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DEVELOPMENT AND OPTIMIZATION OF NANOEMULSION FORMULATION FOR TOPICAL TREATMENT OF CANDIDIASIS

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DEVELOPMENT AND OPTIMIZATION OF NANOEMULSION FORMULATION FOR TOPICAL TREATMENT OF CANDIDIASIS

Master Thesis

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DECLARATION

I hereby declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of thesis until 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 which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patent rights and copyright infringements during the study and writing of this thesis.

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If there is no struggle, there is no progress. Struggling my way from theory to practical work and encompassing all the obstacles has been a long, tiring journey that brings about the sweet joy of achievement. For being able to cross the bumpy road firstly, All praises and thanks to Almighty Allah for granting me the strength and patience to endure and stay determined through all difficulties.

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LIST OF ABBREVIATION

°C	Degree celcius
µg	Microgram
µl	micro litre
µm	Micrometer
API	Active Pharmaceutical ingredient
CEO	Cinnamon essential oil
CLP	Cecal ligation and puncture
CLSI	Clinical and laboratory standards institute
CXB	Celecoxib
DLS	Dynamic light scattering
E.O	Essential oil
FDA	Food and Drug Authority
FTIR	Fourier transform infrared Spectroscopy
GC-MS	Gas chromatography- Mass Spectrometry
GIT	Gastro intestinal tract
GRAS	Generally recognized as safe
HLB	Hydrophile-lipophile balance
Hr	Hour
ISO	International Organization for Standardization
KHz	kilo Hertz
KV	kilo Volts
MIC	Minimum inhibitory concentration
Min	Minutes
Ml	milli litre
MLX	Meloxicam
Mm	Millimeter
MZ	Miconazole
NE	Nanoemulsion

Nm	Nanometer
NSAIDS	Non-Steroidal anti inflammatory drugs
O/W	Oil in water
OA	Oleic acid
PCS	Photon correlation spectroscopy
PDI	poly dispersity index
PIE	Phase inversion emulsion
PIT	Phase inversion temperature
PTA	Phosphotungstic acid
Rpm	rotation per minute
SC	Stratum corneum
SEM	Scanning electron microscopy
TDDS	Transdermal drug delivery system
TEM	Transmission electron microscopy
W/O	Water in oil
ZP	Zeta Potential

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Anabilim Dalı: Farmasötik Teknoloji

ÖZET

Amaç: Candidiasis tedavisi için aktif madde olarakantifungalözellikli uçucu yağ kullanılarak stabil bir nanoemülsiyonformülasyonuoluşturmak ve optimize etmek

MateryalveMetot:Uçucuyağolarakkaranfilyağıvetarçınyağıseçilmiştir. Miktar tayini testi GC-MS kullanılarak yapılmış,Candida türüne karşıantifungal aktiviteyi karşılaştırmak için de mikrobiyolojik çalışma tamamlanmıştır.Formülasyoniçin; noniyonikyüzeyaktifmaddeler (Kolliphor PS® 80, Kolliphor PS® 20 veKolliphor RH® 40) HLB değerlerinegöreseçildiSu fazı olarak saf su kullanılmıştır.Ultrasonikasyonişleminde yüksek enerjili yöntemçubuksonikatöruygulanmıştır.

BulgularveTartışma:

Tarçın yağı, karanfil yağından daha geniş inhibisyon bölgesi vecandidaalbicans'a karşı düşük MIC değeri göstermiştir. Formülasyonumuz;yüzey aktif madde olarak Kolliphor RH 40, yağ fazı olarak tarçın yağı ve sulu faz olarak saf su kullanılarak hazırlanmıştır.Optimum nanoemülsiyonformülasyonu yüksek enerjili ultrasonikasyon tekniği ve çubuksonikatör kullanılarak hazırlanmıştır. Ayrıca formülasyon uygun sonuçlar verendamlacık boyutu, PDI, zeta potansiyeli, viskozite ve pH için karakterize edilmiştir.

AnahtarKelimeler: Topikalformülasyon, Candidiasis, Nanoemülsiyon, Ultrasonikasyon, Tarçınyağı

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SUMMARY

Aim: To formulate and optimize a stable nanoemulsion formulation based on essential oil as an active ingredient with antifungal activity for the treatment of candidiasis

Material and Method: Essential oils selected were clove oil and cinnamon oil. Assay was done by using GC-MS and microbiological study were performed to compare the antifungal activity against *Candida* specie. Non-ionic surfactants were selected for formulation on the basis of their HLB value (Kolliphor PS® 80, Kolliphor PS® 20 and Kolliphor RH® 40) and use of purified water as an aqueous phase. High-energy method for ultrasonication was used, Probe sonicator was used in our study.

Findings and Result: Cinnamon essential oil shows better inhibition zone and low MIC value against candida albicans than clove oil. Our formulation was prepared by using Kolliphor RH 40 as surfactant, cinnamon essential oil as oil phase and purified water as an aqueous phase. Optimum nanoemulsion formulation was prepared using high energy ultrasonication technique, probe sonicator. It was further characterized for droplet size , PDI, zeta potential, viscosity and pH which yield good results.

Keywords: Topical formulation, Candidiasis, Nanoemulsion, Ultrasonication, Cinnamon oil

CHAPTER 1
INTRODUCTION

1.1. Nanoemulsion

Nanoemulsions are defined as two immiscible liquids one of which is dispersed in the second but continuous phase. Nanoemulsions can made into oil in water (O/W) or water in oil (W/O) (Pongsumpun, Iwamoto, & Siripatrawan, 2020). Nano-emulsions are solid in nature, sphere in shape with amorphous surface and lipophilic in nature, having charge (Jaiswal & Dudhe, 2015). Nanoemulsions being submicron in size are of great interest for use as a drug carrier and improving therapeutic efficacy of drugs. They are advanced nano-droplet system for systemic, controlled & target drug delivery systems. (Shaker, Ishak, Ghoneim, & Elhuoni, 2019)

Depending on the method of preparation, difference in droplet size distribution can be achieved, explaining, how preparation techniques can affect the stability of emulsions. Droplet size between conventional emulsions and micro-emulsions with size range of 20-500nm are called as mini-emulsions, ultrafine emulsions, translucent emulsions, nano-emulsions and sub-micron emulsions. Because of the small droplet size nano-emulsions appears continuous and transparent. Its continuous Brownian movement avoids sedimentation & creaming, hence offered high stability. (Fernandez, Rieger, & Angelika, 2004)

Nanoemulsion can be of two types oil-in-water or water-in-oil. As well as double emulsions o/w/o or w/o/w in which dispersed liquid is further dispersed in another liquid.

1.2. Essential Oil

The term "essential oil" was first used by a Swiss reformer named Paracelsus von Hohenheim of medicine in 16th century. He named the effective component as Quinta essential (Macwan, Dabhi, Aparnathi, & Prajapati, 2016). Essential oils are natural compounds obtained from aromatic plants consisting a complex mixture of terpenoids with volatile and non-volatile nature as metabolites(Artiga-Artigas, Guerra-Rosas, Morales-Castro, Salvia-Trujillo, & Martín-Belloso, 2018). E.O are volatile, liquid, colored, limpid and are soluble in organic solvents. The source of E.O's is plants, it can be found in any part of plant like bud, flower, seeds, leaves,

twigs, fruits, root, wood, bark or the stem (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). Generally, the aromatic plants are found in countries with moderate or warm weather's therefore, consist an important part on traditional pharmacopoeia (Nazzaro et al., 2013).

The proved testing of E.O's shows a chance to formulate those essential oils into a formulation because essential oils produce therapeutic effect when used in high concentration, while E.O are known to be skin irritant. In order to reduce the side effects and get better results they have to be formulated in dosage form with supporting fillers to improve stability and reduce side effect (Nazzaro, Fratianni, Coppola, & De Feo, 2017).

The main components of essential oils is terpenes and terpenoids (Hyldgaard, Mygind, & Meyer, 2012). Terpenes are large class of naturally occurring hydrocarbons, with various chemical features and biological properties. They are synthesized in cytoplasm of plant cells through the pathway of mevalonic acid starting from acetyl CoA. Terpenoids are related to terpenes, with some rearrangement or oxygen functionality. Terpenoids can be acidic, alcoholic, aldehydes, ketones or esters depending on their functional groups. Chemical composition of plant essential oils can differ from specie to specie depending on geographical location, environment, the maturity stage and extraction technique (De Martino, De Feo, & Nazzaro, 2009).

1.3. Candidiasis

Candidiasis is defined as overgrowth of candida at certain magnitude to cause inflammation, action or disease. *Candida albicans* is most likely to be found on skin (i.e. 66%) , second most common specie (20%) *Candida tropicalis*, rest of candida species like *Candida parapsilosis, Candida pulucherima,* least common, can also be overgrown leading to candidiasis in high risk patients (Evans & Gray, 2003).

Candida albicans is normally found in GI-tract, oral and vaginal membranes and is also mainly responsible fungal pathogen for a wide range of systemic and mucosal infections. Around (70%) of women around the globe gets vaginal infection caused by *Candida* in lifetime. However, mortality rate has approached 35-60%, and

Candidiasis has proved to be the fourth major cause of hospital acquired blood stream infection in U.S. (Bartlett, 2004; Edmond et al., 1999; Evans & Gray, 2003).

Candida albicans possess some virulence properties that plays role in pathogenicity like, enables it to tightly hold the host cells, secretion of degradative enzymes (e.g phospholipase, aspartyl protease), evade immune system, biofilm formation and switching phenotypes. *Candida albicans* yeast filament transition has been thought of as reason for virulence activity (Kadosh, 2016).

1.4. Aims and Scope

The aim of our study is to prepare nanoemulsion with activity against *Candida albicans* using essential oil. For this study two essential oils Cinnamon essential oil and clove oil were chosed. Both oils were tested for their activity against fungal strains. Depending on the result of antimicrobial assay an essential oil will be use to prepare nanoemulsion along with suitable surfactant at specific concentration and purified water as required. After preparation of optimum formulation, characterization test such as droplet size, PDI, zeta potential, pH and viscosity is done.

CHAPTER 2
GENERAL INFORMATION

2.1. Skin Physiology

Skin is the widest organ of the body consisting 17% of the body weight. Skin provides protection against the environmental factors from penetrating into skin and maintain homeostasis i.e. loss of water. Skin consists of three layers: Epidermis, dermis and cutaneous. (Nastiti et al., 2017)

2.1.1. Epidermis

Epidermis has four distinct layers: stratum corneum, stratum lucidum, stratum spinosum and stratum geminativum. Epidermis mainly functions as a barrier against environmental stress, by preventing the chemicals and other substances from penetrating into body and also avoid the loss of water from skin underlying tissues. Outer most layer of epidermis, stratum corneum consists of dead skin layer made up of keratin, a hard fibrous proteins. This stratum corneum provides huge resistance to percutaneous absorption of chemicals and drugs. Factors affecting drug absorption are hydration of skin and damage to stratum corneum. (Kamath, 1990)

2.1.1.2. Stratum corneum

Stratum corneum is the thicker and outer most layer of skin that is made up of dead component called keratin, a hard fibrous protein. It provides greatest resistance to percutaneous absorption of chemicals and drugs. Factors that can affect drug absorption are hydration of skin and damage to stratum corneum.(Kamath, 1990) Stratum corneum range in size from sub-nano-meters (lipid bilayers) to several tens of microns (glyph lines). Small molecules (<500 Da) can readily penetrate the stratum corneum, but the delivery of larger molecules is challenging but different passive and active approaches can enhance the delivery of large molecules to targeted sites. (Kamath, 1990)

2.1.2. Dermis

Dermis is the second layer after epidermis, it is 1 - 4mm thick and is composed of elastic fibers, collagen fibers and extrafibrillar gel of mucopolysacchirides called glycosaminoglycans. It protects skin from mechanical injuries and support dermal appendages i.e. apocrine, eccrine glands, sebaceous glands and hair follicles as well

as epidermis. Dermis is major mediator between epidermis and subcutaneous layer, it is enriched of nerve supplies, blood capillaries and lymphatic drainage to skin and its appendages. Dermis is responsible for providing nutrition to epidermis and also transmits nerve signals for pain and sensations. Dermis stores water in large amount hence serves as water storage organ. Drugs passing through epidermis via sweat glands and pilosebaceous units directly enters dermis layer and get absorbed via capillaries. (Kamath, 1990)

2.1.3. Subcutaneous layer

It supports both epidermis, dermis and acts as fat storage. It regulates body temperature, provides nutrition and cushion the outer skin layers(Kamath, 1990).

2.2. Skin penetration

The permeation pathway of skin passage is through stratum corneum since it is the first contact of skin to externally applied molecules. Stratum corneum has hydrophobic hindrance against the transport of exogenous chemicals including drugs (Shaker et al., 2019). Flattered corneocytes surrounded by lipid bilayer consisting of ceramides makes stratum corneum a hard layer (Nastiti et al., 2017). Its spread in 10-15 rows i.e. 10µm in thickness, made with keratinized cells known as corneocytes. These corneocytes are enriched of lipid phase, known as intercellular lipid lamellae, and resemble a "brick and mortar" model (Singh & Morris, 2011). Final diffusion through Stratum corneum is a total of lateral diffusion and inter-membrane trans bilayer transport (Shaker et al., 2019)

A penetrating drug applied to skin follows three possible routes across epidermis as shown: **A:** Transcellular route, a lipid domain associated to proteins within corneocytes, **B:** Intercellular route **C:** The appendageal route from hair follicles via associated **D:** sebaceous glands and sweat ducts (Barry, 2001).

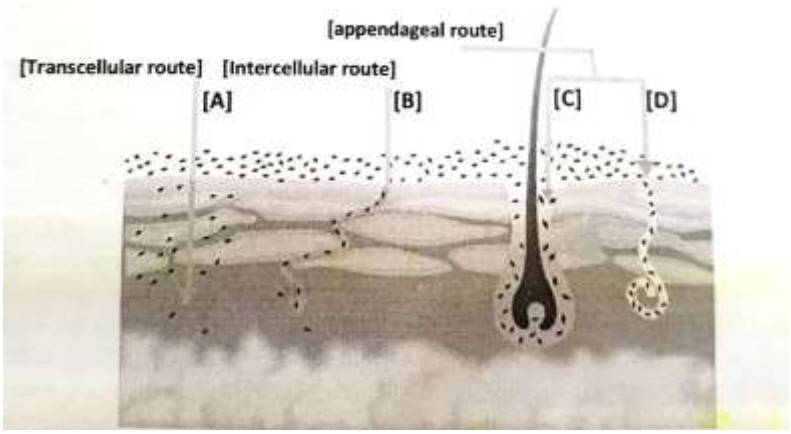


Figure 2. 1 Penetration pathways of first skin layer (Stratum corneum). (A) The transcellular route associated to proteins (B)Intercellular route and appendageal route (C) Appendageal route, hair follicle route (D) via sweat glands (Shaker et al,2019)

The pore pathway is most likely used for the large molecules and ions that are hard to cross an intact structure of stratum corneum (Barry, 2001). Transcellular route is series of partitioning into hydrophilic and lipophilic domains of cells and then diffuse into cells. (Williams-Barry1991_Article_TerpenesAndTheLipidProteinPart.pdf, n.d.). The intercellular route, permeation will occur across the hard path of extracellular matrix without traversing cells. Smaller hydrophilic molecules can easily cross transcellular route than from intercellular route while lipophilic molecules can easily cross the intercellular route than transcellular path. Both, the transcellular and intercellular routes constitute the trans-epidermal gateway. The fused flux of two pathways determines the whole flux across the skin. It is generally acknowledged that trans-epidermal pathway is dominant route for skin permeation and that under skin conditions, diffusion through stratum corneum constitute as rate-limiting step which determines the overall flux of permeant (Shaker et al., 2019).

2.3. Topical drug delivery system

Topical drug agents are aimed to deliver the API active pharmaceutical ingredient on or beyond mucous membranes and skin to get the targeted pharmacological effect. There are various advantages of using topical drug products including target to the localized infectious area, reduced risk of side-effects, increase drug effectiveness and patient compliance(Maha & Masfria, 2020). Topical products are used for local/regional or systemic effects. Products with local effects are applied directly to site of action most likely skin or nails. On the other hand topical products for

regional or systemic effect are meant to penetrate deeper into skin in order to enter blood stream to produce it's therapeutic effect. Most topical drugs are formulated as to provide local effect rather than for absorption into blood stream.(Macwan et al., 2016)

Topical products can be made in variety of dosage forms like creams, lotions and ointments. Lotions and cream are emulsions of o/w or w/o containing an active pharmaceutical ingredient (API) dissolved in either, a water phase or an oily phase, or dispersed as a suspension in the emulsions. (Macwan et al., 2016)

2.3.1. Permeation pathway for NE

Transdermal route is traditional route of drug administration known to enhance the efficacy of therapeutic agents, easy to apply and can stop application, if necessary (Mostafa et al., 2015). This route of administration will bypass hepatic metabolism and reach blood circulation ,(Khopade, Nandakumar, & Jain, 1998) leading to an enhanced bioavailability thus reducing risk of drug related adverse effects. An important mission for transdermal delivery is to deliver sufficient amount of drug at specific rate to reach skin surface (Peira, Scolari, & Gasco, 2001). Drug delivery via skin is appropriate for certain clinical conditions like: Skin infections caused by fungi or skin wounds followed by infection.

2.4. Essential Oils

The term "essential oil" was first used by a Swiss reformer named Paracelsus von Hohenheim of medicine in 16th century. He named the effective component as Quinta essential (Macwan et al., 2016). European pharmacopoeia defined E.O's as " Odorant products with complex composition, obtained from raw extracts of plants, extracted by steam, dry distillation, or an appropriate mechanical method without heat. Generally, physical method is followed for the separation of oil from the water phase which has no specific change in its chemical composition."(Asyikin et al., 2018) Essential oils are natural compounds obtained from aromatic plants consisting a complex mixture of terpenoids with volatile and non-volatile nature as metabolites(Artiga-Artigas et al., 2018). E.O are volatile, liquid, colored, limpid and are soluble in organic solvents. The source of E.O's is plants, it can be found in any part of plant like bud, flower, seeds, leaves, twigs, fruits, root, wood, bark or the stem

(Nazzaro et al., 2013). Generally, the aromatic plants are found in countries with moderate or warm weather's therefore, consist an important part on traditional pharmacopoeia (Nazzaro et al., 2013). E.O's contains various metabolites that are capable of inhibiting the growth of bacteria, moulds and yeasts (Dávila-Rodríguez, López-Malo, Palou, Ramírez-Corona, & Jiménez-Munguía, 2019).

The use of E.O was common in early civilizations, first in Eastern and Middle Eastern then in North Africa and Europe. The International Organization for Standardization (ISO) (ISO/D1S9235.2) define E.Os as "A product made with distillation using water or steam or by processing mechanically or with dry distillation of natural material." (Nazzaro et al., 2017). Hydrosols (aromatics) were used in India for 7000 years. Egyptians increased the use E.O's for treatment of diseases between 2000 and 3000 B.C. While in 1000 B.C, Persians were first ones to use technique of hydro distillation for extracting E.O (Macwan et al., 2016).

E.O are mixtures of complex natural compounds, polar and non-polar. Several studies have been done on antimicrobial, anti-oxidant, anti-viral and anti-fungal activities of E.O's (Dadkhah et al., 2019; Macwan et al., 2016; Nazzaro et al., 2017). However, direct application of E.O's is limited because of low water soluble compounds and also because of excess amount of oil has to be applied for producing effect against bacteria and pathogens (Dávila-Rodríguez et al., 2019).

2.4.1. Chemical composition of EOs

Pure EO contains more than 200 components inside which includes mostly terpenes and phenyl-propanoid derivatives, that are almost similar structurally and chemically. Components of E.O are broadly classified as volatile and non-volatile fractions. Volatile component consists of mono and sesquiterpene components, and several oxygenated derivatives along with alcohols, aliphatic aldehydes and esters. While almost 10% of the isolated E.O comprises of carotenoids, fatty acids, flavonoid and waxes which falls under non-volatile residue . (Asyikin et al., 2018)

Analytical technique used for identifying components of E.O's is (GC-MS) Gas Chromatography - Mass Spectrometry. It is an efficient method for determination of essential oil components. A GC-MS report is considered as fingerprint of any particular E.O and help indicating the purity of E.O. This determination of

components of E.O helps us in understanding the unique properties of oils.(Asyikin et al., 2018) Following could be the components of E.O : (Asyikin et al., 2018)

2.4.1.1. Hydrocarbons

Hydrocarbons are the building blocks of hydrogen and carbon atoms found in essential oils. An example of basic hydrocarbon found in E.O is isoprene as shown:

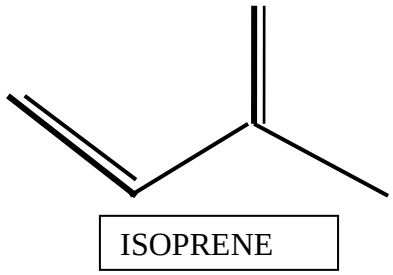


Figure 2. 2. Chemical structure of isoprene

2.4.1.2. Terpenes

Terpenes are mixture of mono, sesqui and diterpenes. Combination of two isoprene units makes up monoterpene, whereas sesquiterpenes are combination of three isoprene units while diterpenes are formed by four isoprene units. (Asyikin et al., 2018)

2.4.1.2.1. Monoterpenes

Monoterpenes are naturally occuring constituent in essential oil plants that contains mostly unsaturated hydrocarbons (C_{10}). Substituents of oxygenated derivatives of monoterpene are alcohol, ketones and carboxylic acids, that collectively makes up a monoterpene. (Asyikin et al., 2018)

2.4.1.2.2. Sesquiterpenes

Sesquiterpenes are combination of three isoprene units. The structure of isoprene can be linear, mono-cyclic or bi and tri-cyclic. Linear structures of sesquiterpenes are branched hydrocarbons with four double bonds. Mono-cyclic structure consists of six carbon ring (C_6). The bi-cyclic represents a pine like structure i.e. cyclobutane ring.(Asyikin et al., 2018)

2.4.1.2.3. Diterpenes

Diterpenes are found in all plant families with C_{20} chemical structure. Diterpenes are combination of four isoprene units. They are too heavy components that cause difficulty in evaporation during extraction process of steam distillation. Therefore, hard to find in isolated aromatic oils. Diterpene derivatives can be found in plant hormones and phytol, where they occur as side chain on chlorophyll. (Asyikin et al., 2018)

2.4.1.3. Alcohol

Alcohol is one of the important component of E.O's. It provides best properties like antiseptic, anti-viral, anti-bacterial and germicidal. Alcohol occurs naturally as a single component or in combination with ester or terpenes. Terpenes attached to oxygen or hydrogen makes up alcohol. A monoterpene containing hydroxyl group inside its hydrocarbon structure is called monoterpenol. Alcohols are considered as safe since its present in low amount and don't have toxic reactions to the skin (Asyikin et al., 2018).

2.4.1.4. Esters

Interaction between the alcohol and acids results in an ester formation. Presence of alcoholic group in ester E.O provides anti-inflammatory effect. Esters are considered to have antifungal and sedative properties with balancing action on the nervous system. (Asyikin et al., 2018)

2.4.1.5. Ketones

Essential oils with ketone group are effective in treating wound healing and improve scar tissues. Ketones possess anti-catarrhal, cell proliferant, expectorent and vulnerary properties and are often found in plants. Thujone is one of the very toxic ketone found in E.O found in sage, mugwort, thuja and tansy. Pulegone is also toxic ketone that is found in pinocamphone and pennroyal oils. However, jasmone, fenchone, carvone and menthone are the non-toxic ketone components found in fennel oil, jasmine oil, spearmint, dill and peppermint oil (Asyikin et al., 2018).

2.5. Role of Essential Oils in Pharmaceuticals

Essential oils has been used for centuries by Middle East society, Persians, Egyptian civilization as well as Indians and African communities. E.O's being used as a treatment for sickness in past history. Therefore, various tests has been performed to understand the effect of essential oils in several diseases. Research has been published regarding several essential oils and their effects against various bacteria, fungi and viruses.

Ancient Egyptians has been using E.O's against bacterial infections. Various studies with strong in-vitro evidence against bacterial activity confirmed it's therapeutic effect against pathogens like *Listeria monocytogenes, Listeria innocua, Salmonella typhimurium, E. coli, Shigella dysentria, Bacillus cereus, Staphylococcus aureus* (Jang, Piao, Kim, Kwon, & Park, 2008). Essential oil consists of various components that have antimicrobial activity depending on the amount component to produce effect on microbes for e.g high concentration of cinnamic aldehyde, euogenol or citral conferring the antimicrobial effect. Tests have shown it's activity against Gr +ve bacteria more, than the Gr -ve bacteria because of difference in their cell wall structure. Monoterpenes and phenols found in sage, rosemary and thyme E.O are found active against viruses, fungi and bacteria(Nazzaro et al., 2013).

Studies performed on *Rosa damascene* E.O showed anti-oxidative and hepatoprotective activity against CLP-induced sepsis(Dadkhah et al., 2019). Several E.O's have been tested for their antifungal activities as well, some of them act as fungicidal some as fungistatic (Nazzaro et al., 2017).

The proved testing of E.O's shows a chance to formulate those essential oils into a formulation because essential oils produce therapeutic effect when used in high concentration, while E.O are known to be skin irritant. In order to reduce the side effects and get better results they have to be formulated in dosage form with supporting fillers to improve stability and reduce side effect (Nazzaro et al., 2017).

2.6. Fungal Infections

Infections have always been a part of human existence and has continued to be a significant problem ever since humans evolved. A significant rise of fungal infections have been witnessed in last three decades. There are number of factors that

implicates the occurrence of mycotic infections such as immuno-compromised diseases like HIV, Immuno suppressing drugs, excessive use of antibiotic or broad spectrum and invasive surgical interventions (Deorukhkar & Saini, 2015).

Fungi are ubiquitous in nature. Of the estimation around 1.5 million species of fungi, around 100 species causes human infections. Those infections include candidiasis, aspergillosis and cryptococcosis. Invasive fungal infections like this have been increased in recent decades, causing substantial morbidity and mortality (Santamaría et al., 2011).

2.6.1. Species of *Candida*

Candida species currently the most common causative agent for fungal infections worldwide. *Candida albicans* has been entitled as frequently causing fungal pathogen by multicenter candidemia (Nawrot et al., 2013; Pfaller et al., 2010). Factors that made *candida* as potentially most pathogenic are formation of invasins and adhesins that mediates invasion and adhesion to host, also secretion of hydrolytic enzymes, transition from yeast to hypha, thigmotropism and contact sensing, phenotypic switching, biofilm formation and metabolic adaptability (Mayer, Wilson, & Hube, 2013). Studies on candidemia shows a significant increase in mortality rate around 25 - 60% due to cross resistance against antibiotics of *Candida albicans*. Therefore, interest towards natural products for treatment has been increased, in order to avoid resistance (Das, Nightingale, Patel, & Jumaa, 2011).

2.6.2. General features and morphology of candida

Above all the pathogenic fungi *Candida albicans* is the only pathogen that cause wide range of clinical manifestation ranging from mucocutaneous overgrowth to disseminated infections. *Candida albicans* has considered as the most persuasive specie among all. In most of the cases the cause of infection has been reported as from *Candida* specie. Generally, member of genus *Candida* is found everywhere in nature in saprophytic and commensal state. *Candida spp.* are frequently isolated from skin and mucosal sites such as gastro intestinal tract and genito-urinary tract of human body (Deorukhkar & Saini, 2015).

Morphologically they are classified as yeast like fungi. (Deorukhkar & Saini, 2015; Jahagirdar, Davane, Aradhye, & Nagoba, 2018). *Candida* cell wall consists of polysacharides, mannan, glucan and chitin. Mannan is distributed throughout the cell wall while glucan and chitin are mainly found in inner cell wall. (Evans & Gray, 2003)

2.6.3. Epidemiology and pathogenicity of candida

Candidiasis is defined as overgrowth of candida at certain magnitude to cause inflammation, action or disease. *Candida albicans* is most likely to be found on skin (i.e. 66%) , second most common specie (20%) *Candida tropicalis*, rest of candida species like *Candida parapsilosis, Candida pulucherima,* least common, can also be overgrown leading to candidiasis in high risk patients (Evans & Gray, 2003).

Candida albicans is normally found in GI-tract, oral and vaginal membranes and is also mainly responsible fungal pathogen for a wide range of systemic and mucosal infections. Around (70%) of women around the globe gets vaginal infection caused by *Candida* in lifetime. However, mortality rate has approached 35-60%, and *Candidiasis* has proved to be the fourth major cause of hospital acquired blood stream infection in U.S. (Bartlett, 2004; Edmond et al., 1999; Evans & Gray, 2003).

Candida albicans possess some virulence properties that plays role in pathogenecity like, enables it to tightly hold the host cells, secretion of degradative enzymes (e.g phospholipase, aspartyl protease), evade immune system, biofilm formation and switching phenotypes. Candida's most important virulence trait is it morphological reversible transition from yeast, single oval budding cells, to hyphal and pseudohyphal filaments, this transition occurs due to several reasons that mimics the host cells to undergo changes like growth in serum, (37°C) body temperature, high CO_2 / low O_2 , normal pH at neutral, some carbon sources and amino acids (example proline). Extension of hyphal filaments of *Candida albicans* also promotes virulence activity such as breakdown of macrophages, biofilm formation, invasion of epithelial cell layers, breaching of endothelial cells and thigmotropism. *Candida albicans* yeast filament transition has been thought of as reason for virulence activity (Kadosh, 2016).

2.6.4. Candidiasis risk factors

Widespread use of immunosuppressive drugs and broad spectrum antibiotics has increased the risk of opportunistic infections, individuals such as recipients of organ transplant, cancer patients on chemotherapy, HIV patients and neonates or critically ill patients. Therefore, Candidiasis is rightly said to be as "disease of diseased".

Chronic atrophic stomatitis, thrush, chronic mucocutaneous candidiasis is extremely common & most likely to occur in healthy individuals (Deorukhkar & Saini, 2015).

2.7. Resistance of Antifungal Drugs Increasing the need of Natural Products

Number of available antifungal drugs is limited compared to antimicrobial agents. Antifungal agents are classifies as azole, polyenes and pyrimidine analogue and echinocandins. Drugs from each class differs in its pharmacokinetic and pharmacodynamic activity as well as route of administration. Nature of these antifungal drugs is either fungistatic or fungicidal and route of administration includes oral, IV and topical. Mode of actions of each drug of class is different such as cell wall inhibitors, cell membrane inhibitors or fungal cell enzyme inhibitors. (Deorukhkar & Saini, 2015) Such limited available pharmacological drugs such as azoles and high rate of candida infections due to immunosuppressive diseases has lead to drug resistance. Empirical use of azole group has increased the incidence of infection due to unknown, unusual and treatment resistance *Candida spp.* (Deorukhkar & Saini, 2015)

Classification of antifungal resistance:

- Microbiological resistance
- Clinical resistance
- Combined resistance

2.7.1. Microbiological resistance

Microbiological or microbial resistance is referred to condition in which minimum inhibitory concentration (MIC) of an antifungal drug exceeds the susceptibility breakpoint for that fungus. It is further divided into two:

I. Primary or intrinsic resistance
II. Secondary or acquired resistance

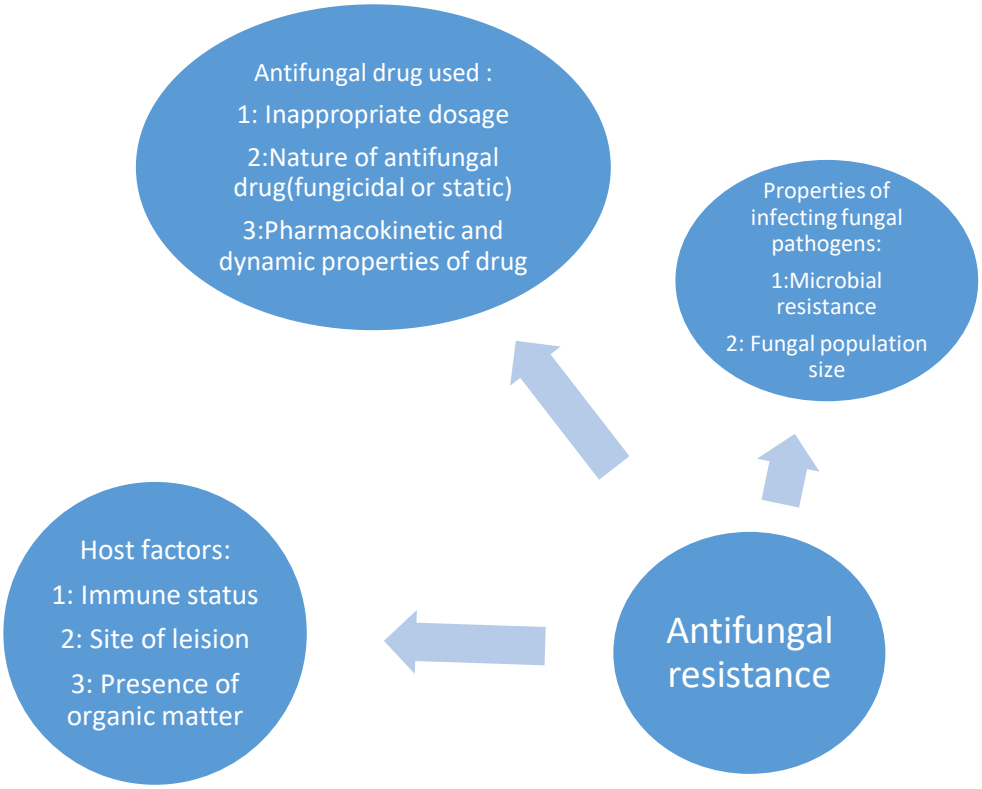
Primary or intrinsic resistance is innate or natural in fungal cell prior exposure to the antifungal drug. Secondary resistance is acquired by the previously susceptible strain of fungal cell that had exposure to antifungal drug, it depends on gene alteration within the fungal cell. (Deorukhkar & Saini, 2015)

2.7.2. Clinical resistance

Clinical resistance is caused by repeated use of antifungal drugs because of repeated prescriptions by clinicians or an inappropriate prescribed dose. Because of repeated exposure cell undergoes genetic adaptations and cause resistance against the drug and it's components (Deorukhkar & Saini, 2015).

2.7.3. Factors causing antifungal resistance

Development of fungal resistance depends on multiple factors like properties of infection causing fungal pathogen, pharmacokinetic and pharmacodynamic properties of antifungals, and host predisposing factors. (Deorukhkar & Saini, 2015)



2.8. Interpretation

Candida albicans is a yeast like fungus commonly found in mucous membranes as normal flora of a healthy person. It's over growth can lead to infections from superfacial mucosal lesions to septicemia. There are various factors that cause the excessive growth of *Candida albicans* that participate in and influence the infection process by adhesion, invasion and destruction of host immune cells. Candidiasis can cause both morbidity and mortality. Reason for this is, limited number of antifungal drugs and their excessive use for treatment leads to drug resistance (Pootong, Norrapong, & Cowawintaweewat, 2017).

The fungal cell wall is the main target for selectivity since it's made up of chitin that is not found in human cell. Chemical treatment is greatly effective for treating fungal infections but the risk of drug resistance makes it complicated. To avoid these strains of resistance and intrinsically developed resistant species made, brings our attention towards some natural products that are promising in treating fungal infections for example Essential oils.(Hu, Zhang, Kong, Zhao, & Yang, 2017; Nazzaro et al., 2017).

2.9. Nanoemulsion

Nanoemulsions are defined as two immiscible liquids one of which is dispersed in the second but continuous phase. Nanoemulsions can made into oil in water (O/W) or water in oil (W/O) (Pongsumpun et al., 2020). Nano-emulsions are solid in nature, sphere in shape with amorphous surface with lipophilic nature having negative charge (Jaiswal & Dudhe, 2015). Nanoemulsions being submicron in size are of great interest for use as a drug carrier and improving therapeutic efficacy of drugs. They are advanced nano-droplet system for systemic, controlled & target drug delivery systems. (Shaker et al., 2019)

Depending on the method of preparation, difference in droplet size distribution can be achieved, explaining, how preparation techniques can affect the stability of emulsions. Droplet size between conventional emulsions and micro-emulsions with size range of 20-500nm are called as miniemulsions, ultrafine emulsions, translucent emulsions, nano-emulsions and sub-micron emulsions. Because of the small droplet size nano-emulsions appears continuous and transparent. Its continuous Brownian

movement avoids sedimentation & creaming, hence offered high stability. (Fernandez et al., 2004)

2.10. Nanoemulsion as Carrier

Most of drugs are hydrophobic by nature that shows low solubility and bioavailability issues, uncertain absorption and dose variations, so a lipid based formulation i.e. nano-emulsion is the best choice for avoiding such problems(Kumar, Bishnoi, Shukla, & Jain, 2019). Nano-emulsion formulation improves bioavailability of hydrophobic drugs by encapsulating it into a lipid based system and masking the irritant effect of skin irritants. Nano-emulsions with smaller particle size provides increased surface area hence improving the drug absorption. They are thermodynamically stable and less energy is used for preparation of nanoemulsions (Jaiswal & Dudhe, 2015). Nanoemulsions are cost-effective, high storage stability and easy to prepare with simple procedure. (Shaker et al., 2019) Nanoemulsions can deliver both hydrophilic drugs and lipophilic through skin providing therapeutic effects. (Shaker et al., 2019)

2.11. Types of Nanoemulsions

NEs are colloidal dispersions of water in oil (W/O) or oil in water (O/W):

2.11.1. Water in oil (w/o)

Water in oil emulsion is formulated for hydrophilic drugs and are non common nanoemulsion formulations than o/w type emulsion for transdermal route. In water in oil type the drug is in water phase not in oil. Since the drug used for this type of formulation are hydrophilic hence, selection of surfactant is based on hydrophile lipophile balance (HLB) value, to bring stability and tension reduction between the oil and water phase (Shakeel & Ramadan, 2010).

2.11.2. Oil in water (o/w)

In most cases, drugs are poorly soluble in water and are thus produced by pharmaceutical industries to formulate it as such making it bioavailable to produce therapeutic effect (Shakeel, Baboota, Ahuja, Ali, & Shafiq, 2008). Nanotechnology has gained higher interest in this regard because of its ability to solubulize drug, improved bioavailability and increase capacity of drug loading. Nanoemulsion is

most acceptable in transdermal drug delivery system because of its enhanced drug permeation to enhance poor soluble drugs bioavailability when compared to other transdermal dosage forms (Kawakami, Yoshikawa, & Hayashi, 2002).

2.11.3. Double emulsion w/o/w type

Double emulsion is a colloidal system in which there is a primary emulsion phase of water in oil dispersed in an aqueous phase using hydrophilic surfactant, and referred as water-in-oil-in-water double emulsion. Double emulsions are thermodynamically unstable because of huge interfacial area that leads to ostwald ripening. o/w/o type emulsion use surfactants twice one as primary surfactant and secondary surfactant, both to maintain the interfacial tension of primary emulsion and secondary emulsion.(Bhattacharjee, Chakraborty, & Mukhopadhyay, 2018)

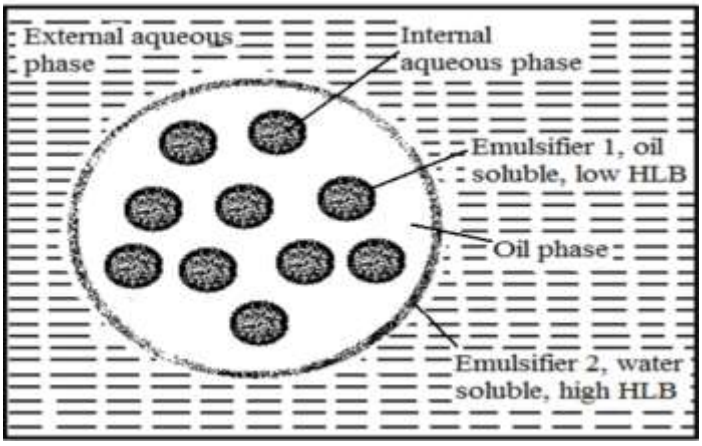


Figure 2. 3. Structure of double emulsion o/w/o(Bhattacharjee et al., 2018)

2.12. Components of Nano-emulsion

Nano-emulsion system consists of following; oil, lipids, surfactants and water soluble co-solvents.

2.12.1. Oils

Oil plays key role in drug bioavailability and it's lymphatic transportation. Lipids generally consists of fatty acid esters or medium-chain, long-chain saturated, partially saturated or unsaturated hydrocarbon chain like triglycerides, diglyceride and mono-acylglycerol, vegetable oil, mineral oils and free fatty acids, soybean oil, lanolin, corn oil or peanut oil etc. Oil selection for development of nanoemulsions is

based on solubility of drug in oil phase and its capacity of drug loading(Kumar et al., 2019) (Mistry & Sheth, 2011) .

OA (Oleic acid) is commonly used oil phase used in formulating NE's. OA is having penetration enhancing property that allows stratum corneum to absorb water and swell up. OA also consists of SC like structurally thus enhancing penetration through thicker and limiting barrier of skin (Kogan & Garti, 2006). Other penetration enhancer oils reported in literature includes capryol 90 (Mostafa et al., 2015) and isopropyl myristate. The high viscosity oil α-tocopherol gives small ranged droplet size, like hexyl laurate (Shaker et al., 2019).

2.12.1.1. Essential oils as oil component of NE

EOs categorized as GRAS " Generally Recognized as Safe" by FDA, thus not harmful and accepted widely by consumers for being of natural origin (Nazzaro et al., 2017). EO's are frequently used in NE for higher stability. Nanoemulsions prepared by using various plant based essential oils and non-ionic surfactants are safe to use, biocompatible as well as stable. Ongoing investigations have shown that use of EO in nanoemulsions are more stable physically when compared to conventionally prepared nanoemulsions (Science, 2018).

There are certain nanoemulsions available prepared by using EO as an oil component.

Table 2. 1 . Nanoemulsions loaded with EO (Science, 2018)

ESSENTIAL OILS	PURPOSE	TARGETTED MICROBES	SURFACTANTS
Eucalyptus	Pharmaceuticals	Proteus mirabilis	Tween 20
Clove	Pharmaceuticals, Disinfectant	E.coli Staphylococcus aureus Salmonella typhi Pseudomonas aurigenosa	Tween 20
Tea tree and Sage	Pharmaceuticals	Trychophyton	Tween 80

		rubrum	
Neem	Pharmaceuticals	Vibrio vulnificus	Tween 20

Essential oils also has activity against fungal strains. Some of EO's along with their mechanism of inhibition are mentioned as follows:

Table 2. 2. Effects of EOs and its components on fungal cell(Nazzaro et al., 2017)

Activity	Essential Oil/Components
Antifungals	*Corriandrum sativum* *Citrus* *Curcuma longa* *Piper nigrum* *Thymus vulgaris* *Commiphora myrra* *Melissa officinalis*
Effects on membranes and walls of fungi	*Cinnamomum* *Citrus* *Mentha piperita* *Thymus* *Corriander sativum* *Anethole* *Benzyl benzoate*
Effect on morphology and cell growth of fungal cell	*Thymus* *Carvacol* *Thymol* *Eucalyptus* *α -pinene* *Citronella*
Inhibition efflux pump	*Cinnamomum* *Citrus* *Eucalyptus* *Mentha* *Thymus vulgaris* *Origanum vulgare*
Action on fungal mitochondrion	*Cinnamomum camphora* *Corriandrum sativum*

	Commiphora myrrha *Origanum compactum*
Synergistic / antagonistic	*Citrus* *Corriandrum sativum* *Cymbopogan nardus* *Eucalyptus* *Rosa domascena* *Citral* *Thymus vulgaris* *Citronellal*
Effect on production of micotoxins	*Cinnamomum* *Cymbopogan* *Cider* *Origanum vulgare* *Citrus* *Eucalyptus* *Mentha* *Thymus*

2.12.2. Surfactants

Surfactants classified as non-ionic, cationic, anionic and zwitterionic (Pandey, 2014). The non-ionic surfactants are mostly acceptable type of surfactant for transdermal NE because of it low toxicity and inertness with NE (Shaker et al., 2019). These surfactants are capable of fluidizing the lipids of SC layer ultimately enhancing the drug absorption (scheuplein and ross 1970). Mechanism of penetration enhancement occurs in two ways, former is the surfactant entering intercellular region of SC, fluidize, solubulize and extraction of lipids takes place. Secondly after extraction of lipids, surfactant permeates into intracellular matrix, bind and interact with the keratin strands, and causes disruption of corneocytes (Breuer, 1979).

Anionic surfactants enhance penetrability through skin for the target, because of a strong interaction between lipids and keratin. Sodium lauryl sulphate alkyl chains have more lipophilic interaction with the skin, by creating additional binding site using sulphate group that leads to increase skin hydration (Shaker et al., 2019).

Cationic surfactants disrupt the cell matrix by interacting with keratin fibers causing disruption. They are capable of creating electronic changes in SC by interacting with

anionic components of skin layer, which enhance the transfer of anionic drugs via skin (Shaker et al., 2019).

HLB value is hydrophile-lipophile balance of surfactant. HLB value on scale ranges from 0 - 60, that shows the affinity of surfactant for water or oil. O/W emulsions required high hydrophilic-lipophilic balance (HLB) value to ensure efficient self dispersibility and stability of SEDDS. Commonly used surfactants for nano-emulsions listed as Spans (fatty acid esters), tweens (Polyoxyethylene), amphiphilic proteins (whey protein isolate), phospholipids (soy, egg or dairy lecithin), lauraoyl macrogolglyceride (Gelusire® 44/14), polysaccharides, and Cremophor® EL (castor oil). In order to get stable SEDDS formulation concentration of surfactant must be in range of 30-60% (w/w) (Mistry & Sheth, 2011).

Surfactants with greater degree of ethoxylation are selected because higher the level of surfactant ethoxylation, its solubility in water will increase and viscosity is is decreased in medium. There is no perfect surfactant combination exist, so possibilities has to be explored (Shaker et al., 2019).

2.12.3. Co-surfactant / Co-solvent

Co-surfactants are used to reduce surface tension and increase flow of liquid over liquid by reducing its bending stress. The interfacial tension is decreased continuously with an increase in concentration of a co-surfactant, until its critical limit, beyond which interfacial tension will increase again. Alcohol concentration required to reach this limit depends on alkyl chain of alcohol, shorter the chain of alcohol higher is the concentration of alcohol required. (Shaker et al., 2019).

Co-surfactants are also called as co-solvents. Co-solvents help in decreasing surfactant related GI-distress and lower interfacial tension to a small negative value. It also improves penetrability of dispersion media and decrease shear required to disperse globules (Kumar et al., 2019). Widely used co-surfactants are: Glycerin, polyene glycerol, polyethylene glycol (PEG), propanol and ethanol (Mistry & Sheth, 2011)

NE prepared using intermediate chain alcohol like n-butanol, n-hexanol and n-pentanol has reduced surface tension between surfactant to water phase. Chain length

of co-surfactant gives effect to NE formulation. Use of alcohols with long chain likehexanol and heptanol as co-surfactant caused separation of closed water domain in continuum of hydrocarbon layer that leads to, less uniform and organized micelle system. While use of medium chain alcohol from stable oil droplets inside system, gives least phase separation (Kegel & Lekkerkerker, 1993). Selection of co-surfactant can control drug release by altering the viscosity, by reducing or increasing the viscosity, without any compromise in stability of NE (Klossek, Marcus, Touraud, & Kunz, 2013). The use of alcohol in NE has strong influence on both, viscosity and density of NE formulation, for e.g Ethanol, reduce the viscosity of overall formulation (Shaker et al., 2019).

The co-surfactants are capable of improving solubility of loaded drug in NE system. Ethanol is less toxic when used topically, hence can be used in combination of various surfactants, increasing the dissolution of API. Optimally selected two or more co-surfactants has tendency to improve the overall flux of the NE formulation, minimizing or ending the use permeation enhancers(Shaker et al., 2019).

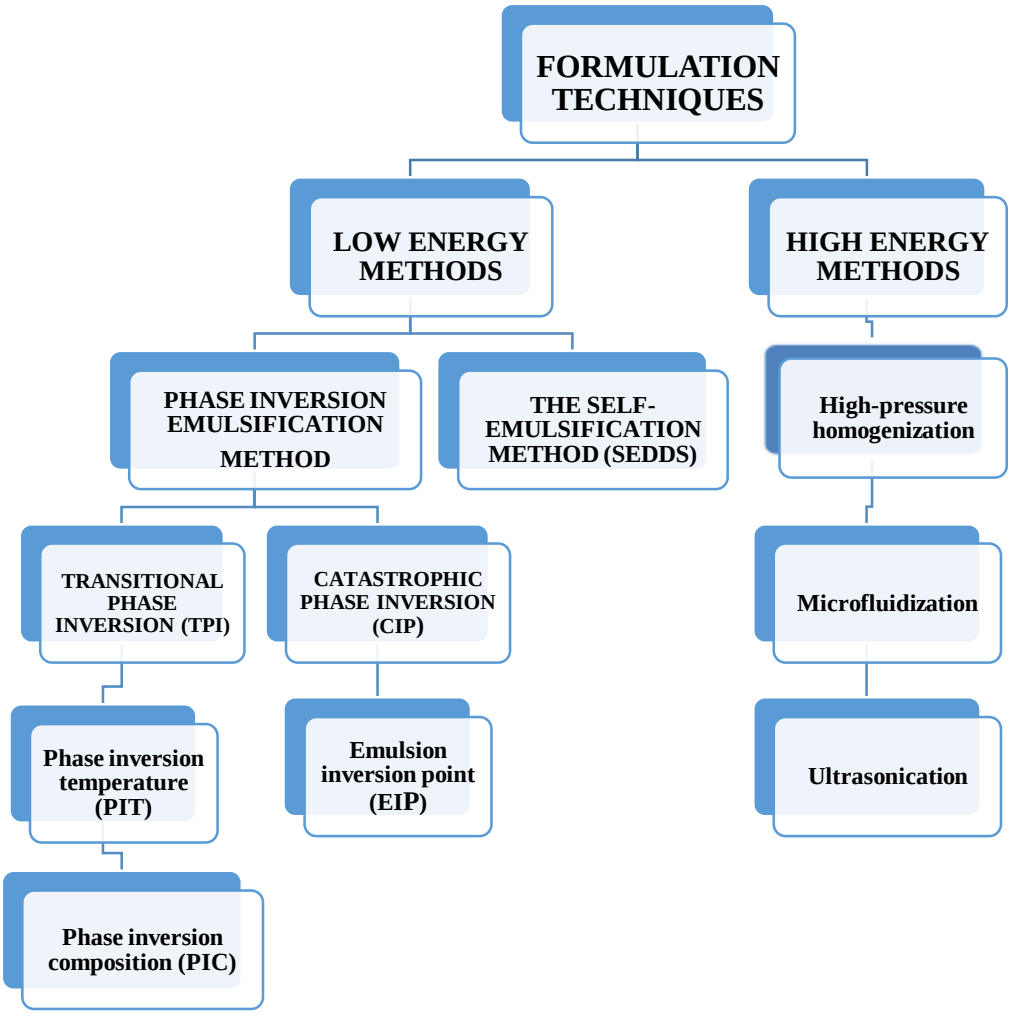
2.13. Nanoemulsion System and Essential Oils

Nanoemulsions has proved to have wide activity against various gram positive and gram-negative bacteria, spores, and enveloped viruses. Myc et al. has reported activity against fungi through novel NE consisting of oil, three surfactants, cosolvent and distilled water (Myc, Vanhecke, Landers, Hamouda, & Baker, 2003). This activity made NE formulation as selective formulation having added feature, with no preservatives for maintaining its stability. This property made it capable of applying directly on skin without disinfecting the skin surface and exert no toxicity to skin cells (Shaker et al., 2019).

Essential oils have low solubility and are unstable in nature against environmental conditions and are susceptible for oxidation. Nano-emulsion formulation is a technique that can resolve both issues of stability and solubility hence keeping it therapeutically active and stable. It consists of oil, water and surfactant and it's particle size ranges from 10-100nm (Pengon et al. 2018).

2.14. Formulation Techniques

Nano-emulsions are classified on the basis of energy requirements, self-emulsification and phase-inversion.



2.14.1. High Energy Method

It is widely used method to formulate nano-emulsions(Mahdi Jafari, He, & Bhandari, 2006). Mechanical energy at high level is used to produce stronger disruptive forces that breaks the larger molecules to smaller size particles. Hence, producing NEs using high kinetic energy. These disruptive forces are produced by mechanical instruments like ultrasonicator, high-pressure homogenizer and microfluidizer (Gonçalves et al., 2018). High energy method helps in controlling the size of particles with type of formulation composition needed. It also helps in managing rheology, stability and colour of emulsion. (Kumar et al., 2019). It involves following methods:

2.14.1.1. High Pressure Homogenization

This method works with high energy, provided to generate homogeneous flow that can break large particles to small size. A high energy homogenizer is attached to create homogeneous flow in the system that produce nanoemulsions. High pressure homogenizer will create intense disruptive force in the system that reduce the size of large particles into nano-sized particles (upto 1nm) (Rai, Mishra, Yadav, & Yadav, 2018). The prepared emulsion is passed through a small slit with high pressure (500-5000psi). The forces involved are hydraulic shear, intense turbulence and cavitation are applied that yields nano-sized nanoemulsions(Floury, Desrumaux, & Lardières, 2000). Efficiency of nano-emulsion produced by homogenizer depends on composition of sample, type of homogenizer, condition for operating homogenizer like energy intensity, time and temperature (Qian & McClements, 2011). High intensity of homogenizer decrease droplet size and forms nano-emulsion.

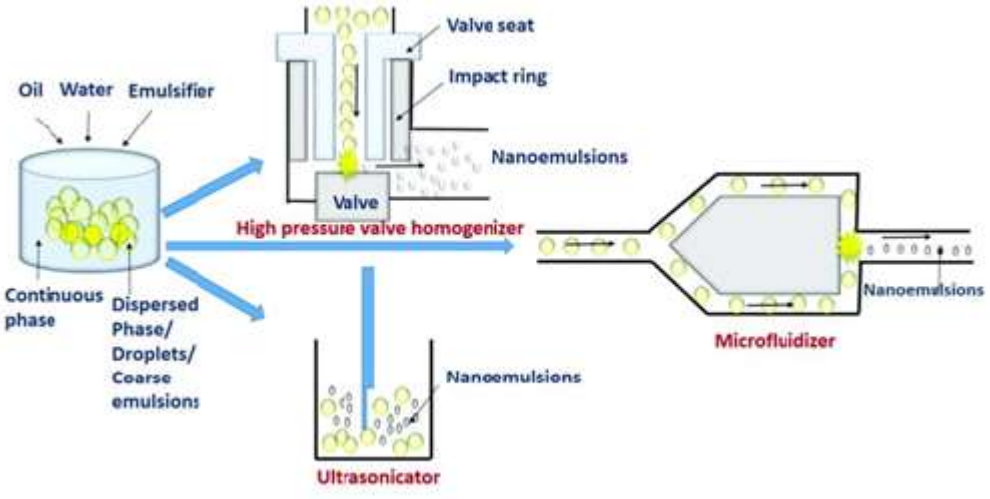


Figure 2. 4.High pressure homogenization (Kumar et al., 2019)

2.14.1.2. Micro Fluidization

It's a technique of mixing of micro sized particles by using a device called micro fluidizer. In this process sample is forced to pass through micro-channels with high pressure (500-20,000 psi). Micro channels are small in size, allowing mixing at micro level(Kumar et al., 2019). The macroemulsion mixture of oil phase and water phase are mixed, then passed through micro fluidizer which provides high pressure are pushed forward to interaction chamber. Inside the interaction chamber, the two macroemulsion molecules strike each other at high speed. The collision produce

cavitation, shearing and impact that produce stable nanoemulsions (Kumar et al., 2019). Microfluidizer produce small and narrow NE particles size distributions then by homogenizer (Marie & Gervais, 2005). Microfluidizer can produce stable nanoemulsions with low concentration of surfactant (Kumar et al., 2019).

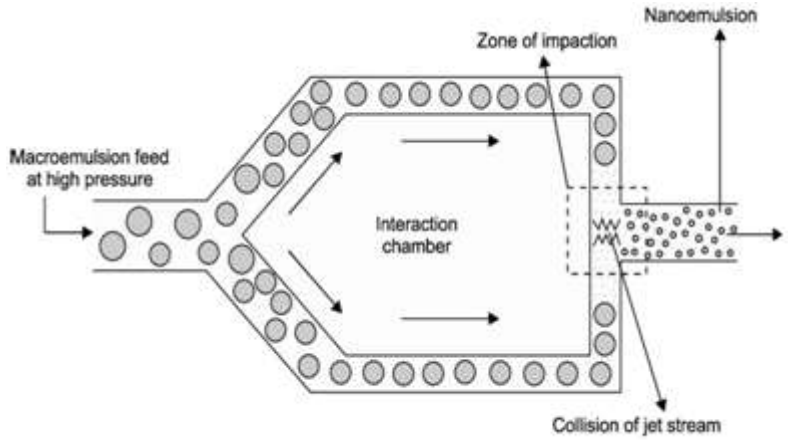


Figure 2. 5. Microfluidization technique(Kumar et al., 2019)

2.14.1.3. Ultrasonication

Ultrasonicator is efficient then other high energy methods in comparison of operation and cleaning (Mahdi Jafari et al., 2006). In this method ultrasonic waves are used to produce cavitation forces to break macro molecules into small nano sized particles. By adjusting the energy of ultrasonic wave, time and input, we can get particle size as desired as well as stable nanoemulsion. In ultrasonication, physical shear is produced by the process of acoustic cavitation (Jayasooriya, Bhandari, Torley, & Arcy, 2004). Cavitation is the process of formation of microbubbles and collapse of microbubbles, caused by the fluctuation in pressure of the acoustic wave. The collapse in micro bubbles cause immense misbalance that forms nano sized droplets (Canselier, Delmas, Wilhelm, & Ablsrnail, 2002).

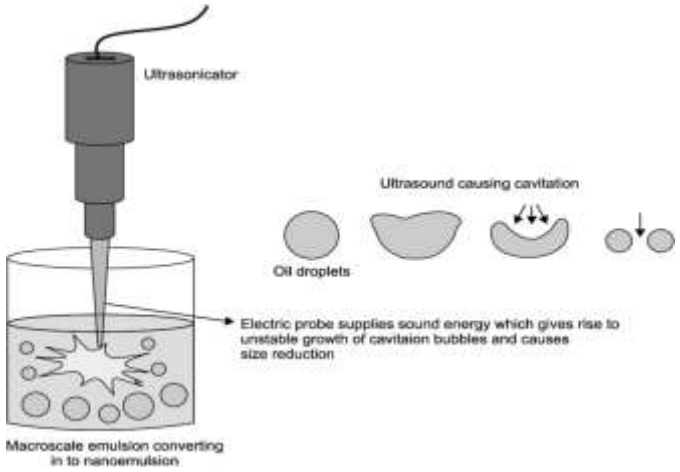


Figure 2. 6. Probe sonication method (Kumar et al., 2019)

Penetration of radiation into oil and water by ultrasound system induce cavitation force thus providing energy formation of new interface, producing nano-sized droplets of emulsion. By using sonication technique NEs can also be formed without using surfactants (Gaikwad & Pandit, 2008; Mahdi Jafari et al., 2006).

2.14.1.3.1. Ultrasonic bath

Ultrasonic bath is an indirect sonication method in which sonic waves or energy are transferred directly to the water bath and then to vessel or multiple tube sample.

An ultrasonic bath can be used to produce nanoemulsion. In bath sonicator the ultrasound waves from water bath are produced at certain temperature and speed which then diffuses through the cell compartment to sample (Jayasooriya et al., 2004).

2.14.1.3.2. Ultrasonic probe

Ultrasonic probe provides sonic energy and is administered into sample with purpose of breaking cells. A probe is inserted directly into sample, so probe is in direct contact to sample, so receive strong energy from the sonicator.

An ultrasonic processor with the tip of probe was inserted in coarse emulsion at frequency, power and amplitude. Sonication is carried out at various temperatures and time to produce disruptive force inside the molecules that reduce the droplet size from simple emulsion to nanoemulsion (Jayasooriya et al., 2004).

2.14.2. Low Energy Method

It needs low energy for producing nanoemulsions. This method is more energy efficient because it use internal chemical energy of system, and demands less stirring for nanoemulsion production. This method requires high amount of surfactant and co-surfactant. It generally involves inversion of phase and self emulsification process (Kumar et al., 2019).

2.14.2.1. Phase Inversion Method for emulsification

During emulsification process abrupt changes in surfactant curvature forms phase inversion from oil in water to water in oil. This change in curvature of surfactant is caused by change in parameters like composition, temperature and mixing etc. (Solè et al., 2010). Phase inversion method is of two types: Transitional phase inversion (TPI) which includes Phase inversion temperature (PIT) and Phase inversion composition (PIC) and Catastrophic phase inversion (CPI) , which involves Emulsion inversion point (EIP).

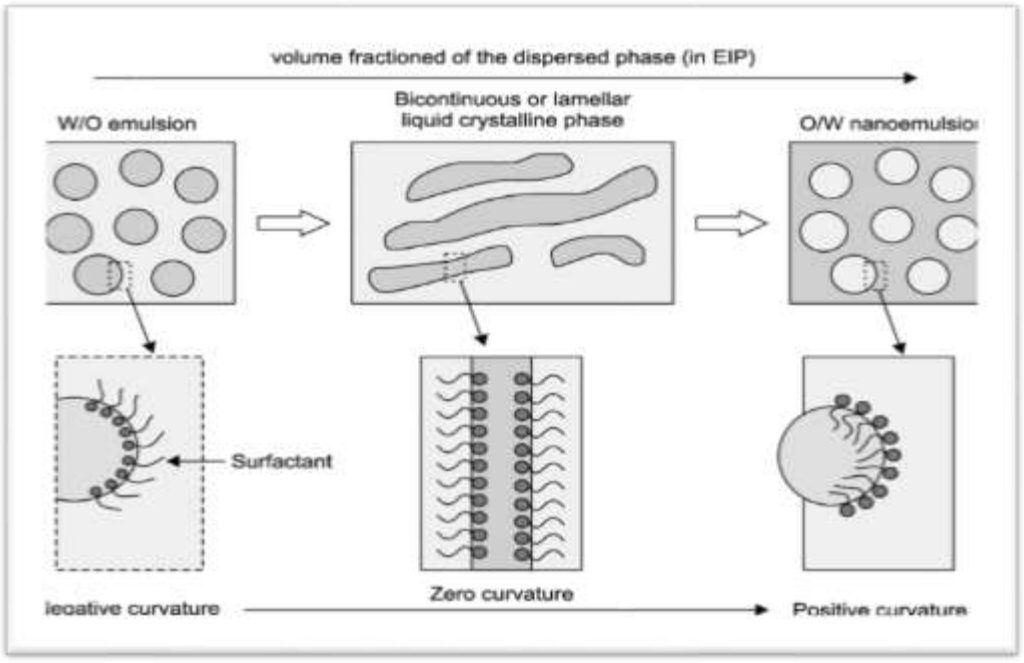


Figure 2. 7. Phase inversion emulsification technique(Solè et al., 2010)

Transitional phase inversion takes place because of change in abrupt affinity of surfactants because of changing parameters like temperature and composition (Kumar et al., 2019; Solè et al., 2010). However, Catastrophic phase inversion occurs

because of flocculation of disperse phase into continuous phase by repeated addition thus, forming a bi-continuous structural phase (Ishak & Annuar, 2016). The catastrophe refers to a sudden change in behavior of system, due to change in conditions. In order to occur the catastrophic phase inversion, surfactant has to be present in dispersed phase to form coalescence in high rate that forms abrupt inversion of phase (Armanet & Hunkeler, 2006). During TIP surfactant affinity or the spontaneous curvature is changed, while surfactant affinity is not changed in case of catastrophic spontaneous curvature.

2.14.2.2. Phase inversion temperature (PIT) method

In this method, the spontaneous curvature surfactant is inverted because of change in temperature. Polyethoxylated surfactant that is non-ionic in nature, undergoes dehydration that makes it lipophilic and cause changes in surfactant curvature. Therefore, phase inversion takes place and nanoemulsions are formed (Kumar et al., 2019; Morais, David, Delicato, & Rocha-filho, 2006).

Process involves mixing of oil, water and non-ionic surfactants at room temperature to make oil in water (O/W) emulsions. Then, an increase in temperature starts dehydration and brings change in curvature of surfactant hence, increasing lipophilicity and surfactant turns its affinity towards oily phase. Inversion of phase occurs because of this and emulsion changes from O/W type to W/O. At Hydrophile-lipophile balance (HLB) temperature (moderate temperature) affinity of non-ionic surfactant is equal toward both oil and water phase i.e. zero curvature of surfactant (Kumar et al., 2019). For better phase inversion, rapid change in temperature such as rapid cooling and heating is required. This rapid change in cool or hot temperature produce nanoemulsions that are kinetically stable (Solans & Solé, 2012).

2.14.2.3.Phase inversion composition (PIC) method

This method is similar to PIT, but differs at one point the phase inversion in PIC occurs due to change is composition of system, not the temperature. In this method, one of the component of mixture is added slowly wither water or oil at a time. If water is added in system slowly the chain hydration takes place in the mixture. The surfactant hydrophile-lipophile balance (HLB) will be balance and curvature of surfactant will be zero, at this point a lamellar or bi-continuous structure will be

formed. If continuous addition of water is done to the system after this point, the curvature of the system will start increase to higher positive curvature. This curvature change causes phase inversion that leads to the formation of nanoemulsions. Thus, phase inversion takes place due to change in composition (Solans & Solé, 2012).

The other parameters like salt addition or changes in pH can produce nano-sized droplets by phase inversion (Kumar et al., 2019; Maestro, Solè, González, Solans, & Gutiérrez, 2008; Reviews & Science, 2007).

2.14.2.4. Emulsion inversion point (EIP) method

In this EIP method, the phase inversion occurs by CPI mechanism. The catastrophic phase inversion is induced by changing the fractioned volume of the dispersed phase rather than the surfactant properties (Fernandez et al., 2004; Mcclements, Rao, Mcclements, & Rao, 2011; Shaker et al., 2019). When water phase will be added, the system starts acting like W/O emulsion, continuous addition of water above level of critical water concentration while stirring continuously, droplets of water will merge into one another until point of phase inversion is reached, it cause the formation of bi-continuous or lamellar structure. Further water addition will cause phase inversion and the phase of system will convert from W/O emulsion to O/W type from bi-continuous microemulsion. The droplet size of formed nanoemulsion, depends on process variables like the rate of adding water and stirring speed. Small molecules of surfactants should be use for catastrophic phase inversion. Surfactants will stabilize both O/W and W/O emulsions (Armanet & Hunkeler, 2006; Fernandez et al., 2004).

Bancroft's rule of stable emulsion a surfactant should be found in continuous phase (Perazzo, Preziosi, & Guido, 2015). So, initially in catastrophic phase inversion, the surfactant is mainly present in dispersed phase that gives unstable emulsion i.e. not obeying Bancroft's rule and then changes from an unstable emulsion to a stable emulsion.

2.14.3. Self Nano-emulsification Method

In Self nano emulsification method no change occurs in curvature of surfactant. The surfactant or co-solvent molecules diffuses rapidly from disperse phase to continuous phase resulting in turbulence, creating nano-sized droplets. The self emulsification method is also referred to as spontaneous emulsification method(Solans & Solé,

2012). SNEDDS are based on self-emulsification process containing hydrophilic content (surfactant and co-surfactant) more than lipid content(Kumar et al., 2019).

Self emulsifying drug delivery system (SEDDS) is defined as an isotropic mixture of oil, surfactant and co-surfactant and sometimes co-solvent that extemporaneously emulsify itself when comes in contact with GI media upon mild stir through peristalsis. Self nano-emulsifying drug delivery system (SNEDD) gives nano sized particles. A nano-emulsified drug if taken orally can efficiently be taken up through lymphatic system where it by-passes the hepatic first pass effect. Large lipid droplets are converted to smaller micelles when comes in contact with bile salts and lipases, these micelles are than absorbed through intestinal villi and micro-villi which is then distributed to body through lymphatic pathway (Gursoy & Benita, 2004).

Nano sized particles of SNEDDS can easily penetrate through skin layers and treat local infection. Process of self emulsification depends on various factors like nature of oil, surfactant / co-surfactant ratio and polarity of emulsion. SEDDS gives coarse emulsions whereas self nano-emulsifying drug delivery system (SNEDDS) makes nano sized particles(Gursoy & Benita, 2004) (Kovvasu & Kunamaneni, 2019).

Two most common methods were reported for mechanism of SNEDDS by diffusing hydrophilic co-solvent from organic to aqueous phase (Pouton, 2000; Solans & Solé, 2012), and the preparation of nanoemulsion negative free energy at transient negative or ultra low interfacial tension(Gurram et al., 2015; Kohli, Chopra, Dhar, Arora, & Khar, 2010).

2.15. Stability of Nanoemulsions

Nanoemulsions may get turbid during storage due to phase separation, flocculation or sedimentation or coalescence that cause instability(Karthik, Ezhilarasi, & Anandharamakrishnan, 2017). Nano-emulsion systems are kinetically stable because it's destabilization kinetic is quite slow (several months)(Kumar et al., 2019). Nanoemulsion being nano size particles as compared to conventional emulsions, Brownian motion effects are dominant than gravitational forces thus, having great stability against gravitational separation. Agglomerates and coalescence occurs because of attraction between droplets / molecules, which is generally quite low in

nanoemulsion system. The nanoemulsions shows better stability towards agglomeration and coalescence (Qian & McClements, 2011).

2.16. Topical Nanoemulsion Characterization

NE characterization is a series of testing procedure that confirms successful formulation of nanoemulsion. It includes stability, droplet size confirmation, compatibility between components of formulation, skin irritation testing and successful delivery of drug through skin (Craig, Barker, Banning, & Booth, 1995; P. K. Ghosh, Majithiya, Umrethia, & Murthy, 2006).

Characterization of transdermal NE is summarized as follows:

2.16.1. Visual inspection

Visual inspection is visualized by naked eye in order to determine successful formulation of NE. We have to observe the sudden turbidity of our transparent and clear NE. Absence of precipitation and phase separation indicates the stability of formulation(Shaker et al., 2019).

2.16.2. Viscosity

Viscosity test is done by using rotational viscometer. The torque required to rotate the paddle in NE is measured. Low viscosity formulation has quick release and rapid permeation to skin than high viscosity NE, nanoemulsion O/W type usually have low viscosity than W/O nanoemulsion(Shaker et al., 2019).

2.16.3. Morphology

To verify the consistency of fabricated droplets shape and size for being nano in size. Equipments used for this test are: Scanning electron microscopy (SEM), transmission electron microscopy (TEM), Neutron scattering, Atomic force microscopy (ATM), Ultrasonic resonating technology and Cryo-electron microscope (Shaker et al., 2019).

The sample of NE is stained with thin layer of 1% solution of phosphotungistic acid and then subjected to carbon or copper coated grid, depends on the equipment used TEM or SEM. Accelerate with voltage of 20kV, and using suitable software and magnification a quantitative measurement of drop size along with the consistency and quality of droplet can be obtained (Shaker et al., 2019).

2.16.4. Particle size

Particle size measurement or Poly-dispersity index (PDI) or Zeta potential (ZP) is used to observe homogeneity of particles, broadness and range of droplet size and surface charge distribution. Lower the value of PDI (<0.2) and higher the value of zeta potential, better is the stability of NE against other destabilizing forces (Shaker et al., 2019).

Equipments used for measuring particle size are photon correlation spectroscopy (PCS) and Dynamic light scattering (DLS)(Shaker et al., 2019).

Size distribution and size of droplets are measured from collective measurements of scattering through DLS of sample i.e. NE. ZP is potential charge difference between the particles and continuous phase and measured using Zetasizer (Shaker et al., 2019).

2.16.5. Electro conductivity

A conductivity meter is used to measure the electro conductivity of NE. The amount of electric current and conductance in the NE sample is measured by using meter. The probe of the meter is inserted into the sample and voltage is supplied between two probes inside the NE sample. Electrical resistance from total disperse particles in sample cause drop in voltage, which is read by the meter (Shaker et al., 2019).

Electro-conductivity is basically the preliminary or initial change in droplet size of NE, although relationship between electrical conductivity and NE stability is not linear (Shaker et al., 2019).

2.16.6. Refractive index

It's an important tool to investigate NE structure by using refractometer. Refractive index is indication of formation of an isotropic mixture and uniformity of NE. Comparison of the refractive index (RI) of NE with refractive index of water (RI of water = 1.333), closer the value of NE to value of water, uniform and transparent is NE sample (Shaker et al., 2019).

2.16.7. In-vitro skin permeation

This test is performed by using Franz diffusion cell apparatus, to evaluate transcutaneous permeation or membrane retention.

The NE sample is placed on donor compartment of any, synthetic or excised skin of model animal, after keeping variety of membranes. Fill the receiver compartment of franz diffusion cell apparatus with a phosphate buffer saline having 7.4 pH, which imitates blood stream. Next step is to stir at 100 rpm at 37°C temperature. sample of 1ml is then taken manually or either automatically, filtered and analyze using UV spectroscopy or HPLC. Once the value of drug released is determined with an hour interval , the steady state flux (Jss) is calculated using formula $Jss = P.C_D$ where P is permeability coefficient, C_D is donor chamber concentration (Shaker et al., 2019).

2.16.8. In-vivo dermato-pharmacokinetic and dynamics

In vivo tests are performed to study the plasma drug concentration profile and pharmacological effect of drug. Equipment used for this test is HPLC and test is performed on intact live animal. Procedure involved administration of nanoemulsion to a shaved skin of animal, and withdraw sample of blood at several time intervals, centrifuge and analyze the plasma by using HPLC. It will help us knowing the amount of drug reaching circulation and pharmacodynamic properties of NE are assessed (Shaker et al., 2019).

2.16.9. Skin irritation

This test is performed to know if formulation will cause irritation to skin. Test is performed on living animals (rats or rabbits).

Steps involves collecting group of animals, formulation is applied to group with hairless skin of testing animals with uniform spread in specific area. Experiment is conducted for seven days and site of application is graded according to score of visual scale. Tested sites are kept for observation for 48 hours to detect edema or erythema formed after applying formulation. Skin irritation score is followed by using Draize method (Shaker et al., 2019).

2.17. Essential Oil and Candidiasis Treatment

Several antifungal drugs are available for the treatment of cutaneous Candidiasis. Excessive use of azole group of antifungal agents that are fungistatic in activity, lead to resistant strains of *Candida albicans* to azole group, which is a challenge to deal with. This challenge draws attention to naturally occurring products that shows

promising results against Candidiasis. Essential oils are one of the natural source of treatment. Essential oils are classified as "Generally recognized as safe" (GRAS) by Food and Drug Administration (FDA), thus are not harmful due to its natural origin and are widely accepted by consumers than "synthetic" agents (Jang et al., 2008). The antifungal activity of essential oils is caused by properties of terpenes/terpenoids, that due to its low molecular weight and lipophilic nature are capable of inhibiting spore formation and germination of fungi(Nazzaro et al., 2017). There have been an increase demand to limit the use of chemical products for treating fungal infections in regard of increased data of resistance against antifungal agents as well as toxicity risks. On this basis compounds derived from plant source like essential oils were highlighted by researchers that can certainly play a fundamental role against candida causing fungi. The use of essential oils is common ever since the earliest civilizations, firstly in the East and Middle East, then in North Africa and Europe (Macwan et al., 2016). The term "essential oil" (EO) was first used in 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim. EO's are complex mixtures of polar and non-polar but natural compounds (Macwan et al., 2016; Masango, 2005), well known for their antifungal activity.

The International Organization for Standardization (ISO)(ISO/D1S9235.2) defines essential oil as a product obtained from distillation of water or steam or by mechanical processing or by dry distillation of natural materials. They appear as liquid, volatile, limpid and colored mixtures of several aromatic compounds. EOs are obtained from all plant parts, mainly from herbs and spices (Ravindran & Jaiswal, 2016; Wu, Jin, Xu, & Yang, 2017). The main components of essential oils is terpenes and terpenoids (Hyldgaard et al., 2012). Terpenes are large class of naturally occurring hydrocarbons, with various chemical features and biological properties. They are synthesized in cytoplasm of plant cells through the pathway of mevalonic acid starting from acetyl CoA. Terpenoids are related to terpenes, with some rearrangement or oxygen functionality. Terpenoids can be acidic, alcoholic, aldehydes, ketones or esters depending on their functional groups. Chemical composition of plant essential oils can differ from specie to specie depending on geographical location, environment, the maturity stage and extraction technique (De Martino et al., 2009).

Essential oils and their components has variety of targets, particularly the membrane and cytoplasm and in certain situations they completely alter the morphology of cells (Nazzaro et al., 2013).

2.18. Cinnamon Essential Oil

Cinnamon oil is an essential oil obtained from bark, leaves, twigs of genera *Cinnamomum* that belongs to family Laureaceae. The active antifungal component found in cinnamon oil is cinnamaldehyde, cinnamyl cinnamate and benzyl cinnamate (Boniface et al., 2012). Cinnamon oil works against cytoplasm activity of fungal cell causing membrane rupture and destruction of intracellular and extracellular enzyme (Gupta, Garg, Uniyal, & Kumari, 2008). The activity of cinnamon oil is because of cinnamaldehyde an aromatic aldehydes that inhibits amino acid decarboxylase activity and has proved from studies to be active against microbes (Gupta et al., 2008). Cinnamon is rich with cinnamaldehyde (50.5%) which is highly electronegative and its electronegativity is interferes in biological processes involving electron transfer and react with nitrogen containing components like proteins and nucleic acid (Gupta et al., 2008). Cinnamon oil has low lipophilicity (Miastkowska, Michalczyk, Figacz, & Sikora, 2020). Cinnamon essential oil has shown good results in various studies against candida albicans, hence its use as an antifungal is promising. Essential oils being alcoholic and acidic in nature can be irritant to skin when applied directly. Cinnamon essential oil has to be formulated in an acceptable dosage form. CEO being hydrophobic in nature can be formulated with some lipophilic carrier and nano-emulsions is the best technique to encapsulate the hydrophobic drug into a system of lipid carriers and surfactants. Various essential oils have shown their respective activity against fungal cells. Following table shows the number of E.O with antifungal activity as well as effects of certain E.O against fungal cell (Nazzaro et al., 2017).

2.19. Studies Based on Nanoemulsions Using Cinnamon Essential Oil

Various successful stable nanoemulsions using Cinnamon oil as an oil phase have been prepared for certain use. Some of those preparations are mentioned as follows:

Zhang et al. prepared nanoemulsion preparation using a blend of clove and cinnamon essential oil as an oil phase, Tween 80 as surfactant and ethanol as co-surfactant.

Objective of this study was to investigate the antimicrobial activity of formulation. Prepared formulation was stable (Zhang, Zhang, Fang, & Liu, 2017).

Another study was performed by Ghosh et al to investigate bactericidal activity of prepared nanoemulsion using cinnamon essential oil as an oil phase, Tween 80 as a surfactant and water by ultrasonication. Studies shows that the surfactant concentration is directly related to stability of NE formulation and inversely proportional to droplet size. Emulsification time has direct relation with droplet size and stability(V. Ghosh, Saranya, Mukherjee, & Chandrasekaran, 2013).

Miastkowska et al prepared nano-emulsion using four essential oils, including cinnamon essential oil. Aim of study was to see fungicidal effect of CEO against fungal strains. Results shows better fungicidal activity from prepared nanoemulsions using essential oils than from pure oils (Miastkowska et al., 2020).

Kumar et al. prepared nanoemulsion using CEO and objective of this study was to evaluate antifungal activity of prepared formulation. Prepared NE consists a blend of cinnamon essential oil and usinic acid. in vitro and in vivo tests were performed using candida strains and dermatophytosis models. Prepared formulation was meant for topical treatment of candidiasis and dermatophytosis (kumar, Ramteke, Pandey, & Pandey, 2019).

Another study performed by Pongsumpun et al. by preparing a nanoemulsion using cinnamon essential oil as oil phase and Tween 80 as surfactant. The obtained nanoemulsion were stable (Pongsumpun et al., 2020).

Katarzyna et al. studied various essential oils including thyme, lemon, clove, geranium, basil and cinnamon oils for testing fungicidal and fungistatic activity against *Candida albicans* and *Candida glabrata*. Result shows that the highest antifungal activity was from cinnamon essential oil (Gucwa, Milewski, Dymerski, & Szweda, 2018).

CHAPTER 3
MATERIAL AND METHOD

3.1. Materials

Cinnamon oil and Clove essential oil was kindly gifted from (Bazmialem Vakif University, Istanbul, Turkey). Surfactants used Kolliphor PS 80, Kolliphor PS 20 and Kolliphor RH 40 were from BASF® chemical company (Ludwigshafen, Germany) and purified water was used as required throughout the study. Digital balance (Mettler Toledo AB204-S/FACT)

Figure 3. 1. Digital balance

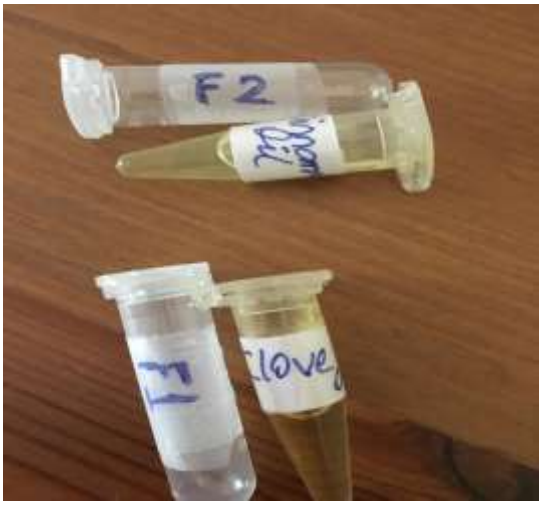


Figure 3. 2. F1 as Clove oil and F2 as Cinnamon oil

Figure 3. 3. Surfactants used Kolliphor PS 80, Kolliphor PS 20 and Kolliphor RH 40

3.2. METHODS

3.2.1. Assay of Essential oils

Essential oils were subjected for analytical study using GC-MS and constituents of cinnamon oil and clove oil were analysed. EOs were also assayed for antifungal activity. Method used for antimicrobial test was disc diffusion method and broth dilution method.

3.2.1.1. GC-MS of Essential Oils

The constituents of both E.O's (Cinnamon oil and Clove oil) were analyzed and provided by using Gas chromatography-mass spectrometry (Bezmialem Vakıf Üniversitesi Fitoterapi Eğitim Uygulama ve Araştırma Merkezi, Istanbul, Turkey) (Agilent Technologies). Total constituents in essential oils were detected by using peak normalization method. Chromatogram achieved in the end of GC-MS shows the peak for constituents and their respective percentage.

3.2.1.2. Antifungal Activity Test for Essential oils

3.2.1.2.1. Disc diffusion method

Disc diffusion method was carried out using Amphotericin B disc was used as control. strains of *Candida albicans* were used in experiment *Candida albicans* ATCC 32033. Yeast was incubated on Dextrose Agar for certain time period. A blank disc was impregnated with essential oil. Petri plates were incubated at specified temperature and time. Later on, the zone diameter on plates was measured.

3.2.1.2.2. Broth microdilution

Broth microdilution method includes formation of dextrose medium as broth. Yeast was incubated on agar at sepcified time and temperature, and subject to incubation, the concentration in the last well without microorganism growth was read as Minimum Inhibition Concentration (MIC) (µg/ml)

3.2.2. O/W Nanoemulsion Preparation Using Probe Sonicator

3.2.2.1. Nanoemulsion Preparation With Cinnamon Essential Oil and Three Different Surfactant Percentage

Surfactants (Kolliphor PS80, Kolliphor PS 20 and Kolliphor RH 40) were dispersed in purified water with specified amounts. And then were mixed by using magnetic stirrer (IKA Werker RT 15 Power). Specific amount of cinnamon essential oil was added and homogenized. This mixture of water-surfactant was homogenized again at specific speed for certain time using homogenizer (IKA T25 Digital Ultra Turrax).

After homogenization process formulation was subjected to probe sonicator (Sonic VibraCell) to get nanoemulsion.

Table 3. 1. Preparation of Nanoemulsion with different surfactant and oil ratios

Formulation code	Cinnamon oil	Kolliphor PS 80: CEO	Kolliphor PS 20:CEO	Kolliphor RH 40:CEO	Water
C1	50 µl	Start from 1:1	-	-	5 ml
C2			-	-	5 ml
C3			-	-	5 ml
C4			-	-	5 ml
C5	50 µl	-	Start from 1:1	-	5 ml
C6		-		-	5 ml
C7		-		-	5 ml
C8		-		-	5 ml
C9		Start from	-	-	5 ml

C10	50 µl	2.5:1	-	-	5 ml
C11	50 µl	-	-	Start from 3:1	5 ml
C12		-	-		5 ml
C13		-	-		5 ml
C14		-	-	Start from 4:1	5 ml
C15		-	-		5 ml
C16	50 µl	Start from 7:1	-	-	5 ml

3.2.2.2. Nanoemulsion Preparation at different Sonication Amplitude Percentage

For amplitude study the three formulations were selected from each surfactant formulation depending on their droplet size and PDI. These selected formulation were than homogenized (IKA T25 Digital Ultra Turrax) and sonicated (Sonic VibraCell), time was fixed as standard. Oil was added during homogenization, whole process was carried out at specific temperature.

3.2.2.3. Nanoemulsion Preparation at different Sonication Time

Time study was performed on selected formulations with better droplet size and PDI of each surfactant and was prepared by homogenization (IKA T25 Digital Ultra Turrax) then subjected to sonication (Sonic VibraCell) and amplitude of sonication was kept fixed as standard and time was varying. Oil was added during homogenization, whole process was carried out at specified temperature.

3.2.3. Characterization of Nanoemulsion

3.2.3.1. Droplet size, PDI and zeta potential

Nanoemulsion formulation was checked for droplet size, PDI and zeta potential by diluting with purified water. ZetaSizer is based on Dynamic light scattering (DLS).

The prepared mixtures that fulfilled the criteria of nanoemulsions as stable and transparent dispersion were then taken to measure droplet size and PDI using ZetaSizer Nano ZS (Malvern 1000 HS®, Worcestershire, UK). Each sample was measured thrice. Test was carried out and results were recorded.

3.2.3.2. Visual inspection

Prepared formulation was inspected visually by naked eye for clarity of nanoemulsion formation. Also observed for sudden turbidity of transparent and clear formulation or phase separation, if any. Clear formulation confirms the formation of stable nanoemulsion.

Figure 3. 4. Visual appearance of NE formulation C11, C12 and C13

3.2.3.3. Viscosity

Viscosity is an important factor that influence the stability and drug release of NE formulation. Viscosity is collectively depending on oil, water and surfactant concentrations. Excess amount of water content will reduce the viscosity of formulation, while decrease in surfactant concentration will increase the interfacial tension between both oil and water phase, leading to increased viscosity.

Viscosity of prepared optimum nanoemulsion was measured by using RheoStress RS1 rheometer (HAAKE^{TM}, US).

3.2.3.4. pH

pH-meter (Mettler Toledo) was used to check the pH of our formulation. The probe of pH meter was inserted into formulation and pH was measured.

Figure 3. 5. pH-meter equipment (Mettler Toledo)

CHAPTER 4

FINDINGS

4.1. Essential Oil Assays

Essential oils were subjected to GC-MS for eluting components and finding there percentage amount and antifungal activity test to check its effect against fungal strains. Following were the findings for each performed tests

4.1.1. GC-MS Analysis Result

Essential oils consists of complex mixture of terpenes and terpenoids. In order to analyse the components of oils we use gas chromatography and following were the results from clove oil and cinnamon oil.

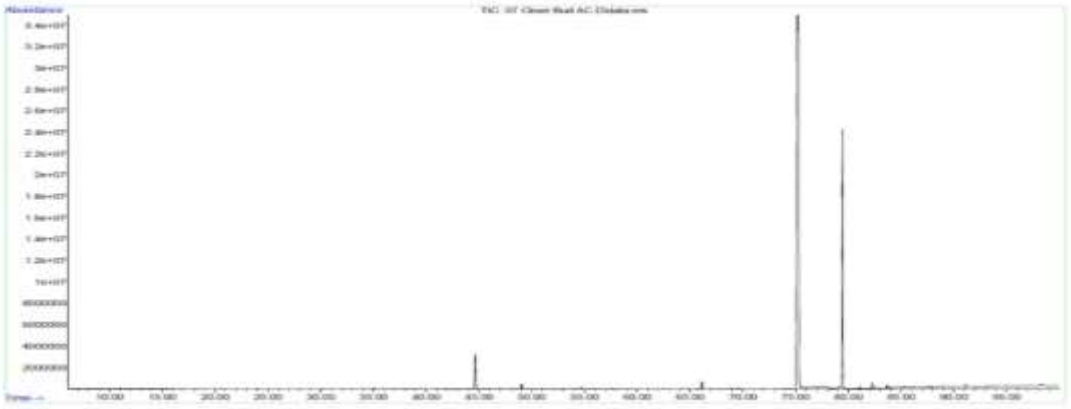


Figure 4. 1. Clove oil chromatogram from Gas chromatography

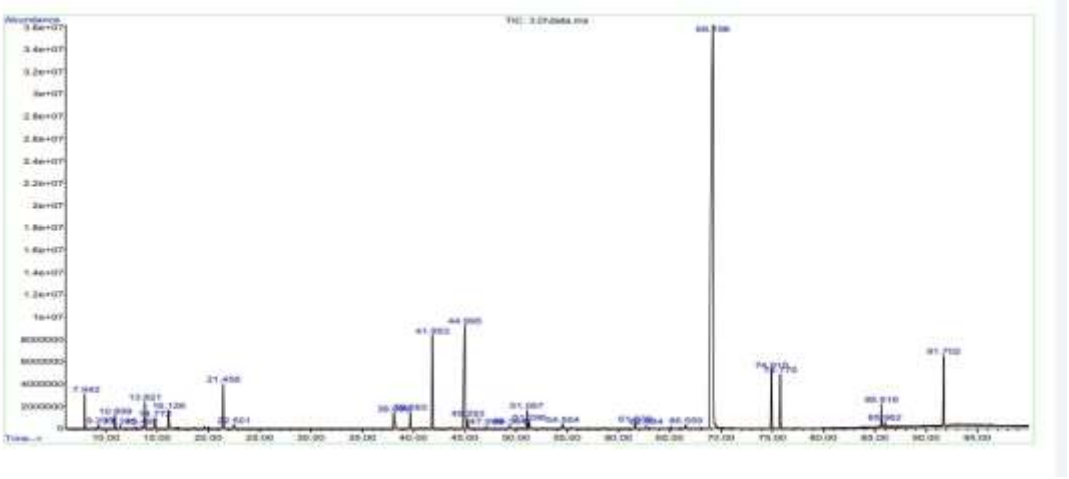


Figure 4. 2. Chromatogram for Cinnamon oil components

4.1.2. Antifungal activity tests for Essential oils

To study antifungal activity for essential oils disc method was used to check the inhibition zone of both oils against three different fungal strains and compare the activity. Other test of broth dilution was performed to check the minimum inhibitory concentration of essential oils. MIC shows the minimum concentration of oil enough to inhibit the fungal growth.

4.1.2.1. Disc diffusion test

Candida fungal strain was used to check the inhibitory activity of cinnamon essential oil and clove essential oil. After incubation the zone of inhibition was measured and compared to see the most active oil.

Table 4. 1. Zone in diameter inhibition for CEO and Clove oil

Disc diffusion zone in diameter (mm)			
Yeast strain	**F1**	**F2**	**Amphotericin B**
Candida albican ATCC 32033	27 mm	66 mm	14 mm

4.1.2.2. Broth dilution test

Broth microdilution test was performed to compare the minimum inhibitory concentration of essential oils against fungal specie. Following were the findings

Table 4. 2. MIC result for CEO and Clove oil

Minimum inhibitory concentration (µg/ml)		
YEAST STRAINS	**F1**	**F2**
Candida albican ATCC 32033	0.018 µg/ml	0.004 µg/ml

4.2. O/W Nanoemulsion Preparation with three Surfactants

Preparation of formulation with three different surfactants at various surfactant to oil ratio. These formulations were selected on the basis of visual appearance and droplets size, PDI values. Clarity was the challenge in preparation, formulations shows acceptable droplet size and PDI but visually turbid. In order to avoid this turbidity we prepared more formulations using Kolliphor PS 80 and Kolliphor RH 40

with periodic sonication and better results were obtained in the end yielding us optimum formulation.

4.2.1. Nanoemulsion Preparation With Cinnamon Essential Oil and Three Different Surfactant Percentage

Sixteen formulations were made using three different surfactants and cinnamon essential oil as oil phase and purified water as and aqueous phase.

4.2.2. Nanoemulsion Preparation at different Sonication Amplitude Percentage

Formulations prepared from sonication with different surfactants at various surfactant to cinnamon oil ratio, two formulations from above prepared formulations C3 and C5 were selected depending on their droplet size and PDI value and subjected to sonication with different sonication amplitude percentage. Finding showed C3 with lowest droplet size and PDI values than C5

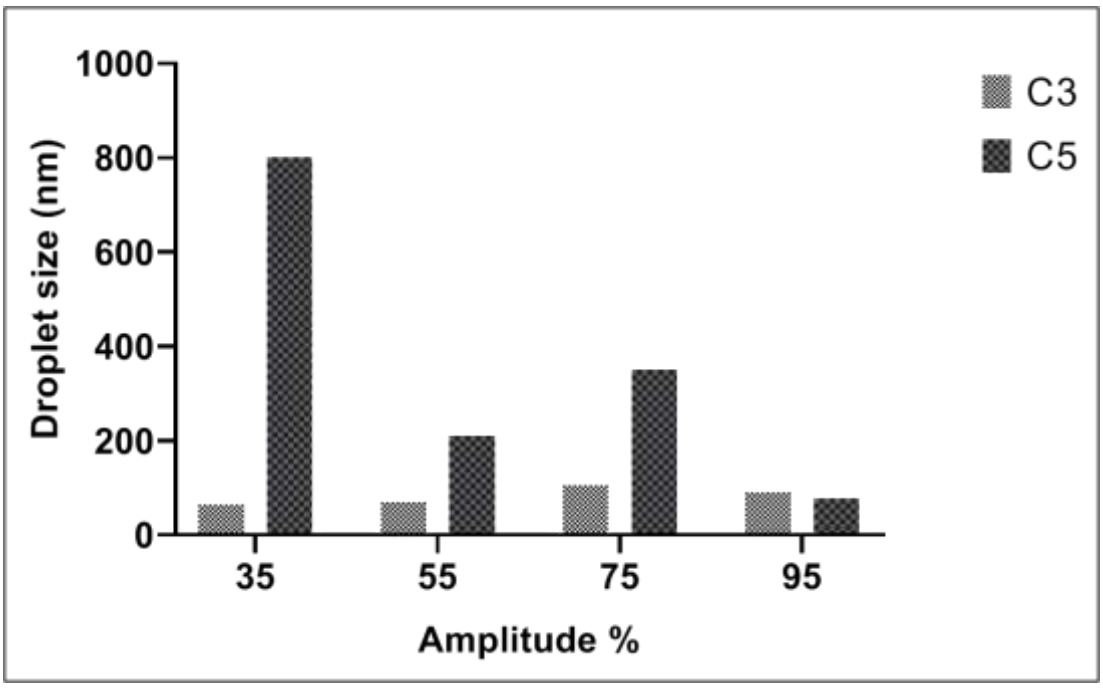


Figure 4. 3. Different sonication Amplitude percentage study for droplet size using Kolliphor PS 80 and Kolliphor PS 20

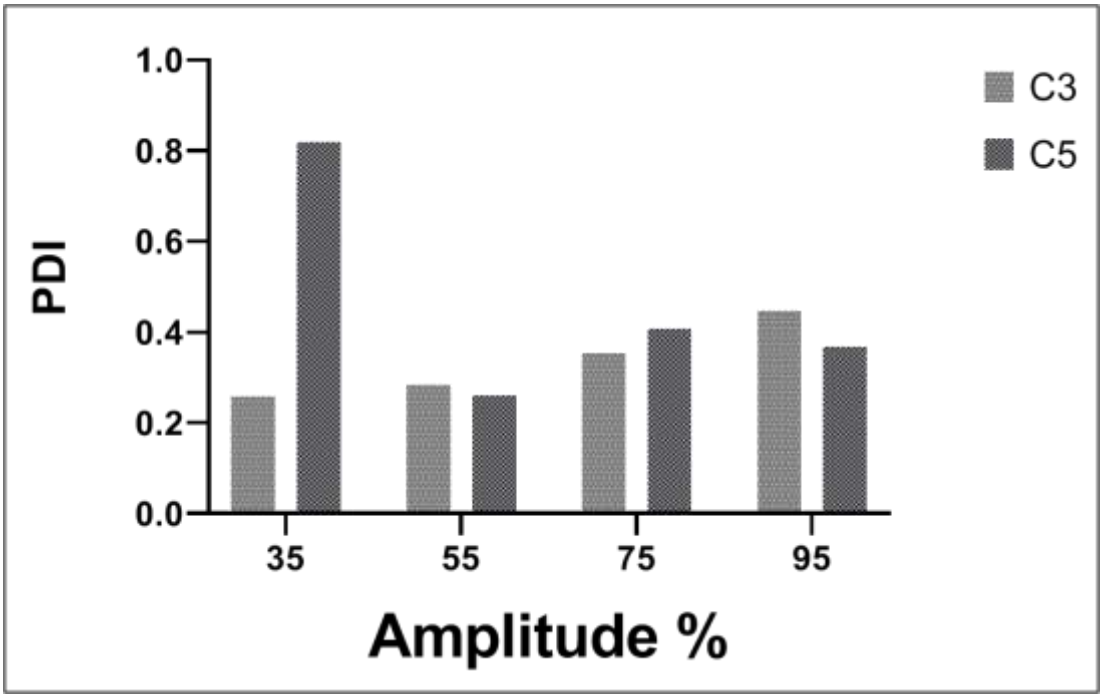


Figure 4. 4. Different Amplitude sonication study for PDI using Kolliphor PS 80 and Kolliphor PS 20 formulations

4.2.3. Nanoemulsion Preparation at different Sonication Time

The selected C3 and C5 formulations were subjected to study time parameter. In this we changed time of sonication keeping amplitude as fixed. Following were the findings of this study.

C3 after sonication at (A2) 55% amplitude was the appropriate formulation but blurry in appearance. therefore, time of sonication was applied differently to it as 24

minutes (T5) and at 55% amplitude.

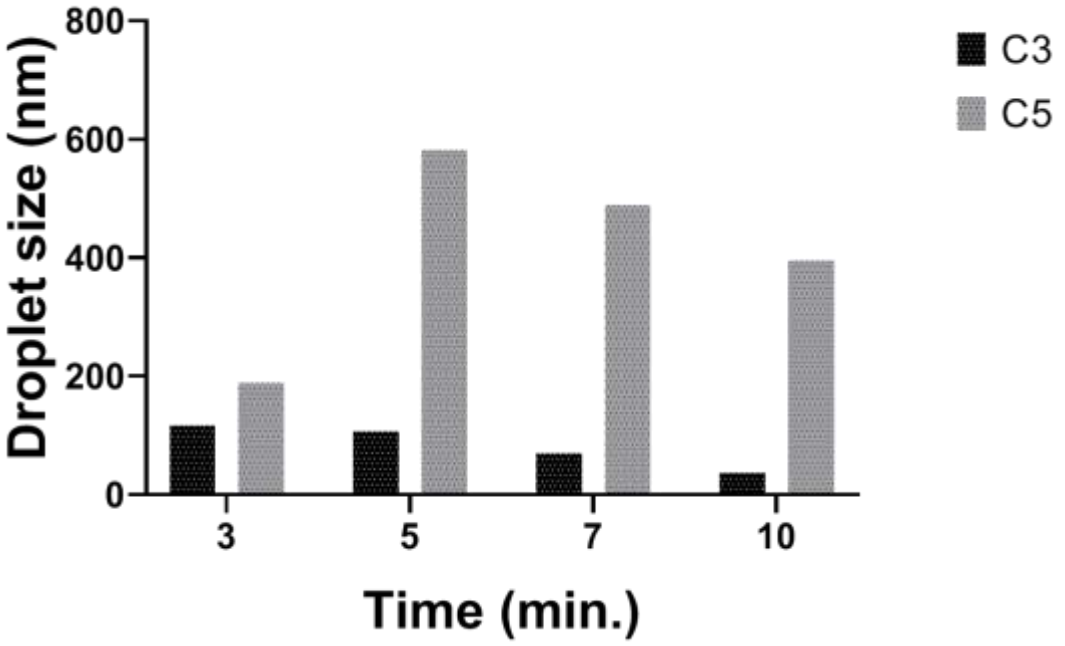


Figure 4. 5. Different time of sonication study for droplet size using Kolliphor PS80 and Kolliphor PS 20 formulations

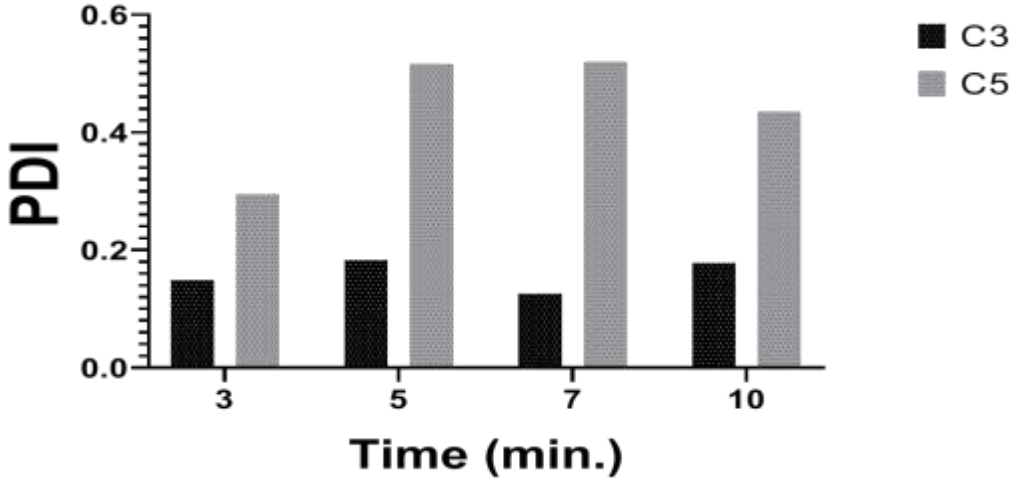


Figure 4. 6. Different time of sonication study for PDI using Kolliphor PS80 and Kolliphor PS 20 formulations

4.3. Characterization of Prepared Nanoemulsion

Characterization of prepared nanoemulsion was performed such as droplet size, PDI, Zeta potential, visual assessment, viscosity, storage and pH.

4.3.1. Droplet size, PDI and ZetaPotential

Prepared formulations were subjected to study their distinctive features such as droplet size, PDI and zeta potential using ZetaSizer. Findings for our formulation were as such. Droplet size and PDI or optimum formulation was 154.6 nm, PDI as 0.206 and zeta potential of was measured as -10.0 mV

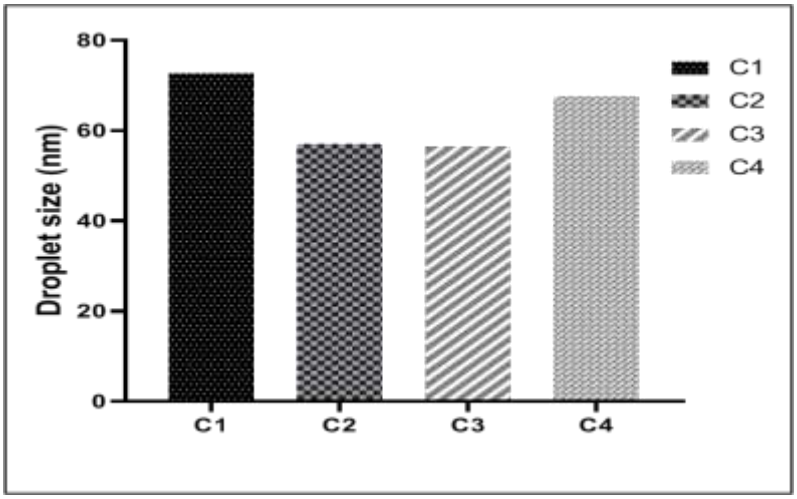


Figure 4. 7. Droplet size for formulations with Kolliphor PS80

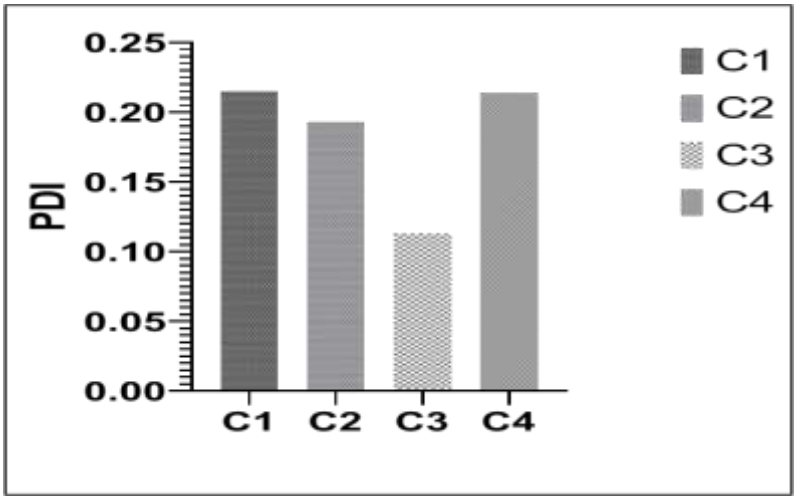


Figure 4. 8. Graphical representation for PDI of formulations with KolliphorPS80

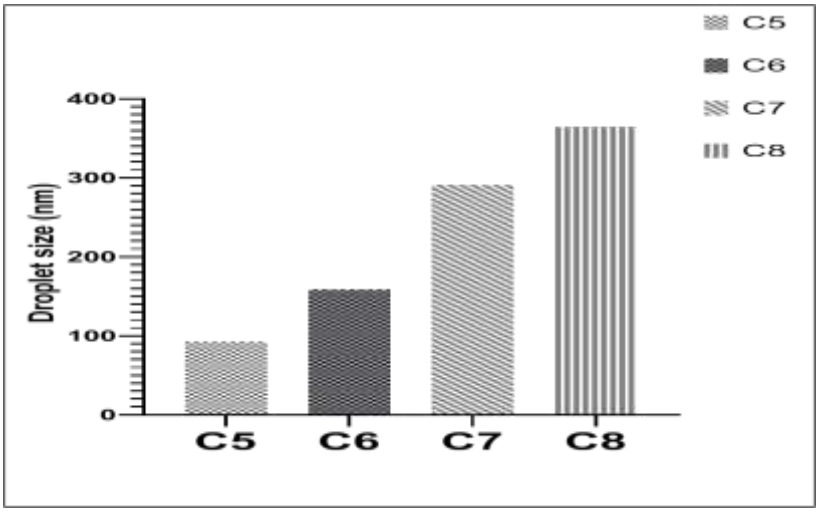


Figure 4. 9. Droplet size graph for formulations with Kolliphor PS 20

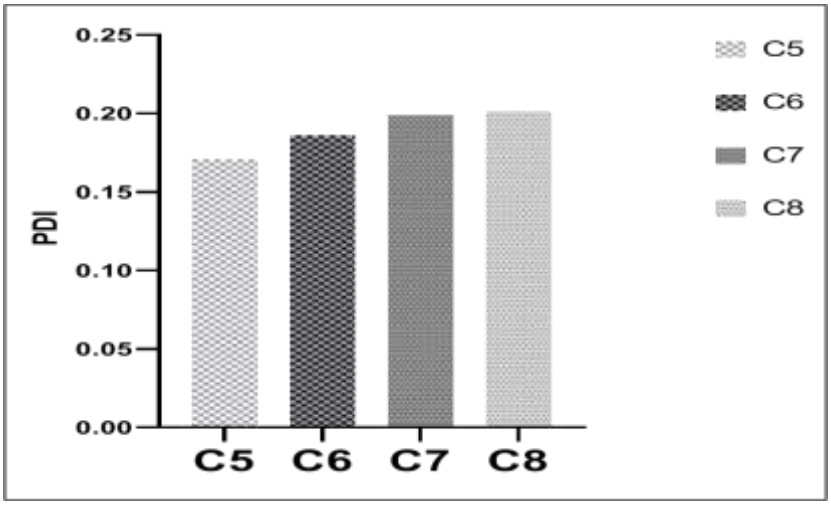


Figure 4. 10. Graphical representation for PDI of formulation with Kolliphor PS 20

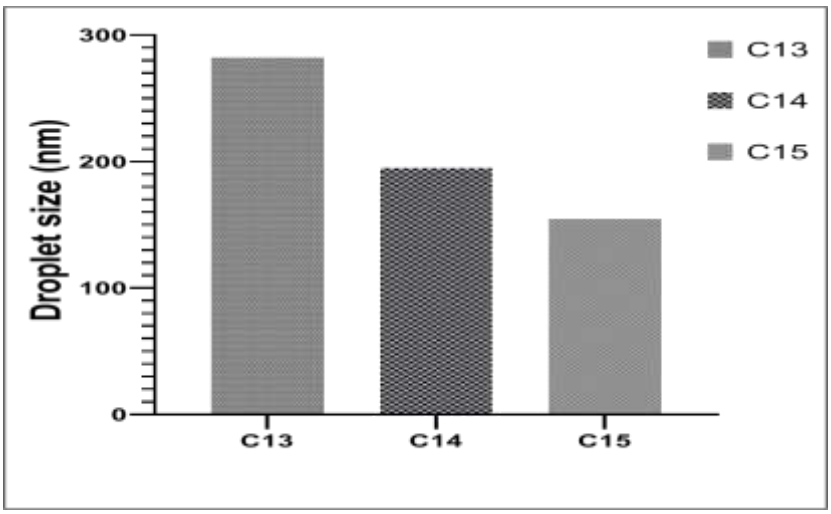


Figure 4. 11. Droplet size distribution for formulations made with Kolliphor RH 40

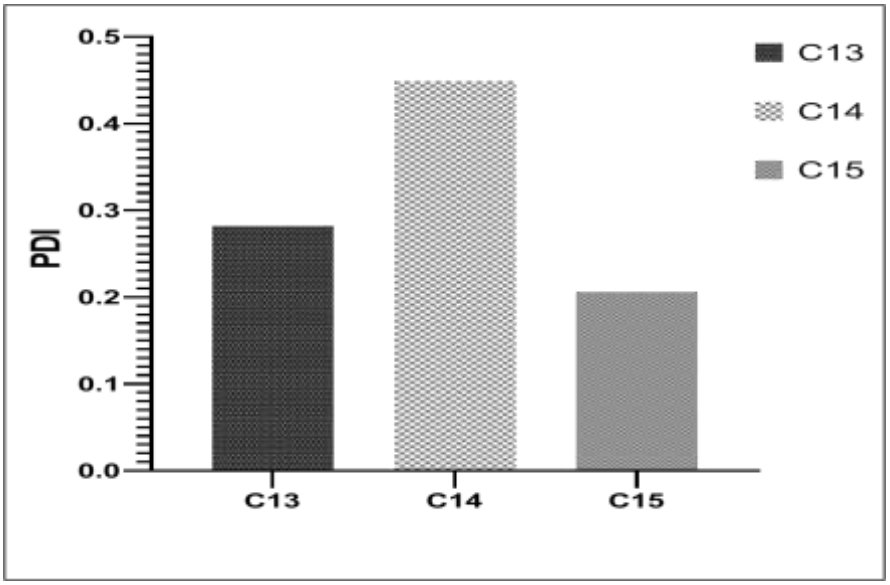


Figure 4. 12. PDI value for formulations prepared with Kolliphor RH 40

4.3.2. Visual Assessment

Visual appearance of formulation by naked eye. Following was the appearance of prepared formulations.

Figure 4. 13. Visual appearance of C3 at different time of sonication

Figure 4. 14. Visual appearance of C5 at different time of sonication

Figure 4. 15. Visual appearance of C9, C10, C11 and C12

Figure 4. 16. Visual appearance of C11, C12 and C13

4.3.3. Viscosity

Viscosity of optimum formulation was recorded as 0.8872 cP

4.3.4. pH

pH of optimum formulation C15A1T4P was measured as 5.57 using pH-meter.

Figure 4. 17. pH-meter

CHAPTER 5
DISCUSSION AND RESULTS

5.1. Assay for Essential Oil

Two essential oils cinnamon oil and clove oil were selected. Essential oils have been used to treat various antimicrobial infections ever since in history but cannot be used as treatment dose due to its hydrophobic nature and low absorption. To overcome this issue essential oils can be encapsulated in a carrier system to improve its absorption and make it available at site of action. Nanoemulsion formulation is best carrier system in this regard.

First step to start formulation study is to confirm the activity of selected essential oils against candida species. For this purpose analytical study was done using gas chromatography and complex mixture of EO were separated and identified along with their concentration. After this analysis results we use EOs to undergo antifungal activity test by using disc method and broth dilution method and results obtained from both oils were compared and chosed depending on their activities to use in our formulation.

Gas chromatography results in elution of constituents from complex mixture of terpenes and terpenoids of CEO and CO and their identification. As shown above chapter of findings, the components found in clove oil at various concentration but euogenol was found in abundance, similarly in cinnamon oil result shows many constituents in different concentration but maximum concentration of an aldehyde named cinnamaldehyde was found.

5.1.2. Activity of EOs against fungal strains

In order to check activity of cinnamon oil and clove oil against fungal species, these two tests were performed and results were compared to know the better activity of one oil over the other. Tests performed were disc diffusion and broth microdilution. Findings are mentioned in previous chapter.

5.1.2.1. Diameter zone of inhibition

Disc diffusion test result shows that both essential oils (CEO and clove oil) are active against candida species. Test was performed using Amphotericin B as standard and

after incubation period, the diameter of inhibition zone on petri dishes were measured and findings shown in previous chapter shows cinnamon oil as strong inhibitor against *Candida albicans*. Hence, cinnamon oil is strong inhibitor of fungal growth as compared to clove oil.

5.1.2.2. Minimum inhibitory concentration

Broth microdilution test was performed to find minimum inhibitory concentration of essential oils. Finding shows that both Cinnamon oil and clove oil yield low concentration for inhibiting three different fungal strains but in comparison cinnamon oil has lowest MIC value of 0.001µg/ml. MIC is the minimum concentration of essential oil use to inhibit the growth of fungi. Hence, cinnamon oil shows better result than clove oil so was used for formulating our nanoemulsion formulation against candidiasis.

5.2.1. O/W Nanoemulsion preparation with different surfactant ratios

Three surfactants on the basis of their HLB values were selected for preparing nanoemulsion. Kolliphor PS 80 (HLB-15), Kolliphor PS 20 (HLB-17) and Kolliphor RH 40 (HLB-13), four formulations were prepared using each surfactants at specified concentrations to cinnamon oil and purified water as aqueous phase with continuous sonication at specific speed and time. Depending on transparency results of sixteen formulation C3 and C5 were better formulations and were chosed to prepare with different parameter changes in order to optimize the formulation.

In total sixteen formulations were made using Kolliphor PS 20, Kolliphor PS 80 and Kolliphor RH 40. First 8 formulations C1 - C8 were prepared first a coarse emulsion was made using surfactants (Kolliphor PS 80 and Kolliphor PS 20) and water, homogenized with addition of oil and sonicated continuously. Prepared formulations failed to give transparency which was a challenge during preparation and was due to the surfactant oil ratio. Therefore two better formulations C3 and C5 were chosed to undergo parameter study in order to get better droplet size, PDI as well as transparent and clear in appearance.

5.2.2. Nanoemulsion preparation at different sonication amplitude percentage

Selected concentrations C3 and C5 were subjected to parameter study in which amplitude of sonication was varying 35%, 55%, 75% and 95% while time of sonication was kept constant as 3 minute. Study result shown in 4.2.2. C3 has good droplet size and PDI range at 35% A1 but couldn't give transparent appearance whereas C5 showed higher droplet size and PDI with turbid appearance. The graph shows that C3 has shown increase in droplet size with increase in amplitude percentage while PDI was also increased with increase in amplitude percentage. In case of C5 droplet size was fluctuating but stayed higher in range throughout except at 95% amplitude, droplet size was smallest in range with good PDI value but appeared turbid. This turbidity of formulation is due to oil to surfactant concentration.

5.2.3. Nanoemulsion preparation at different sonication time

Second parameter for optimizing our formulation was to change the sonication time while keeping the amplitude of sonication fixed at 35%, graph shows that by increasing the time of sonication C3 shows decrease in droplet size while the smallest droplet size was yielded by C3 at T4 with 36.58 nm and PDI 0.178. In case of C5 by increasing sonication time the droplet size was increased and lowest droplet size was observed at T1 with 188.8nm and PDI of 0.295. All results obtained from this time parameter study were not transparent visually only C3 at T4 showed little transparency as compared to others. Since T4 shows visual improvement we decided to increase the time of sonication with this formulation C3A2 at 24 minutes T5, yielded result shows good range of droplet size and PDI yet turbid appearance.

5.3. Characterization of Prepared NE

5.3.1. Droplet size, PDI and Zeta potential

Droplet size plays an important role for assessing nanoemulsion. Smaller the size of particles larger is the interfacial surface for permeation. PDI is the ratio of droplet size distribution in system. Polydispersity index indicates the homogeneity and stability of droplet size in emulsion. Surfactant concentration also plays role in size of droplet size, by increasing the amount of surfactant the diameter of droplets decreases (Aqil, Kamran, Ahad, & Imam, 2016; V. Ghosh et al., 2013). Droplet size

for the C3 formulation with surfactant 3% surfactant concentration yields droplet size 56.53nm while C5 at 1% surfactant concentration yields 92.42nm. We can see that increase in surfactant concentration in case of Kolliphor PS 80 reduced the droplet size but at certain point after C3 formulation, 4% of surfactant concentration at C4 reached CMC (critical micelle concentration) where surfactant has no effect on droplet size anymore but have negative effect. At C4 we see increase in droplet size. In formulation made with Kolliphor PS 20, we notice that fixed 1% surfactant concentration increased the droplet size in each formulation which shows that compatibility between our surfactant and other ingredients is not good enough to make colloidal dispersion.

Polydispersity index indicates the homogenized size distribution within the system. PDI closer to zero shows the best homogeneity within colloidal system. In our formulations we can see that PDI values were in range of zero showing homogeneity of system. Homogenized size distribution avoids the coalescence in the system hence making it stable.

Zeta potential shows the charge distribution within the system. Every particle in system has individual charge at surface. In order to have a uniform formulation there should be an even charge distribution at surface to avoid agglomeration and stabilizing the nanoemulsion. The zeta potential of our optimum formulation C15A1T4P was measured as -10.0 which proves the stability and uniform charge distribution in surface to avoid flocculation in nanoemulsion.

5.3.2. Clarity test for NE

Nanoemulsions have to be clear in appearance. Formulations we prepared were having difficulty in giving clarity to formulation. We observed first eight formulations with different surfactant to oil concentration yielding turbidity except C5 showed little clarity than turbidity so we increased the time of sonication from 10 to 24 minutes and yet not clear. We prepared four more formulations in order to obtain clear results but we changed sonication from continuous to periodic sonication. Periodic sonication yields better result in clarity. It shows that sonication has effect on transparency of nanoemulsion.

5.3.3. Viscosity

Increase in viscosity can effect the penetration of formulation through skin. Viscosity of formulation is affected by the sonication amplitude. Lower the viscosity better is the permeation. According to our optimum formulation result viscosity i.e. 0.8872 is in good range, so we can assure that our formulation have good permeation due to ideal viscosity range.

5.3.4. pH

pH of formulation has important role for compatibility of formulation with skin. Our formulation has pH value of 5.57 that is compatible to pH of our skin. Skin pH range (4.7 - 7.5).

CHAPTER 6

CONCLUSION

To conclude this study, cinnamon oil was tested for antifungal activity and findings shows better activity of cinnamon oil over fungal strains than clove oil. Therefore, we proceed our work with cinnamon essential oil as dual ingredient having active effect against fungi as well as oily phase excipient for our nanoemulsion formulation.

For preparing an optimum nanoemulsion formulation we incorporated cinnamon oil into emulsion system using water as an aqueous phase and surfactant to overcome interfacial tension and maintain stability of colloidal system.

Formulations with different ratios were prepared with Cinnamon oil as an oil phase and API, the surfactants used were Kolliphor® PS 20, Kolliphor® PS 80 and Kolliphor® RH 40. Purified water was used as an aqueous phase throughout the study. The most compatible NE formulation was with Kolliphor® RH 40 with periodic sonication gave an optimum result among other formulations.

This optimum formulation was then tested for its characterization such as droplet size, PDI and zeta potential for stability of our nanoemulsion system and was accepted depending on findings and result.

The final formulation as Cinnamon EO-based nanoemulsion for the treatment of candidiasis wasn't tested due to the pandemic situation of COVID-19, but according to the good results obtained from the whole study, we can ensure the effectiveness of our NE formulation.

Further study will be continued to get final dosage form for candidiasis treatment.

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CURRICULUM VITAE

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E-mail	Dr.asma_shahbaz@hotmail.com		

Educational Level

	Name of the Institution where he/she was graduated	**Graduation year**
Postgraduate/Specialization	-	-
Masters	Pharmaceutical Technology/Near East University	2020
Undergraduate	Pharm-D/University of Peshawar, Pakistan	2014
High school	Pakistan International School, Riyadh. Saudi Arabia	2009

Job Experience

Duty	**Institution**	**Duration (Year - Year)**
Production Pharmacist	Leads Pharmaceutical Industry, Islamabad. Pakistan	Dec 2014 - Oct 2016
Ambulatory care pharmacy (Volunteer work)	King Faisal Specialist hospital and Research Center, Riyadh. Saudi Arabia	Jan 2014-Feb 2014
Trainee pharmacist, I.V -dispensing for in-patients	King Saud Medical Complex, Riyadh. Saudi Arabia	Jan 2012 - March 2012

Foreign Languages	**Reading comprehension**	**Speaking***	**Writing***
English	Very good	Very good	Very good
Arabic	good	good	Good
Turkish	good	good	Good

Foreign Language Examination Grade								
YDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
-	-	6.5	-	-	-	-	-	-

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

Computer Knowledge

Program	**Use proficiency**
SPSS	Very good

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