2T3 NE sample.

CHAPTER FIVE

5. DISCUSSION AND RESULT

5.1 Antibacterial Activity of Oregano and Rosemary EOs

5.1.1 Bacterial inhibition zone

Regarding the bacterial inhibition zone, the test's results on *Staphylococcus aureus* bacterium showed that**A1** oil (Oregano) has an inhibition zone much wider than **A2** oil (Rosemary) which informs us that **A1** oil is more effective against the selected bacterium that associated with wounds than **A2** oil. The inhibition zone diameter in the disc diffusion was illustrated in Figure 4.3.

5.1.2 Bacterial minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) for a given antibacterial agent is the lowest concentration dose of this antibiotic that inhibits the growth of the bacterial strain at this dose. Therefore, from the results that given previously, for example; from this study, when we say that the MIC of **A1** and **A2** oils against *Staphylococcus aureus* bacterium is 0.024 μg/ml and 0.48 μg/ml, respectively, it means that the minimum concentration of the oil that will inhibit the growth of this bacterium is 0.024 μg/ml for **A1** and 0.48 μg/ml for **A2**, and so on for the other bacterial strains. **A2** oil was found to be more effective on Gram positive bacteria than Gram negative ones.

As a conclusion, from the previously given results, we can observe that **A1** oil is more effective than **A2** oil, in which a very small dose concentration of **A1** oil is needed to inhibit the growth of the bacterial strains in comparison to the **A2** oil and the standard.

5.2 The Preparation of Nanoemulsion with Different Surfactant : Oil Ratio at Different Time of Sonication and Sonication Amplitude Percentage

As understood from Figure 4.4 and Figure 4.5, we can conclude that by increasing the surfactant concentration; the size of the droplets is decreased. The reason behind

the necessity of increasing the surfactant concentration is that in the beginning when the selected surfactant is added, the droplet size is decreased, therefore, smaller droplets are going to have a larger surface area and thus, the addition of more surfactants will be required in order to stabilize the system. So, in general, increasing the concentration of the surfactants will lead the interfacial area to be increased, hence the interfacial tension will be decreased providing more stable NE formulation (Komaiko & Mcclements, 2016). Attention should be paid not to add the surfactants in excess amounts, in which these excess amounts more than the required will result in the formation of large clumps that are difficult to dissolve, besides, it was observed that extra amounts of surfactants more than needed may also increase the size of the droplets existing in the system due to the formation of highly viscous liquid crystalline phase. All these issues will lead to alteration of the production of an ideal NE formulation or even the stability of the system might be disrupted which are undesired results (Komaiko & Mcclements, 2016).

In conclusion, according to the overall results that are given previously, NE formulations that were prepared with Kolliphor RH® 40 (**O8**) had better and acceptable results of visual appearance, droplets size, and PDI values in any time of sonication and sonication amplitude over the NE formulations that were prepared with Kolliphor PS® 80 (**O4**).

At the end, we can extract that **O8** NE formulations are more suitable to be taken over **O4** formulations due to the results obtained from the whole previously performed studies. **O8 (**55 A2**) (**7 T3**)** samples were chosen to be the optimum NE formulations for our study among all tested samples due to the acceptable results provided by these two samples.

The reasons behind these fluctuations in the prepared NE formulations are related to many factors, these factors could be related to the type of surfactants, the sonication time used to prepare the samples, or the amplitude percentage applied on the samples. So, by looking generally on the results in Figure 4.4 and Figure 4.5, we will find that the type of surfactants used along with the concentration added have noticeable effects on the prepared samples. Therefore, when the ultrasonic cavitation is applied on the prepared coarse emulsion, the droplets will start to collapse forming

smaller droplets. The formation of smaller droplets will lead to the creation of a fresh interface. This newly formed interface will disrupt the stability of the system and the surface tension will increase, where the function of the surfactants starts in this point which they will adsorb onto the freshly created interface and cover the newly formed small droplets leading to the decrease in surface tension with increasing in surface area. Therefore, the capability of the surfactants to cover the newly formed droplets is critical to maintain the stability and surface tension of the system, in which if the newly formed droplets are not covered totally with the surfactant; the risk of coalescence formation will be high, which then will affect the PDI, and as a consequence, the whole system will be affected (Mahdi Jafari et al., 2006).Among the prepared eight NE formulations, we noticed that the smallest droplets were formed in the samples when the concentration of the surfactants (Kolliphor PS® 80 (**O4**) and Kolliphor RH® 40 (**O8**)) was at their maximum amounts, this is an evidence for the previously discussed paragraph.

Talking about sonication time and amplitude percentage; sonication time was found to have the largest effects on droplets size, while the sonication amplitude had its effects on the viscosity of the prepared sample, but at specific sonication time and amplitude, an optimum result will be carried out and increasing the parameters may have adverse effects on the system's components (Ngan et al., 2019). Extended sonication time may also have positive effects on the viscosity due to the long exposure to cavitation. Generally, the sonication time alone or the sonication amplitude alone will be insufficient to produce optimum NE results, in which these two parameters should work together to exert synergistic effects on the prepared NE formulation (Ngan et al., 2019). So in general, when we compare the droplet size of the prepared NE samples before and after applying the time of sonication and the sonication amplitude parameters, we will notice that there are extreme differences in the droplet size of those prepared NE formulations before and after applying the sonication parameters. Another important reason behind the differences that found in the prepared samples is the compatibility between the surfactants and the other components in the NE system, therefore, we can notice that (Kolliphor RH® 40 (**O8**)) was more compatible with the NE components than the other used surfactant.

5.3 Characterization of The Prepared NEs

The size of the particles in the NE systems has a crucial role in the production of optimum results that correspond to the criteria of an ideal NE formulation. The stability of the NE system is maintained mainly by the nano-sized droplets of the formed emulsion, therefore, nanoemulsions have the superiorities over the other systems by their long-term stability. This good stability is due to the small size of the droplets which prevent the formation of physical instabilities in the NE system.

The nano-sized of the droplets in the NE system also enables a great penetration of the applied formulation onto the skin. The stratum corneum which is the outermost layer of the skin and the most predominant barrier across any applied formulation, and the intercellular spaces are considered to have dimensions between 50-100 nm (Yokota & Kyotani, 2018). Therefore, since the droplet size result of our final optimum NE formulation (**O8A2T3**) was very low; the penetration of the prepared NE formulation into the skin will be ensured without any challenges. The ZP value for a given NE sample is critical in the preparation of an optimum NE formulation, in which this value indicates the repulsion between particles that exist in the system. Since each particle has a surface charge and they are subjected to the Brownian motion in the NE system, therefore, as the repulsion between these particles increases, the better ZP values will be recorded, and as a consequence, the better NE formulation will be produced (Abolmaali et al., 2011). The ZP value of our prepared optimum NE formulation (**O8A2T3)** was relatively high, this value indicates a good repulsion between the NE particles which refers to the successful production of an ideal NE formulation. The value of the PDI in the prepared NE samples is absolutely critical in achieving an ideal NE formulation. PDI is the measurement of the homogeneity uniformity of distribution of the droplets throughout the system, in which the lower the PDI value (closer to zero), the higher uniformity of the particles and the narrower size of distribution of those particles, resulting in an optimum NE formulation. The high value of PDI was related to the coalescence of some particles that didn't cover fully with a surfactant used in the preparation as mentioned earlier (Kotta et al., 2015). Therefore, since the value of our final optimum NE formulation

(**O8A2T3**) was minimized, that means the particles in the system are more homogeneous and uniform in distribution.

CHAPTER SIX

6. CONCLUSION

At the end of our study, when the antimicrobial activity of Oregano and Rosemary essential oils was compared; Oregano oil showed very effective antibacterial activity over Rosemary oil and therefore, it was chosen to be the model oil of the study over Rosemary oil due to this superiority of antibacterial activity.

Oregano EO was then incorporated in the NE system as an Oregano EO-based nanoemulsion formulation. Oregano oil was the oil phase of the system and the surfactant used was Kolliphor RH® 40 with a specific surfactant : oil ratio and the purified water was the aqueous phase of the system.

The whole components of the NE system were compatible to each other and the results of droplet size, zeta potential, PDI, visual appearance, viscosity, and pH proved this compatibility.

Therefore, as a conclusion, the aim of this study was to form an EO-based nanoemulsion formulation for the acceleration of wound healing process. We were not able to evaluate the activity of the final formulation as Oregano EO- based NE formulation on the acceleration of the wound healing processes due to COVID-19 issues, but according to the results provided by this study starting by the great antibacterial activity of Oregano oil and ending by the successful production of an optimum NE formulation; the synergistic activity between these factors will offer us outstanding results in the inhibition of any microbial growth, and by this activity; the whole process of the wound healing will be accelerated leading to the achievement of our study's goal.

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8. CURRICULUM VITAE

Name	Abdulkader	**Surname**	Rawas
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Nationality	Syrian	**Tel**	+905338642390
E-mail	rawas94sy@gmail.com		

	Name of the Institution where he/she was graduated	**Graduation year**
Postgraduate/Specialization	-	-
Masters	Pharmaceutical Technology/ Near East University	2020
Undergraduate	Pharmacy/ Al-Ahliyyah Amman University	2017
High school	Al-Rabea High School	2012

Educational Level

	Duty	**Institution**	**Duration (Year - Year)**
Lab Assistant		Near East University	2019
Pharmacy Training		Al-Hayah Pharmacy	2018
-		-	-

Job Experience

Foreign Languages	**Reading comprehension**	**Speaking***	**Writing***
English	Very good	Very good	Very good
Turkish	Moderate	Moderate	Moderate

Foreign Language Examination Grade*								
YDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
-	-	-	-	-	-	-	-	-

	Math	**Equally weighted**	**Non-math**
ALES Grade	-	-	-
(Other) Grade	-	-	-

Program	**Use proficiency**
SPSS	Good

Computer Knowledge

***Evaluate as very good, good, moderate, poor.