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Phase behavior of polyphenols in olive leaves extract

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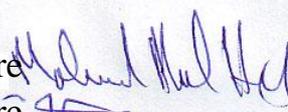
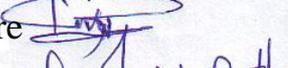
Thesis Approval

Phase behavior of polyphenols in olive leaves extract

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1436/2015

Dedication

I dedicate this thesis to the memory of my beloved father, it is your shining example that I try to emulate in all that I do. Maybe you are not here but you are alive in my heart and soul.

Throughout my life one person has always been there during those difficult times. I would like to dedicate this thesis and everything I do to my amazing mother, who has always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

To my big brother who has been there for me all the time, for his creative ideas and for all the love, care and kindness.

To my sisters, for their love and support throughout the years. Thank you for the laughing and the fighting, and everything in between!

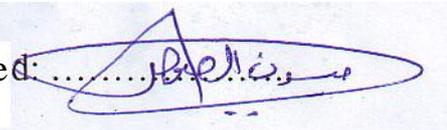
To my life partner, I would not reach today without your love and motivation, which has been a constant source of support and encouragement during the challenges of this journey.

Maisoon ALSous

Declaration:

I certify that this thesis submitted for the degree of Master is my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for higher degree to any other university or institution.

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Abstract

This study aims to investigate emulsification of a triglyceride oil, olive oil, and formulating an environmentally friendly biocompatible microemulsion that can be used in food, pharmaceuticals, cosmetics; using olive leaves extract and nonionic sorbitan ester and ethoxylated sorbitan ester surfactants that are known for their good cutaneous tolerance, low irritation and potential and toxicity. The sugar based surfactants in this study are polyoxyethylene sorbitan monooleate (Tween 80), polyoxyethylene sorbitan monolaurate (Tween 20) sorbitan monostearate (Span 60). The oil phases are olive oil as the main oil and isopropyl myristate (IPM). Propylene glycol was used as a cosurfactant. And olive leaves extract (OLE) was used as the water phase.

This study also aims to explore the role of phenolic compounds in OLE as a cosurfactant in enhancing the microemulsion formation.

At first, we studied the effect of using OLE that has been extracted using ethanol as a solvent on the phase behavior of olive oil/Span 60/OLE, olive oil/Tween 20/OLE, olive oil/Tween 80/OLE, olive oil/mixed surfactant/OLE and olive oil: Propylene glycol /Tween 80 /OLE.

Then we investigated in the effect of using 32% OLE that has been extracted using acidified water as a solvent on the phase behavior of olive oil/Span 60/OLE, olive oil/Tween 20/OLE and olive oil/Tween 80/OLE. After studying the previous phase behaviors, we have chosen the best sugar based surfactant with the most emulsification properties and high solubilization capacity in order to be used in the following phase behaviors.

Then we explored the effect of using 22% OLE that has been extracted using acidified water as a solvent on the phase behavior of olive oil:IPM/Tween 80/OLE and studying the effect of using IPM as a co-oil on the emulsification process. Also with the same OLE we studied its effect when propylene glycol was used as a cosurfactant on the phase behavior of olive oil:Propylene glycol/Tween 80 /OLE. Then we investigated the effect of using 34% OLE that has been extracted using acidified water as a solvent on the phase behavior of olive oil:IPM/Tween 80/OLE and IPM/Tween 80/OLE. Anisotropy was detected using visual inspection and cross polarizers. Total phenolic content was determined using UV-Visible spectrophotometer for 22% OLE and 34% OLE. The hydrodynamic diameter of microemulsion micelle also was determined using dynamic light scattering.

It was found that using ethanol as a solvent to extract phenolic compounds from the olive leaves didn't help in enhancing the microemulsion formation. OLE that was extracted using acidified water gave better results. Tween 80 has the most emulsification properties of olive oil between the three surfactants. Phenolic compounds helped in the formation of microemulsion and acted as a cosurfactant. Total phenolic content (as mg Gallic acid/g of dry olive leaf) for 43% OLE equals 23.49 mg/g, and 13.53 mg/g for 22% OLE. The average hydrodynamic diameter of microemulsion micelle of olive oil:IPM/Tween 80/OLE system equals 25.88 nm at 25°C and 12.78 nm for microemulsion micelle of IPM/Tween 80/OLE system.

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Abbreviations, Symbols and Terminology

HLB: Hydrophile–Lipophile balance.

CMC: Critical micelle concentration.

L1: Spherical normal micelles.

L2: Reversed micelle.

H1: Normal hexagonal phase.

H2: Reversed hexagonal phase.

L α : Lamellar phase.

I1: Normal micellar cubic liquid crystalline phase.

I2: Reversed micellar cubic liquid crystalline phase.

V1: Normal bicontinuous cubic phase.

V2: Reversed bicontinuous cubic phase.

O/W: Oil in water.

W/O: Water in oil.

CPP : Critical packing parameter.

Tween 80 : Polyoxyethylene sorbitan monooleate.

Tween 20 : Polyoxyethelene sorbitan monolaurate.

Span 60 : Sorbitan monostearate.

OO : Olive oil.

MQ water : Deionized water supplied by a Milli-Q water purification system.

PG : Propylene Glycol

ME : Microemulsion

nm : Nanometer.

Ppm : Part per million.

IFT : Interfacial tension.

OLE : Olive leaves extract

BHT : Butylated hydroxytoluene.

BHA : Butylated hydroxyanisole

IPM : Isopropyl Myristate.

DNA : Deoxyribonucleic acid.

LDL : Low-density lipoprotein.

HDL : High-density lipoprotein.

HPLC: High Pressure Liquid Chromatography.

SFE : Super Fluid Extraction.

LC-MS : Liquid Chromatography-Mass Spectrometry

pH : Negative log of the activity of the hydrogen ion in an aqueous solution.

AA : Antioxidant activities.

TPC : Total phenolic content.

TFC : Total flavonoid content of olive leaves.

UV-Vis : Ultra violet-Visible.

DLS : Dynamic light scattering.

dH: Hydrodynamic diameter.

EOs : Essential oils.

mg : Milligram.

g : Gram.

LC : Liquid crystal.

Chapter One

Introduction

1.1. Surfactants

Surface-active agents or simply surfactants are molecules that have a chemical structure, which makes it particularly favorable for them to reside at interfaces [Goodwin, 2004].

They are widely used in agriculture, pharmaceutical, biotechnology, nanotechnology, cosmetic, detergent, printing, recording, microelectronics, petroleum, mining and other industries. They exist in both natural and synthetic forms [Farn.2006].

Surfactants consist of two parts. One is soluble in a specific fluid (the lyophilic part) and the other is insoluble (the lyophobic part) [Holmberg, et al. 2002].

When the fluid is water then one is talking about hydrophilic and hydrophobic parts, respectively. The hydrophobic part is usually a hydrocarbon, and the polar hydrophilic head group may be anionic, cationic, nonionic or zwitterionic. Fig.(1.1).

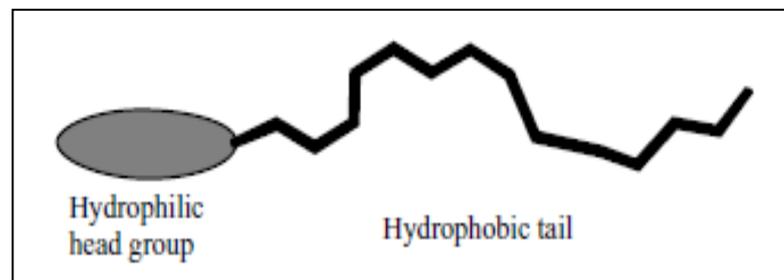


Fig.(1.1) Schematic illustration of surfactant molecule.

The surfactant molecules are adsorbed in the interface of two immiscible phases. They destroy the cohesive forces between polar and non-polar molecules and replace them at the interface. The molecular interaction at the interface occurs between the hydrophilic head of the surfactant and the polar phase molecules and between the hydrophobic tail of surfactant and the non-polar phase molecules. This phenomenon lowers the tension across the interface [Farn.2006]. The formation of this type of low interfacial energy surface is the basis of the stability of most oil and water emulsions and all microemulsions.

Surfactants are classified according to the nature of their headgroup. Anionic surfactant have negatively charged headgroups such as carboxylate (RCOO^-M^+), cationic surfactants have a positively charged headgroups like quaternary ammonium halides ($\text{R}_4\text{N}^+\text{X}^-$), while zwitterionic surfactants are both positively and negatively charged (usually dependent on pH) such as the sulfobetaines ($\text{RN}^+(\text{CH}_3)_2\text{CH}_2\text{CH}_2\text{SO}_3^-$). Nonionic surfactant headgroups carry no charge, but derives its water solubility from highly polar groups such as R-Polyol groups including sugars. [Kjellin, 2002].

When facing a problem of making an emulsion, hundreds of emulsifying agents are available. To help save time in emulsifier selection, HLB System "Hydrophile-Lipophile Balance" is used. It is a measure of the degree to which it is hydrophilic or lipophilic, and its determined by calculating values for the different regions of the surfactant molecule. [Tadros,2013].

1.2. Surfactant self –assembly

A fundamental property of surfactants is their ability to form aggregates when mixed with water. Common types of aggregates are micelles. They begin to form at a specific concentration called the critical micelle concentration, CMC, which is dependent on the surfactant structure. Below the cmc the surfactants are solubilized as monomers in the solution. Micelles begin to form at the cmc and all additional surfactant added above the cmc forms or goes into the micelles. Thus, this implies that the monomer concentration is constant at and above the cmc. The micelles consist of a rather limited number of surfactants, typically 50-150, forming a closed structure in order to minimize the contact between the surfactants hydrophobic part and the water. The mechanism behind this is called the hydrophobic effect. The surfactant tailgroups will constitute the liquid-like hydrophobic interior of the aggregates while the headgroups form an outer hydrophilic layer towards the water phase [Kjellin, 2002].

Acting surfactants at oil/water interface formed a large number of self-assembled structure depending on the type of surfactants [Borne, 2002]. Spherical normal micelles (L_1) are formed at high water content (oil/water), while reversed micelle (L_2) are formed at low water content (water/oil), between these two extremes different isotropic and anisotropic liquid crystalline phases with decreased water content or increased temperature or electrolyte concentration may be formed. The following sequence of anisotropic liquid crystalline phase may take place for the surfactant systems. Normal hexagonal phase (H_1) \rightarrow lamellar phase ($L\alpha$) \rightarrow reversed hexagonal phase (H_2). Isotropic discrete reversed micellar cubic liquid crystalline phase (I_2) and normal (I_1) can appear between the micellar and the hexagonal phases of both the reversed and normal types.

The variation and complexity of these structures has led to intensive research on potential industrial applications. However, microemulsion is the most important structures in which single low viscous isotropic phase formed in L_1 and L_2 regions of the phase diagram.

The shape of the micelles is dependent on the structure of the surfactant, typically the relative size of the head-group and tail-group. This is often described with the critical packing parameter, CPP, defined as:

$$CPP = v/al$$

Where a is the optimal head-group area and v and l is the volume and length of the surfactant hydrophobe, respectively. Spherical micelles will be formed if $CPP < 1/3$. As CPP increases, meaning that the relative size of the hydrophobic part increases, the curvature of the aggregates will decrease and disc-, tablet-, and rod like micelles are formed. As the concentration of surfactant increases, the micelles often grow more or less in size and at even higher concentrations various types of phases can be formed Fig. (1.2). For example:

hexagonal phase.....($1/3 < CPP < 1/2$)

lamellar phase.....($CPP \approx 1$)

cubic phase.....($CPP \geq 1$).

The different phases can have very different physico-chemical properties, e.g. viscosity and the ability to scatter light [Kjellin, 2002].

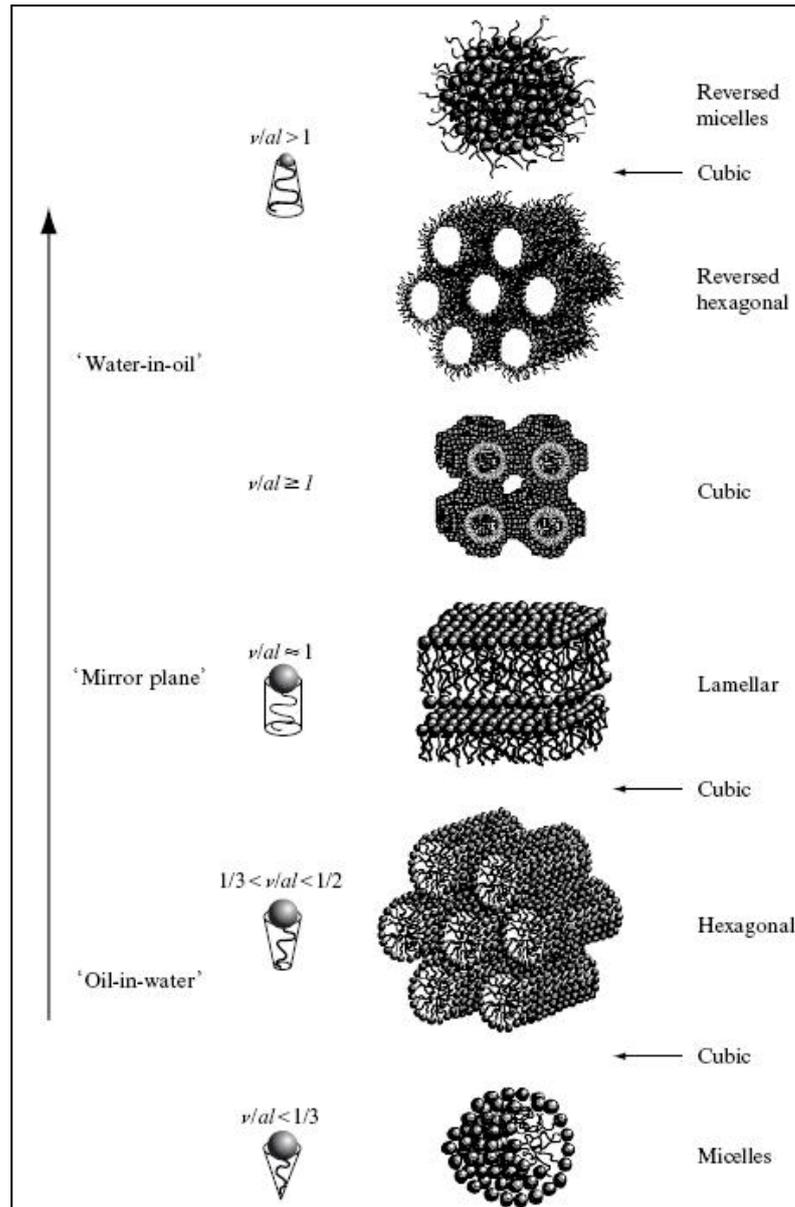


Fig.(1.2) Dependence of aggregate morphology on the packing parameter [Holmberg, et al. 2002].

1.3. Sugar based surfactants

Sugar-based surfactants are the final result of a product concept, which is based on the greatest possible use of renewable resources. While the derivatization of fats and oils to produce a variety of different surfactants for a broad range of application has a long tradition and is well established; the production of surfactants based on fats, oils, and carbohydrates on a bigger industrial scale is relatively new.

Sugar-based surfactants such as sorbitan esters, sucrose esters, alkyl poly glycosides, and fatty acid glucamides are compounds of a very broad range of structural diversities. Sugars (monosaccharides, disaccharides etc.) may be connected to the hydrophobe at any of its hydroxyl groups and by different types of connecting linkages. The hydrophilicity of sugar can be changed by oxidation, reduction or addition of hydrophilic groups, such as a sulfonic acid residue or a polyoxyethylene chain. Thirdly, the hydrophobic moiety or moieties can also be subjected to a wide array of changes [Piispanen, 2002].

This type of surfactant is being produced on an industrial scale and both the hydrophobic and hydrophilic moieties of the surfactant stem from cheap renewable raw materials, and consequently are biodegradable and nontoxic.

These properties make them interesting as substitutes of other surfactants, which are potentially damaging to the environment.

In addition, they have found application in personal care products since they exhibit favorable dermatological properties, as liquid dishwashing agents, hard surface cleaners, agrochemicals, and for industrial and office cleaning.

The cosurfactants can lead to destruction of a microemulsion upon dilution due to partitioning of them out of the interfacial region into the continuous phase. For these reasons, several nonionic surfactants have been studied for their emulsifying ability as they do not require necessarily a co-surfactant for microemulsion formation [Polychniatou, et al. 2011].

1.4. Sorbitan Esters

Sorbitan esters have been known for a couple of decades when the first industrial chemical processes were established for their manufacturing. They can be produced by a two-step

Process showed in Fig.(1.3). Which includes dehydration of sorbitol in the presence of acid (e.g., NaH_2PO_3) to form 1,4-sorbitan as main isomer, which is subsequently esterified with fatty acids in a second reaction step with an alkaline catalyst (e.g., K_2CO_3) at 200°C – 250°C [Ruiz, 2009].

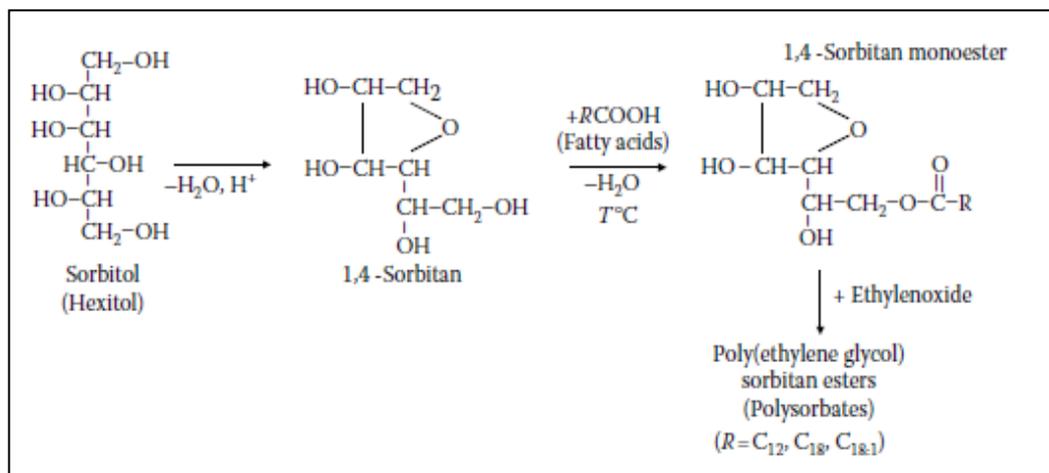


Fig.(1.3) Synthesis of sorbitan esters by intramolecular dehydration of sorbitol and subsequent base-catalyzed esterification with fatty acids [Ruiz, 2009].

Depending on the type and amount of fatty acid used, different product compositions, consisting of mixtures of mono-, di-, or trisorbitan esters (e.g., laurates, oleates, or stearates) are produced with hydrophilic/lipophilic balance (HLB) values in a typical range of 1–8. To modify these relatively hydrophobic materials, a common technology is to further derivatize

the sorbitan esters by reaction with ethylene oxide to produce sorbitan ester ethoxylates—or polysorbates—with HLB values of 10–17, depending on the number of ethylene oxide units attached, showed in Fig.(1.4) .

Sorbitan esters and the ethoxylated products are mainly used as emulsifiers in pharmaceuticals, foods, cosmetic products, pesticides, for emulsion polymerization and explosives and other technical applications.

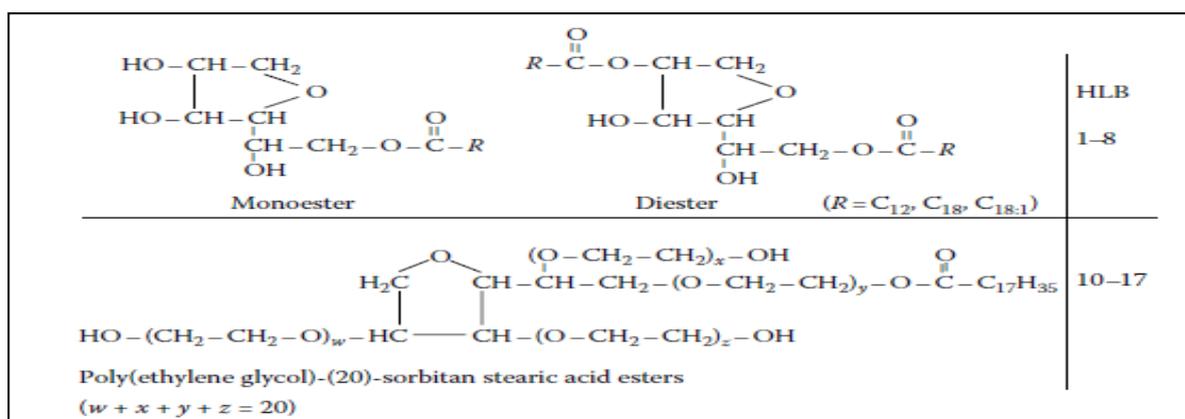


Fig.(1.4) Hydrophilicity of sorbitan esters [Ruiz, 2009].

1.4.1. Tween 80 (Polyoxyethylene sorbitan monooleate)

Polysorbate 80 (commercially also known as Tween 80) is a nonionic surfactant and emulsifier with HLB=15 derived from polyethoxylated sorbitan and oleic acid. It is a viscous, yellow liquid. It is hydrophilic in nature because the hydroxyl groups on the sorbitan ring are replaced and substituted with bulky polyoxyethylene groups. This substitution makes Tween 80 more soluble in water, so it tends to form oil-in-water emulsions. Such surfactants are usually ecofriendly and biodegradable and hence can be used in several cosmetics, dish washings, pharmaceuticals and food industries. For example, it can be used for various purposes namely; tween-80 was used as sensitizer to determine trace of As(V) in human hair and tea samples. Tween 80 is used in the formulation of biotherapeutic products for both

preventing surface adsorption and as stabilizers against protein aggregation Fig.(1.5) [Jadhav, *et al.* 2012].

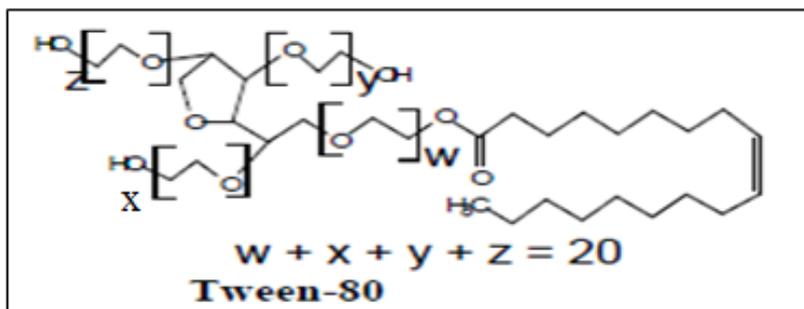


Fig.(1.5) Chemical structure of Tween 80 [Jadhav, *et al.* 2012].

Ternary phase diagram of Tween 80, olive oil and water with HLB value of 15 was studied. A large area of transparent/clear solution was formed in the oil rich regions. The microemulsion area were found to be attached to the borders of the diagrams where water and oil ratio was low and the surfactant ratio was high, as showed in Fig.(1.6) [Syed, *et al.* 2014].

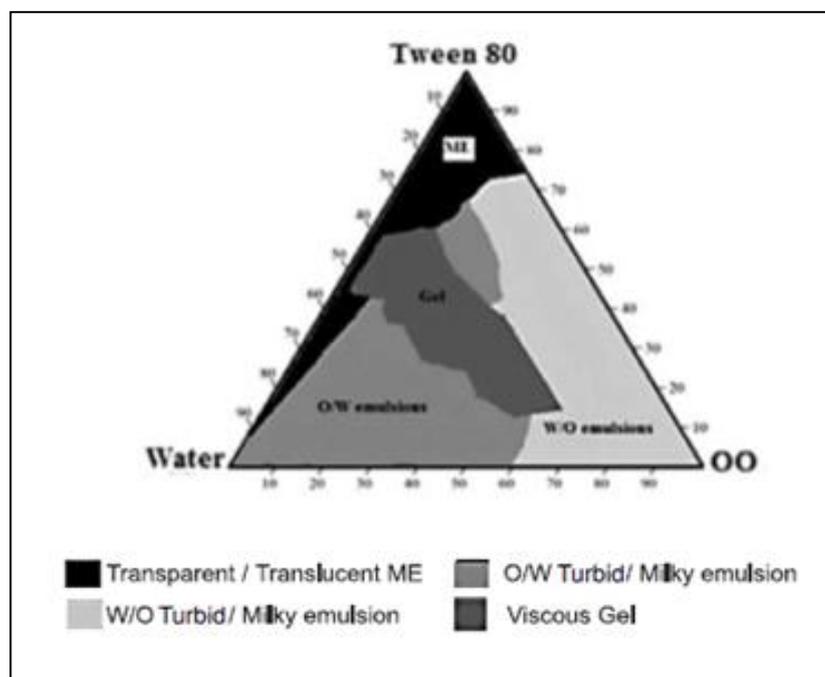


Fig.(1.6) Ternary phase diagrams of olive oil (OO) with Tween 80 and water [Syed, *et al.* 2014].

1.4.2. Tween 20 (Polyoxyethelene sorbitan monolaurate)

Tween 20 is a nonionic detergent with HLB 16.7 widely used in biochemical applications. It has been used as an emulsifying agent for the preparation of stable oil-in-water emulsions Fig.(1.7).

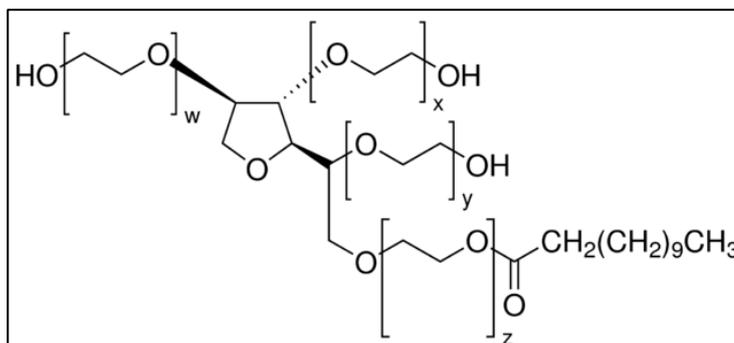


Fig.(1.7) Chemical structure of Tween 20 where $x+y+z+w=20$

<http://www.sigmaaldrich.com/catalog/product/sigma/p9416?lang=en®ion=IL>

1.4.3. Span 60 (Sorbitan monostearate)

Span 60 is an ester of sorbitan (a sorbitol derivative) and stearic acid and is sometimes referred to as a synthetic wax with HLB 4.7. It is a non-ionic surfactant with emulsifying, dispersing, and wetting properties. It is primarily used as an emulsifier to keep water and oils mixed. sorbitan monostearate is used in the manufacture of food and healthcare products Fig.(1.8).

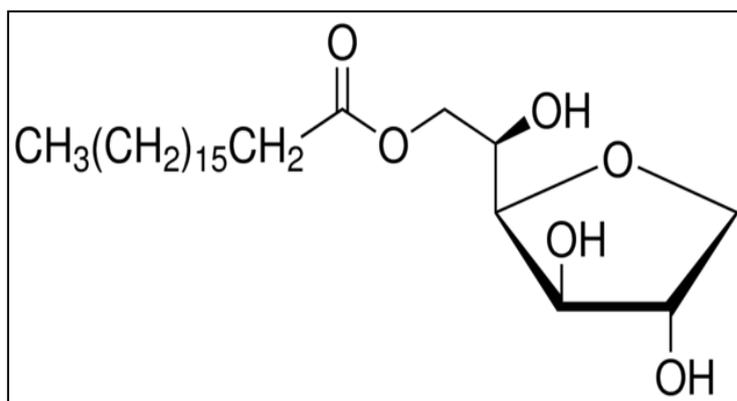


Fig.(1.8) Chemical structure of Span 60

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1.5. Propylene glycol

Propylene glycol (PG) that is of HLB=4.45 and short-chain alcohols are known to act as cosurfactants. Hence, it is presumed that a considerable part of PG is incorporated into the surfactant layer and will increase the interfacial fluidity, and the other part of PG will decrease the polarity of the water because PG is mainly soluble in water. PG is one of the least hydrophilic simple polyols, that is soluble in water but practically non-soluble in the oil phase. In comparison with other alcohols, PG is relatively tolerable by the skin Fig.(1.9).

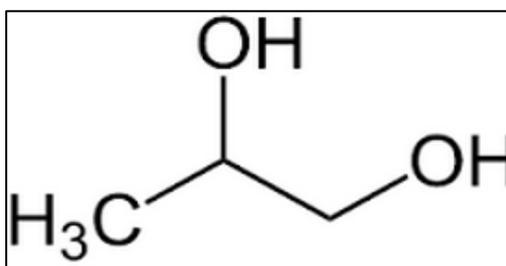


Fig.(1.9) Chemical structure of Propylene glycol .

Fanun investigated the pseudo ternary phase diagram of the water + propylene glycol/ sucrose laurate/peppermint oil + ethanol systems at 25°C [Fanun, 2009]. The mixing ratios (w/w) of water/propylene glycol and that of ethanol/oil equal 1 and 2, respectively. It was found that the presence of Propylene glycol influences the extent of the microemulsion regions and their internal structure. The roles of propylene glycol in microemulsions is to delay the occurrence of liquid crystalline phases, to increase the fluidity of the interfacial layer separating oil and water, to decrease the interfacial tension between the microemulsion phase and excess oil and water, and to increase the disorder in these interfacial layers as well as their dynamic character.

Ternary phase diagram of Tween 80:PG(Surfactant mix 1: 1), Olive oil and water with HLB value of 9.72 was studied. Attempts to obtain microemulsion using olive oil was not successful Fig.(1.10) [Syed, et al. 2014].

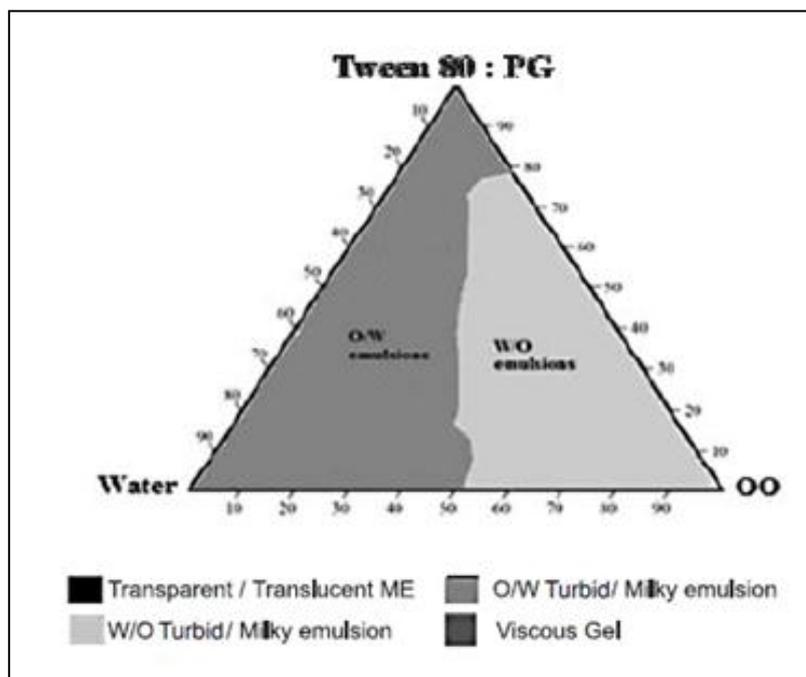


Fig.(1.10) Ternary phase diagrams of olive oil (OO) with Tween 80 : Propylene glycol(PG) (Surfactant mix 1 : 1), and water.

1.6. Microemulsion

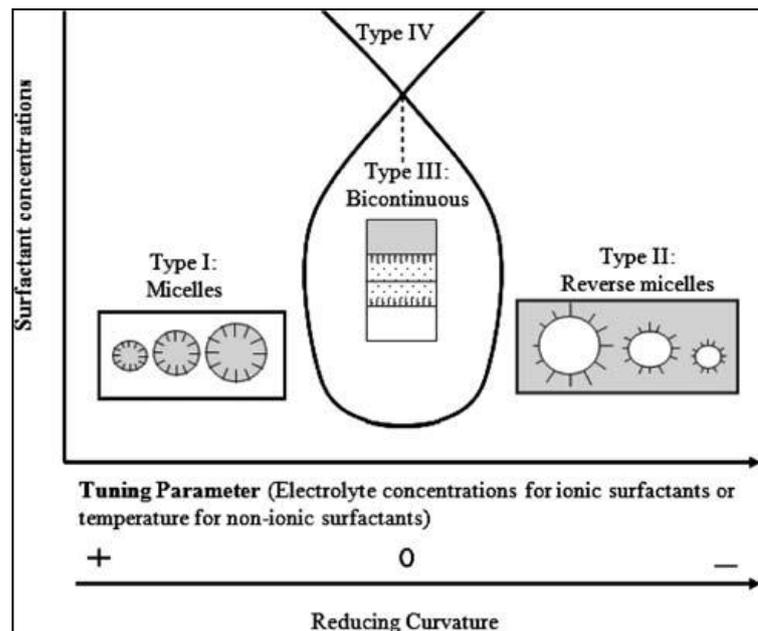
In 1959, Schulman *et al.* visualized the existence of small emulsion-like structures by electron microscopy and subsequently coined the term “microemulsions”. Microemulsions are macroscopically isotropic mixtures of at least a hydrophilic, a hydrophobic and an amphiphilic component. Their thermodynamic stability and their nanostructure are two important characteristics that distinguish them from ordinary emulsions which are thermodynamically unstable [Cosima, 2009].

The interfacial tension of microemulsion has to be ‘ultra-low’, with typical values being in the range from 10^{-4} up to 10^{-2} mNm^{-1} , while the particle size range from 5–50 nm [Goodwin, 2004].

In the current research sugar based nonionic surfactants are used. They are considered to be temperature dependent. At low to moderate surfactant concentrations, three microemulsion types can be produced. As denoted by Winsor, Winsor Type I microemulsions are normal

micelles in equilibrium with the excess oil phase, Winsor Type II microemulsions are reverse micelles in equilibrium with the excess water phase, and Winsor Type III microemulsions are a bicontinuous phase containing oil, water and surfactant in equilibrium with the excess water and excess oil phases. The microemulsion Type I–III–II transition can be achieved by increasing the temperature for nonionic surfactants (Fig.1.11). Increasing temperature causes the surfactant solution to become more hydrophobic and thus segregate more towards the oil–water interface, thereby reducing the surfactant film curvature and interfacial tension. At net zero curvature, a Winsor Type III system is formed.

A Winsor Type IV microemulsion occurs when the surfactant concentration is increased in a Type III system, thereby increasing the volume of the middle phase until it becomes a single phase.



Fig(1.11) Microemulsion phase behavior presented as a “fish” diagram showing change in curvature with surfactant concentrations and tuning parameters [Sabatini, et al. 2008].

Microemulsions are prepared by the spontaneous emulsification method (phase titration method) and can be depicted with the help of phase diagrams. Construction of phase diagram is a useful approach to study the complex series of interactions that can occur when different

components are mixed. Microemulsions are formed along with various association structures (including emulsion, micelles, lamellar, hexagonal, cubic, and various gels and oily dispersion) depending on the chemical composition and concentration of each component [Talegaonkar, et al. 2008].

Microemulsions are usually characterized by ternary phase diagram, which three edges are the components of a microemulsion; namely oil, water and surfactant. Any cosurfactant used are usually grouped together with the surfactant at a fixed ratio and treated as a pseudo-component [Kai, 2010]. Pseudo ternary phase diagram is often constructed to find the different zones including microemulsion zone, in which each corner of the diagram represents 100% of the particular component Fig.(1.12). The region can be separated into w/o or o/w microemulsion by simply considering the composition that is whether is oil rich or water rich [Talegaonkar, et al. 2008].

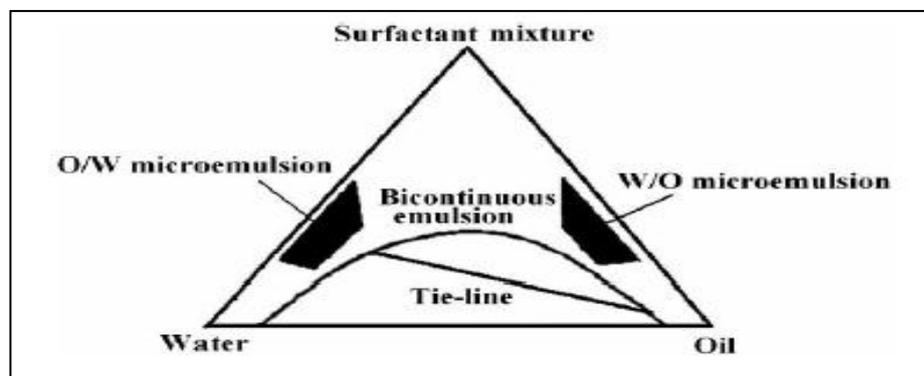


Fig.(1.12) Pseudoternary phase diagram of oil, water and surfactant showing microemulsion region [Talegaonkar, et al. 2008].

The primary aim of microemulsion research is to find the conditions under which the surfactant solubilizes the maximum amounts of water and oil. When starting from a pure surfactant system, it is generally easy to solubilise oil or water or both in the microemulsion structure. However, very often large amounts of surfactants (up to 50%) are needed to

solubilize equal amounts of oil and water. Thus, the challenge is to attain a single phase with a surfactant content as low as possible [Cosima, 2009].

Also, it is important to find the chemical type of surfactant which best matches that of the oil, because the chain length compatibility of a surfactant and oil is critical for the formation of emulsion systems. Surfactant type plays a major role in determining the rheological properties and droplets size of the systems. Choice of surfactant is crucial to obtain the desired formulation. Each oil and surfactant has a specific HLB value. The HLB of the selected surfactant or surfactants and cosurfactant that matches the HLB of the selected oil provides the lowest interface tension between the oil and water phases. The HLB of the selected surfactant(s) reflects the stability of the system can be obtained when the HLBs of the surfactant and oil are similar [Syed, *et al.* 2014].

Temperature, salinity, and type of alcohol are the common variables used to manipulate phase behavior and microemulsion structure, because these parameters modify the interactions between components with the polar head of the surfactant. Pressure provides a completely different mechanism of control of the microemulsion, due to the main effect being created on the hydrophobic tail; however, the effect of pressure is weak compared with that of temperature, so it is usually kept constant [Prieto, *et al.* 2008].

Characterization of microemulsions is very important especially for industrial processes. This includes both macroscopic measurements and microenvironment methods. Macroscopic measurements involving viscosity, conductivity, as well as dielectric measurements, cross polarizers for birefringence and polarizing microscope. On the other hand, microenvironment studies can involve pulsed field NMR, scattering methods such as light scattering, neutron scattering and X-ray scattering.

Visual evaluation helps to differentiate between microemulsions and other two phase systems such as emulsions. Microemulsions appear to be translucent or transparent, whereas emulsions are turbid. They are isotropic and nonbirefringent in nature whereas lamellar liquid crystals are anisotropic and birefringent in nature. Cross polarizers and polarizing light microscopy can be used to distinguish between microemulsion and different types of surfactant association structure that appear during the construction of phase diagram. Particle size analysis can be performed using Dynamic Light Scattering (DLS) [Parekh, 2011].

Anisotropy for systems containing sodium bis (2- ethylhexyl sulfosuccinate) (AOT), with the cationic hydrotrope tetraethyl ammonium chloride (TEAC), in the presence of water and heptane was detected using cross polarizers and polarized microscopy [Kanan, *et al.* 2012].

1.7. Alcohol conventional effects

There are two effects of using alcohol as an additive. First, it contributes to the general formulation as a cosurfactant (slightly hydrophilic contribution for methanol and ethanol; lipophilic contribution for *n*-butanol and longer linear alcohols) and second, as a co-solvent. The alcohol co-adsorbs with the surfactant at the interface and thus changes the overall interaction of the amphiphilic film with the adjacent solvents. It is worth noting that the lipophilicity of the cosurfactant increases with the carbon chain length (*n*-butanol < *n*-pentanol < *n*-hexanol < *n*-heptanol). The longer the alcohol, the lower its tendency to act as cosurfactant, because it is rather solubilised in the oil phase. Consequently, the cosurfactant effect may be said to fade away, and to vanish for octanol or longer alcohols, depending on the nature of the oil phase. As the alcohol mostly partitions into the water or oil phase it behaves either as a co-solvent or a linker. When such alcohol co-solvents are present in small proportion, they might not mix uniformly in the bulk of the oil or water phase and they could exhibit a third effect so-called lipophilic linker Fig.(1.13) [Cosima, 2009].

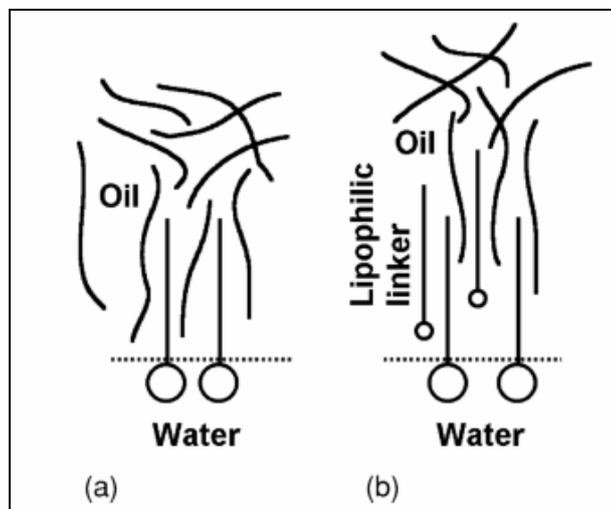


Fig.(1.13) Solubilisation improvement of a conventional surfactant (a) by a lipophilic linker (b) [Cosima, 2009].

Cristina Prieto and Lourdes Calvo investigated the phase behaviour and the structure of the *n*-hexane/water emulsions based on a nonionic, nontoxic and biocompatible surfactant, Tween 80. 1-butanol and ethanol were used as cosurfactants of Tween 80 in the ratio of 1 : 1 v/v, a better solubilisation between phases and a decrease of the mixture viscosity were reached, achieving a higher stabilisation of the system. The emulsion region diminished, increasing the microemulsion one, and the gel-like region disappeared.

It is believed that the alcohol joins the interface of the micelle, placing itself among surfactant heads, creating an increase of the dielectric constant and the ionisation degree. The penetration of the alcohol into the interfacial film reduces the repulsion of the long hydrophobic tails of the surfactant at the interface, favouring its dissolution in the oil phase. Consequently, an increase in the length of the cosurfactant hydrocarbon chain increases the superficial activity (Traube's rule) so that the micelle reaches a greater degree of stabilisation.

At the molecular level, this effect could be explained as follows: the chain length of the surfactant must be equal to the sum of the cosurfactant chain length and the oil chain length in order to minimise disruption in the interfacial region. When chain lengths are not the same, the resulting monolayer film is disrupted easily.

The presence of the alcohol also affects the physical properties of the water. The alcohol disrupts the water structure, creating an increase in the lipophilic character of the Tween 80. Furthermore, the alcohol provokes a decrease in the mixture viscosity; therefore, the big molecules such as Tween 80 can reach the interface faster. However, the presence of the alcohol in the mixture also promotes a decrease in the cloud point of the surfactant [Prieto, et al. 2008].

The high surfactant concentration required to formulate a microemulsion, usually 20% or more, remains a major concern for the user. In addition, much of the work on microemulsions has employed alcohols as cosurfactants and as co-solvent in order to decrease surfactant film rigidity, thus promoting microemulsion formation and delay the occurrence of liquid crystalline phases. Alcohol can also reduce the time needed for equilibration to be reached in multi-phase systems [Kayali, *et al.*2012].

1.8. Application of microemulsion

The outstanding properties of microemulsions such as thermodynamic stability, high solubilizing capacity and ultralow interfacial tension (IFT) make them desirable for applications in many fields [Kanan, *et al.* 2012]. Microemulsions used in enhanced oil recovery, fuel, coatings and textile finishing, lubricants, cutting oils and corrosion inhibitors, detergency, cosmetics, agrochemicals, food ,pharmaceuticals, environmental remediation and detoxification, analytical applications ,Microporous media synthesis (microemulsion gel technique) ,liquid membranes and in biotechnology [Moulik, *et al.*2006].

1.9. Formulation of microemulsion

Although oil and water are not miscible at ambient temperature, a small amount of surfactant is able to co-solubilise them. Formulation is important because the properties of surfactant–oil

water systems in general and the formation of microemulsions in particular, are very sensitive to it and slight deviations from a ‘proper’ formulation may result in drastic changes of the properties. Consequently, formulation has to be controlled accurately, which is quite challenging because of the high number of degrees of freedom in any practical case. This is why formulation is sometimes considered as ‘magic business’. Formulation essentially relates to the content of the systems and generally not to the way it is attained if thermodynamically stable systems are considered. The simplest microemulsion system would contain an organic oil phase, an aqueous phase generally referred to as water, and a surfactant at a given temperature and pressure [Cosima, 2009]. Investigations the phase behavior of OLE with olive oil and a biocompatible surfactant introduce the use of OLE in different formulations.

1.10. Olive oil/Olive leave extract/Nonionic surfactant microemulsion

Many plants, herbs and processed foods are suitable candidates for supplying functional molecules to food and pharmaceutical industry: natural products like olive oil and olive leaves are a good source of polyphenols and other antioxidants as well as by-products. The general demand of food, pharmaceutical industry and customers to replace synthetic additives in foods and drugs with potent plant extract having antioxidant and antimicrobial properties is becoming an urgent need.

The health promoting properties of olive leaves extract that is rich in polyphenolic antioxidant compounds, as well as its potential application as natural food additives have led to a great scientific and commercial interest. As mentioned earlier, there is a consumer demand for food products free of artificial food additives, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), since these chemically synthesized preservatives have been reported for carcinogenesis. Consequently, a lot of effort has been expended on the extraction, isolation and separation of those natural secondary metabolites. For this purpose, several

extraction techniques (liquid-solid phase, liquid-liquid phase, supercritical fluid, accelerated pressurized, ultrasound and microwave-assisted extraction, soxhlet) have been developed [Stamatopoulos ,*et al.* 2014].

the preparation of olive leaf extract microemulsion that is rich in polyphenols can be widely applied to the fields of medicines and food. For example, the olive leaf extract microemulsion can be applied to edible fat and oil. And it is used for the prevention of fats and oils rotting.

The Mediterranean diet, which is characterized by a high consumption of fruit and vegetables and by the use of extra virgin olive oil as the main fat dressing, is considered healthy for the high content of natural antioxidants. Polyphenol compounds have demonstrated antioxidant properties due to their redox potential, so they can act as hydrogen donors, reducing agents, nascent oxygen quenchers and chelators of metal ions in numerous food applications. So, their high potential as food preservatives can be exploited for the inhibition of lipid oxidation. Also, by consuming polyphenols through foods or pharmaceutical drugs positive effects on body health are expected: the anticarcinogenic, antiviral, immune-stimulating and anti-inflammatory [Mosca, et al. 2013].

Microemulsion formation of triglyceride oils like olive oil at ambient conditions (temperature and pressure) and without the addition of co-oil and/or alcohols is challenging at best. Undesirable phases, such as macroemulsions, liquid crystals and sponge phases, are often encountered when formulating triglyceride microemulsions. Vegetable oils like olive oil are considerably more difficult to solubilize in microemulsions. Many attempts have been made to form vegetable oil microemulsions at ambient conditions and without the addition of co-oil or alcohols, but without success. Co-oils such as isopropyl myristate can easily form microemulsions with conventional surfactants; therefore, it is often mixed with triglyceride oils like olive oil to enhance microemulsion formation. The reason that olive oil microemulsion is elusive appears to be the unique structure of triglyceride molecules.

Triglycerides are esters of fatty acid with glycerol, contributing to their complicated behavior. Their long and bulky alkyl chains make triglycerides highly hydrophobic, while the ester region in the molecule causes high polarity; in combination, these lead to poor solubilization by the surfactant. Therefore, conventional surfactants are not able to produce low interfacial tension (0.1 mN/m) with vegetable oils at ambient conditions without alcohol or co-oil addition [Sabatini, et al. 2008].

Also, Syed, et al has also reported that olive oil contains predominantly long-chain triglycerides of oleic acid. Thus, the molecular weight of olive oil is most probably too high to assist in the formation of microemulsion [Syed, et al. 2014].

Oil/lipid based formulations have been developed in the past by using phase diagrams. The use of phase diagram for developing oil based drug delivery systems, their identification and characterization is very important.

Numerous oils are considered as satisfactory food grade materials or also being used in the pharmaceutical industry. In this research olive oil was used for its marvelous health benefits.

Short and medium chain alcohols like polyols can function as cosurfactants that can reduce surface tension and increase the flexibility of the interfacial film.

Sugar based surfactants were selected (Tween 80 ,Tween 20 and span 60), Propylene glycol as a co-surfactant, and oils (olive oil and IPM) using HLB values, and then, investigated the ternary phase diagrams behavior of oils, with different sugar based surfactants/ and cosurfactant, with OLE extract. Ternary phase diagrams were constructed in order to identify the types of dispersion systems formed by the mixtures at different concentrations of their components. My interest was in investigating the microemulsion regions mainly.

1.11. Objective of the Research

The main objective of this Research is to study the phase behavior of polyphenols in olive leaves extract, in order to formulate a stable , biocompatible microemulsion between OLE, olive oil and a sugar/nonionic surfactant like span 60, tween 80 and tween 20. This microemulsion could be used in the formulation of pharmaceutical, food or cosmetic products.

To obtain this formula there are specific objectives needed to be achieved:

- 1) To develop a ternary and pseudoternary phase diagram between OLE/olive oil/surfactant with the best components concentration for microemulsion preparation.
- 2) To choose the most efficient surfactant and determine the surfactant ratios which have high solubilization capacity as well as high surfactant efficiency.
- 3) To study the role of polyphenols in OLE as cosurfactants.
- 4) To study the effect of using OLE that has been prepared using ethanol as a solvent and OLE that has been prepared using acidified MQ water as a solvent on the microemulsion formation.
- 5) To compare the effect of using IPM oil as a co-oil on the microemulsion formation, with the effect of using olive oil as the main oil.
- 6) To compare the effect of using IPM as the main oil on the microemulsion formation, with the effect of using olive oil as the main oil.

Chapter Two

Literature review

2.1. *Olea europaea* L

The olive tree, botanically-classified as *Olea europaea* L., is one of the most important fruit trees in Mediterranean countries [Yateem, et al. 2015].

It grows throughout the entire Mediterranean region and in most of the Southern European countries. In the Mediterranean area, there are nearly eight million hectares of cultivated olive trees. Both the oil and the dried green-grayish colored leaves are used medicinally [Altinyay, et al. 2006].

2.2. Olive oil

Olive oil is used by humans for food since prehistoric times. Olive oil not only contains oleic acid (18:1n-9), but also small amounts of other fatty acids, such as palmitic, palmitoleic, stearic, linoleic, and α -linolenic acids and squalene (Fig.2.1). In addition to fatty acids, olive oil also contains phenolic compounds. Oleic acid, a monounsaturated nonessential fatty acid, belongs to n-9 family of fatty acids. Olive oil is unique because it has high oleic acid content. In contrast, majority of other cooking oils. The presence of one double bond makes oleic acid not only less susceptible to oxidation, but also contributes to the high stability and long shelf life of olive oil [Farooqui, 2012].

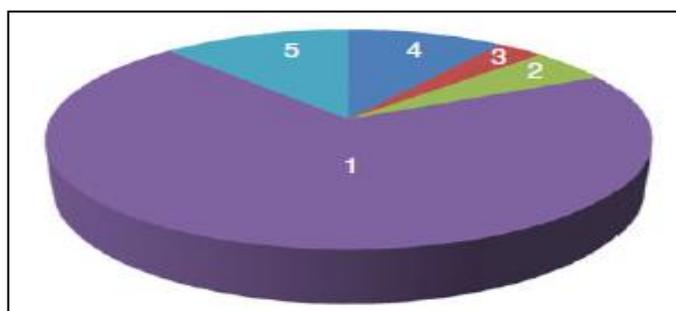


Fig.(2.1) Proportions of various fatty acids found in extra virgin olive oil. 1.Oleic acid ;2.stearic acid; 3.palmitoleic acid;4. linoleic acid; 5. palmitic acid [Farooqui, 2012].

Particular attention has been focused on the nutraceutical properties of phenolic compounds provided with antioxidant activity. The most abundant antioxidants in virgin olive oil are lipophilic and hydrophilic phenols, which are physiologically produced in the plant to react against various pathogen attacks and/or insect injuries [Bulotta, et al. 2014].

The phenolic compounds of olive oil can be divided into three categories: simple phenols, secoiridoids, and lignans. All these components inhibit autooxidation. Major nonfatty acid components of olive oil include hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin, pinosresinol, caffeic acid, vanillic acid, syringic acid, p-coumaric acid, o-coumaric acid, protocatechuic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid. The olives mainly contain the polar glycosides oleuropein and ligstroside (Table 2.1) [Bulotta, et al. 2014].

Hydrophilic		Lipophilic
Phenolic alcohols	Flavonoids	Tocopherols
Hydroxytyrosol	Apigenin	(α , β , γ , δ)
Tyrosol	Luteolin	
Secoroidoids	Phenolic acids	Tocotrienols
Oleuropein	Gallic acid	(α , β , γ , δ)
Ligstroside aglycon	Vanillic acid	
Lignans	Benzoic acid	
(+)-1-pinosresinol	Cinnamic acid	
(+)-1-acetoxypinosresinol	Caffeic acid	
	Coumaric acid	

Table (2.1) The main Phenolic compounds in virgin olive oil [Bulotta, et al. 2014].

Oleuropein is the ester of elenolic acid with 3, 4 - dihydroxyphenylethanol (hydroxytyrosol), and ligstroside is the ester of elenolic acid with 4-hydroxyphenylethanol (tyrosol). Oleuropein and ligstroside are the parent compounds of the less polar oleuropein- and ligstroside-aglycones. Oleuropein- and ligstroside-aglycones are produced by the removal of the glucose

moiety from the oleuropein- and ligstroside-glycoside by β -glucosidase during olive ripening process (Fig 2.2). Those aglycones and their various derivatives are the most abundant phenols in olive oil. Thus, hydroxytyrosol and tyrosol are simple phenols and oleuropein is a secoiridoid (Fig 2.3) [Farooqui, 2012].

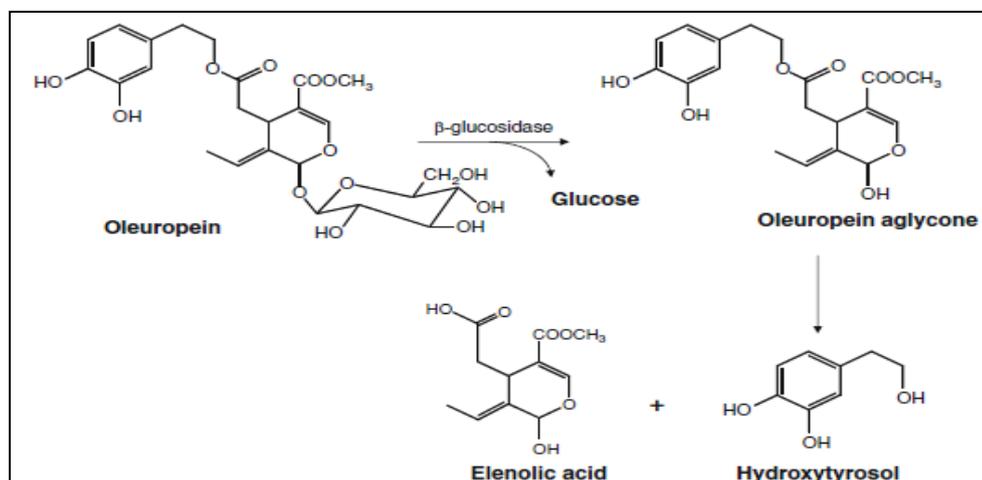


Fig.(2.2) Scheme showing the generation of hydroxytyrosol from oleuropein [Farooqui, 2012].

2.2.1. Antioxidant activity of Oleuropein and Hydroxytyrosol

Defense against reactive oxygen species is fundamental to protect cellular molecules as lipids, proteins or DNA and avoid the development of degenerative diseases. When the defensive mechanisms are overtaken by the action of the free radicals, the subsequent cellular damage may lead to several diseases, including atherosclerosis, cardiovascular diseases, skin and neurodegenerative diseases, diabetes mellitus and metabolic syndrome. Finally, physiological processes such as aging have been associated with disequilibrium between the action of reactive oxygen species and that of antioxidants.

Phenolic compounds in general and Oleuropein derivatives in particular, act as natural antioxidants acting as: a. free radical scavengers and radical chain breaking; b. anti-oxygen radicals; c. metal chelators. With their catecholic structure, they are able to scavenge the peroxy radicals and break peroxidative chain reactions producing very stable resonance

structures. Also they are important for the food stability and protect against the oxidation occurring naturally during virgin olive oil storage owing to reaction with air [Bulotta, et al. 2014].

2.2.2. Protection against cardiovascular diseases

Supplementation of olive oil in human diet improves the major risk factors for cardiovascular disease, such as the lipoprotein profile, blood pressure, glucose metabolism, and antithrombotic profile. In addition, olive oil contains many other components, such as phenolic acids, lignans, and flavonoids, which may promote many beneficial effects on human health. Multiple mechanisms have been proposed to explain beneficial effects of Mediterranean diet. These mechanisms include: decrease in LDL cholesterol, increase

HDL-cholesterol, and reduction of oxidative stress due to polyphenols and flavonoids, which may act as scavengers and protect heart tissue and LDL from free radical damage.

Olive oil was chosen for the oil phase of the microemulsion, because of its high nutritive value and it has been also recognised as a functional food product for human health. Moreover, olive oil contains a variety of compounds with antioxidative activity presenting great stability against oxidation compared to other vegetable oils. Some of these antioxidant compounds appear to have amphiphilic properties and thus may be suitable as surfactants. Studies carried out mainly with phenolic compounds have confirmed that quercetin, in olive oil emulsions has both an emulsifying and antioxidant ability [Polychniatou, et al. 2011].

2.3. Isopropyl myristate (IPM)

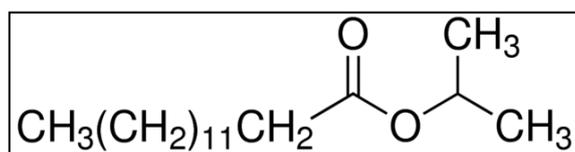


Fig.(2.3) Chemical structure of Isopropyl

A polar liquid with low viscosity that is practically colorless and odorless and decomposed at 208 °C, withstands oxidation and does not readily become rancid. Used in cosmetics and topical medicinal preparation as an emollients, lubricant and enhance absorption through the skin [Fanun, et al. 2006].

Since vegetable oils are considerably more difficult to solubilize in microemulsions. IPM was used as a co-oil with olive oil in some phase diagrams because it can easily form microemulsions with conventional surfactants; therefore, it is mixed with triglyceride oils like olive oil to enhance microemulsion formation [Sabatini, et al. 2008].

The ternary phase diagram of Tween 80, IPM and water of HLB 15 was studied. A large area of transparent/clear solution was formed in the oil rich regions. The microemulsion area was found to be attached to the borders of the diagrams where water and oil ratio was low and the surfactant ratio was high as shown in Fig.(2.5) [Syed, et al. 2014].

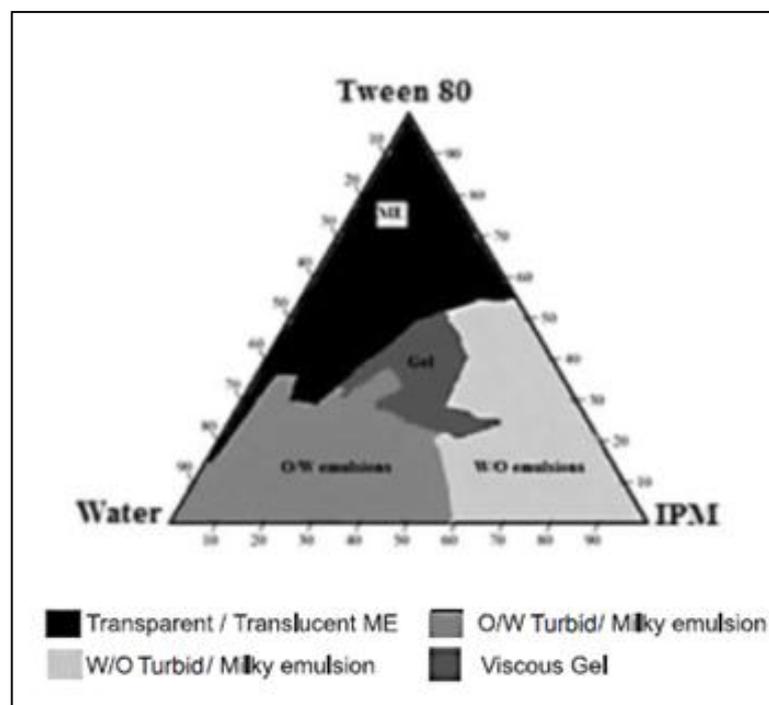


Fig.(2.4) Ternary phase diagrams of IPM with Tween 80 and water [Syed, et al. 2014].

2.4. Olive leaves

In the Mediterranean area, olive leaves are one of the by-products of farming of the olive grove; they can be found in high amounts in the olive oil industries (10% of the total weight of the olives). During olive tree cultivation, the pruning step generates a considerable volume of olive leaves, which are usually used as animal feed, and which could also be used for antioxidant or olive-leaf extract production [Lazou, A. et al.2013]. Leaves of the tree became important when olive leaf extract was reported to be potent in treating fever and malaria in 1854. Since then, several researchers demonstrated hypotensive, hypoglycemic, coronary dilatatory, antiarrhythmic, antiuricaemic, antioxidant, anticomplementary, antimicrobial, thyroid stimulatory, antiviral and anti-HIV activities of olive leaf extract [Altinyay, et al. 2006]. The leaves of the tree consist of secoiridoids, phenolic compounds, flavonoids and volatiles.

Olive leaves are considered as a cheap raw material which can be used as a useful source of high-added value products (phenolic compounds) [Abdelmelek, H et al.2012].

The active constituent of olive leaf is oleuropein, which has reported to be a potent antioxidant endowed with anti-inflammatory properties. It has pharmacologic and health promoting properties including hypoglycemic, antioxidant, antimicrobial, antimycoplasmal, antiviral, anti-tumor and angiogenic activities. It was found to inhibit androstenedione 6 β -hydroxylase activity, a cytochrome P450 3A marker in human liver microsomes, prevent lipid peroxidation on rat liver microsomes. Oleuropein has been also shown to inhibit LDL (Low Density Lipoprotein) oxidation and possess vascular protection activity by inhibiting platelet aggregation induced by platelet-activating factor [Altinyay, et al. 2006].

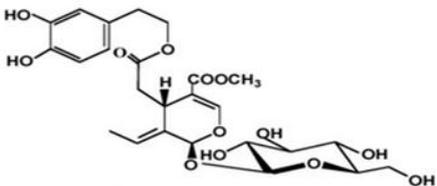
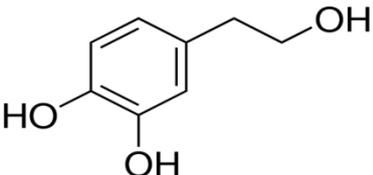
Oleuropein is generally the most prominent phenolic compound in olive cultivars and can reach concentrations of up to 140 mg g⁻¹ on a dry matter basis in young olives and 60–90 mg

g⁻¹ of dry matter in the leaves. In the leaves, oleuropein makes up 19% (w/w) and flavonoids make up 1.8% (w/w), of which 0.8% is luteolin 7-glucoside. The leaves are considered to be the richest part that contains oleuropein.

Oleuropein is the major phenolic compound of olive leaves and varies from 17% to 23% depending upon the harvesting time of the leaves. Fresh olive leaf generally needs drying and milling before extraction. As a preservation method, drying is carried out to remove the water from the leaves to protect the leaves against spoilage and degradation of oleuropein by enzyme action [Yateem, et al. 2015].

2.4.1. Olive leaf extract polyphenols compounds structures

Separation and analysis of olive leaf extract (OLE) by using HPLC at wavelength 280 nm revealed the following phenolics, hydroxytyrosol; tyrosol; luteolin-7-O-glucoside; verbascoside; apigenin-7-O-glucoside; oleuropein [Hayes, et al.2011] . Olive leaf extract is rich of phenolic compounds and contains, also caffeic acid quercetin and chryseriol [Dekanski , et al 2009]. Fig.(2.7) shows the OLE chemical structures [Omar, 2010].

Name	Structure
Oleuropein	 <p>The chemical structure of Oleuropein is a complex polyphenol. It features a luteolin core (a flavone with two hydroxyl groups at positions 5 and 7) linked via an ether bridge to a glucose molecule. This glucose is further substituted with a hydroxytyrosol moiety and a methyl ester group (COOCH₃).</p>
Hydroxytyrosol	 <p>The chemical structure of Hydroxytyrosol consists of a benzene ring with two hydroxyl groups (HO) at the 3 and 4 positions and a 2-hydroxyethyl group (-CH₂-CH₂-OH) at the 1 position.</p>

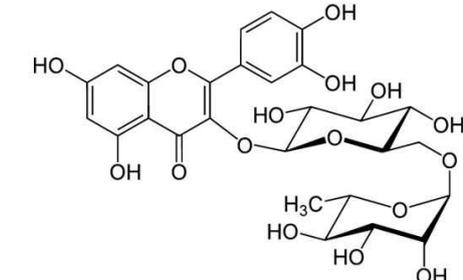
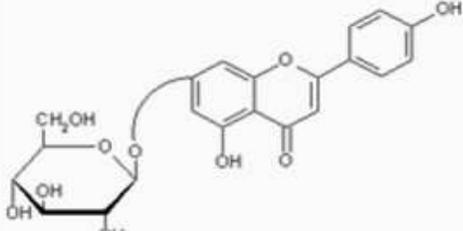
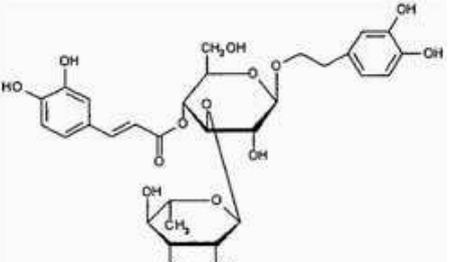
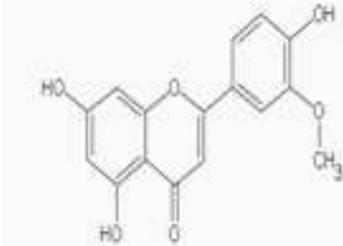
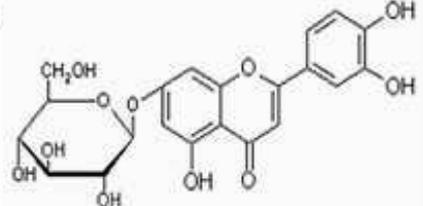
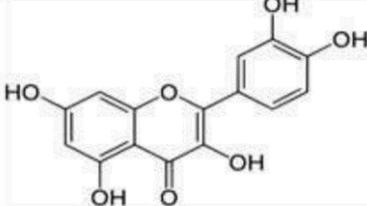
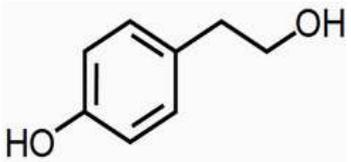
<p>Rutin</p>	
<p>Apigenine-7-O-glucoside</p>	
<p>Verbascoside</p>	
<p>Chryseriol</p>	
<p>Luteoline-7-O-glucoside</p>	
<p>Quercetin</p>	
<p>Tyrosol</p>	

Table.(2.2) OLE polyphenol compounds structure.

Scientists has reported about how olive leaves extract (OLE) found to be useful for many acute and chronic diseases, Due to the high content of high polyphenolics content [Zari et.al, 2011, Susalit et.al, 2011, Yamada at.al 2009, Soni et.al, 2006].

2.4.2. Pretreatment of olive leaves

The active ingredients in olive plants are accompanied by certain enzymes (β -glucosidase) capable of affecting hydrolysis of the active fraction, these enzymes must be inactivated before or during extraction of the active fraction. This is particularly important when fresh plant materials are used. At first the raw materials (olive leaves) must be dried at room temperature to remove the water content from the leaves. The olive enzymes may be inactivated by placing the dried plant material (olive leaves) into boiling Water or boiling alcohol for 30 minutes to inactivate the enzymes. Use of boiling alcohol is preferred over use of boiling water; because the boiling alcohol treatment is carried out at lower temperature and also alcohol denatures the enzymes even at cold temperatures. The alcohol can also be removed faster from the plant materials than can water, and therefore the boiling water treatment needs to be followed by another period of oven drying. Followed by grinding olive leaves to a fine powder after they have been dried then used for the extraction [Pinnell, et al.2004 , Fredrickson, 2000].

2.4.3. Extraction of phenolic compounds from olive leaves

Because of the huge health benefits of OLE, it became an important subject. So regarding extraction method, soxhlet, super critical fluid extraction, liquid- liquid extraction, and dynamic ultrasound-assisted extraction can be used [Ansari ,et al 2011].

2.4.3.1. Sampling preparation

After harvesting, most herbs have a moisture content of 60%-80% and cannot be stored without drying. Otherwise, important compounds can breakdown or micro-organisms may contaminate the material. Drying of the herbs in shade in a thin layer is generally preferred [Handa ,et al 2008].

Fresh olive leaf generally needs drying milling before extraction. Milling the dried leaves can reduce particle and facilitate solvents entering into the cells of leaves. It also improves extraction efficiency or extractability. Many different drying approaches have been explored by researchers, but air drying, microwave drying and freezing drying have been mostly reported in literature [Malik ,et al 2008].

There is little literature reporting on extraction of phenolics from fresh olive leaves, and it was reported that increased levels of oleuropein found in dried leaves than in fresh leaves probably due to the conversion of oleuropein glucoside into oleuropein by β -glucosidase present in fresh leaves [Siva, et al 2006].

2.4.3.2. Methods of extraction

Traditionally, there are number of methods to make herbal remedies such as infusions, tinctures ad decoctions, and many substances have been used as a base for extracts. Infusion is the simplest way to prepare leaves and flowers for use as a medicine. By simply pouring boiling water into handful of dried herbs and infuse for certain length of time, water based extracts are obtained. Tinctures are made by soaking dried or fresh herbs in alcohol for 10-14 days, and then removing the herbs [Chevallier, 2000].

Solvent extraction was the main methods adapted by most researchers to extract phenolics from olive leaves. This is a process designed to separate soluble compounds by diffusion from a solid matrix using a liquid matrix. This process takes place by two steps, which are adsorption of solvent into the solid phase by osmotic forces, by capillary and by solvation of the ions in the cells, then followed by diffusion from the solid phase .

The aim of the extraction is to concentrate antioxidant component of raw materials; the extraction process involves a more or less vigorous agitation of the ground raw materials with extraction solvent at ambient or elevated temperatures and subsequent separation of the residue by filtration. Repeated extraction steps may be accomplished to increase the extract yield. Alternatively, a packed bed of the ground material can be used which is leached by the extraction solvent under refluxing conditions [Oreopoulou, 2003].

A comparison between two methods of olive leaves extraction, namely Soxhlet and Supercritical Fluid Extraction (SFE) methods had been done. For investigating the effect of solvent type on extraction efficiency, the Soxhlet method was carried out by hexane, water, ethanol, methanol, and methanol/hexane (3:2, v/v) mixture. The effects of process variables such as temperature, pressure, and co-solvent type (water, ethanol, and methanol) through (SFE) were also evaluated. The two methods were compared with each other. It was found that the use of 20% co-solvent (v/v) increased the solubility sharply refers to that of supercritical CO₂ used alone. Although ethanol is preferable as co-solvent for its non-toxicological and environmental considerations, its efficiency through (SFE) was considerably behind water and methanol in terms of the maximum oleuropein content (2.91mg/g dried leaf versus 10.9 and 14.26 mg/g dried leaf, respectively). Furthermore, in the Soxhlet method, methanol showed almost 122 times better recovery of oleuropein than that of ethanol. The maximum oleuropein yield of 14.26 mg/g dried leaf obtained by CO₂ modified with 20% methanol at 300 bars and 100 CO was followed by that of CO₂ modified with water at the same

conditions. On the other hand, water in contrast to methanol, is cost effective and also superior regarding toxicity, flammability, and availability; consequently, it can be highly recommended as co-solvent in (SFE). With respect to extraction methods, although the highest yield was achieved by Soxhlet, a general comparison between (SFE) and the Soxhlet method cannot be established, where (SFE) considers short processing time and low solvent consumption. The Soxhlet method could have disadvantages from the point of view of the products quality leading to target compounds with unpleasant aromas because of the long extraction time [Sahin , et al 2011].

Various processing and extraction methods were investigated to evaluate stability and recovery of oleuropein and other polyphenols from olive leaves. Brief thawing of frozen leaf samples (5 minutes) caused a sharp reduction in extractable oleuropein levels(57.7%), and 53.5% loss in oleuropein occurred when frozen leaf powder was thawed for only 2 minutes. Simple drying of fresh leaves at room temperature (25°C) fully preserved oleuropein and verbascoside levels while drying at an elevated temperature of 60°C resulted in losses at various levels of all polyphenols studied. While extraction in 80% methanol is the most effective method for olive leaf polyphenols for laboratory use, boiling of dried leaves was also a very efficient method for extracting oleuropein and verbascoside that gave 96 and 94% recoveries of these compounds, respectively, when compared with the methanol extract. Oleuropein was quite stable in aqueous extracts for 7 days when stored at room temperatures but degraded after 17 days. Other polyphenols were less stable in aqueous extracts and started to show some degree of degradation after 7 days (little change occurred during the first 24 h storage at room temperature) and were completely degraded after 17 days. On the other hand, oleuropein and other polyphenols in methanol extract were quite stable for 30 days when stored at room temperature [Malik ,et al 2008].

2.4.3.3. Effect of Extraction Solvent on Oleuropein Content

Various types of solvents were used to extract oleuropein from olive leaves. Higher oleuropein levels were observed regarding the solvent containing deionised water at 60 °C Fig.(2.8) because it is a water-soluble phenolic compound whose solubility can be increased by elevating the temperature. Addition of more lipophilic solvents such as methyl or ethyl alcohol to the water decreases water efficiency in extracting oleuropein from the leaves. As can be seen clearly in Fig.(2.8), the use of lipophilic solvents such as n-hexane and dichloromethane is of no help to extract oleuropein from the olive leaves.

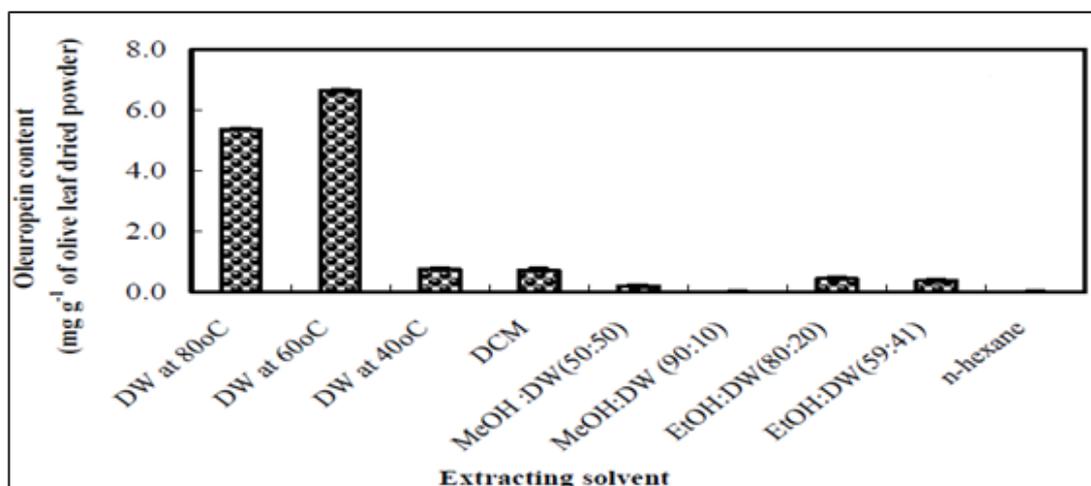


Fig.(2.5) Effect of extracting solvent on extraction yield of oleuropein from olive leaves [Ansaria, et al 2011].

Also some methods of extraction concern in the environmental side besides the cost. Ansaria et.al, optimized a green and inexpensive water-based method of extraction. They found that deionised water adjusted to pH 3, at 60 °C for 4 h had the highest maceration extraction method has high efficiency to extract olive leaves [Ansaria, et al 2011].

Both the extraction yield and antioxidant capacity of extract are strongly influenced by the solvent, due to the different polarity and different antioxidant potential of compounds extracted. Therefore, organic solvent of higher polarity are more effective in quantitative recovery of phenolic compounds than non-polar solvent and methanol was reported in many

studies as a good solvent for extraction of phenolics from the plants including olive leaves. However, it may lead to unacceptable levels of toxic residues in the final extract; ethanol and water are the most widely employed solvents for safety and abundance reasons. Ethanol alone was not effective as a solvent for extraction of phenolic compounds from olive leaves, and water has an important role in the extraction process by increasing the diffusion of extractable polyphenols through the plant tissues [Lou, 2011].

The effect of extraction solvent on oleuropein content was investigated by extracting a fixed quantity of olive leaves in different solvents (types) and using different compositions (volume fraction). To this end, seven extraction procedures were performed (each time using 10 g of olive leaves) using 100 ml of seven extraction solvents (water, 80% methanol, 100% methanol, 50% ethanol, 80% ethanol, 100% ethanol, and 20% acetonitrile) for 4 hours. Results showed that the highest oleuropein content was obtained when the olive leaves were extracted with 80% ethanol (13 mg/g) followed by 20% acetonitrile (10.0 mg/g), and 80% methanol (5.31 mg/g), and 50% ethanol (2.57 mg/g). Regarding pure water, pure methanol and pure ethanol, the amount of oleuropein extracted using these solvents is low (0.16, 0.10, and 0.02 mg/g respectively). Statistically, there is a significant difference in the oleuropein content with change of solvent (type and composition) which is indicated by different small letters (a, b, c, d, e, f, and g) Fig.(2.9).

These results show that a mixture of an organic solvent with water is needed to effectively extract oleuropein from olive leaves as very low amounts of oleuropein were extracted using pure solvents compared to solvent mixtures. These results showed that using water as a co-solvent with organic solvents increases the amount of oleuropein extracted from olive leaves. Additionally, the solvent mixtures are better as they deactivate the enzymes which are

responsible for conversion of oleuropein into other compounds which have high protein-denaturing, and protein- cross linking activities [Yateem, et al. 2014].

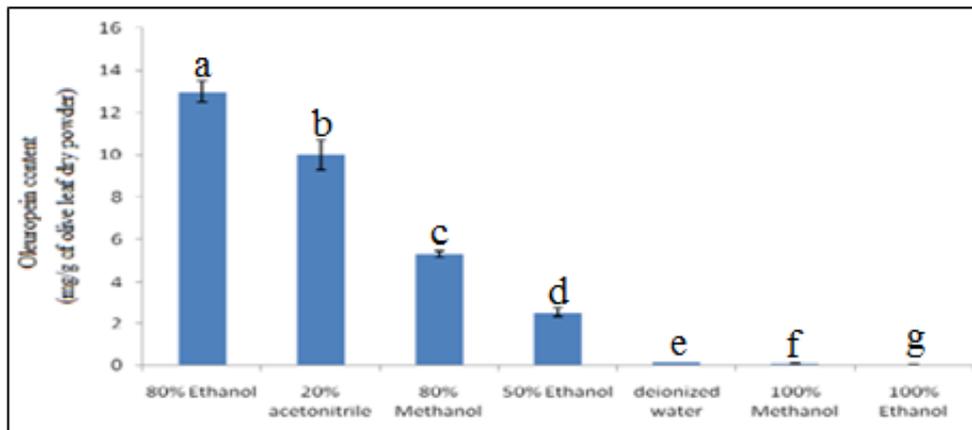


Fig.(2.6) Oleuropein content in olive leaves extracted by using different solvents [Yateem, et al. 2014].

2.4.3.4. Effect of pH on Oleuropein Extraction

To optimize the extraction of oleuropein by aqueous solvents, olive leaves powder was extracted with deionized water at 60 °C and various pH values. Higher yields of oleuropein were observed at an optimum pH of 3 that may be related to lower degradation of oleuropein at this pH, while the use of higher or lower pHs, caused a significant decrease in the yields of oleuropein. Fig.(2.10) shows that optimum pH of extraction can be obtained at pH 3. The extraction of oleuropein at this pH is about 1.5 to 10 times greater [Ansaria, et al 2011].

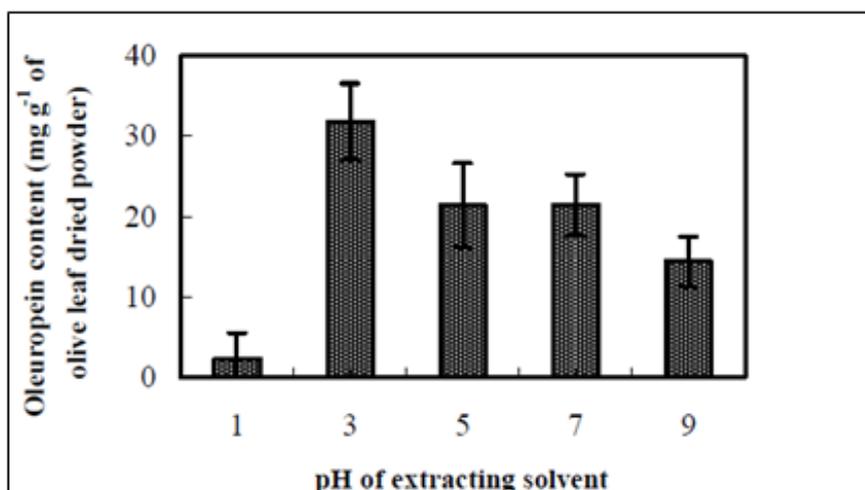


Fig.(2.7) Effect of pH on extraction yield of oleuropein from olive leaves [Ansaria, et al 2011].

The effect of pH of extraction solvent on oleuropein content was studied by extracting fixed amount of olive leaves (10 gram) in 100 ml distilled water (at room temperature for 4 hours) adjusted to different pH's (3, 5, 7, and 9, pH adjusted with 0.1N hydrochloric acid or 0.1N sodium hydroxide). Results showed that the highest amount of oleuropein was got when olive leaves are extracted with water at acidic medium, pH 3 (6.85 ± 0.17 mg/g). With increase of pH to 5, oleuropein content was sharply reduced (0.26 ± 0.02 mg/g) which is only 3.8% of oleuropein amount extracted at pH 3, and with further increase of pH to 7 and 9, oleuropein content further decreased sharply (0.16 ± 0.01 , and 0.09 ± 0.01 mg/g, respectively). This decrease in oleuropein content with pH increase can be attributed to the ionization of the hydroxyl groups of oleuropein with pH increase which results in poor oleuropein recovery from the leaves [Yateem, et al. 2014].

2.5. Analyzing Methods

2.5.1. Total Phenolic Content

Total antioxidant activity, phenolic contents and reducing power in six Iranian olive cultivars were determined. The highest antioxidant activity (28.699 mmol FeII/100 g dry plant), total phenolic contents (2997 mg gallic acid/100 g dry plant) were determined by the Folin-Ciocalteu procedure. Reducing power (8.331 g Vitamin E/100 g dry plant) were detected in Mishen and the lowest in Conservalina. A linear positive relationship existed between the antioxidant activity, total phenolic compounds ($r^2 = 0.976$) and reducing power of the tested olive pulp ($r^2 = 0.848$). This result suggests that 97% of the antioxidant capacity of Iranian olive pulp results from the contribution of phenolic compounds. Also, it can be concluded that antioxidant activity of pulp extracts is not limited to phenolics. Activity may also come from the presence of other antioxidant secondary metabolites, such as volatile oils carotenoids and vitamins, which in this case contributed to 3% of the antioxidant capacity. On the other hand,

about 85% of the antioxidant activity of phenolics is due their redox properties, which allow them to act as reducing agents, hydrogen donors and the others may also have a metal chelating potential. So, Iranian olives possess relatively high antioxidant activity due to contribution of phenolic compounds [Hajimahmoodi, et al 2008].

The effect of geographical region and harvesting date (seasonal change) on antioxidant activities (AA), total phenolic content (TPC) and total flavonoid content (TFC) of olive leaves obtained from different geographical regions of Palestine (north, middle, and south) at different maturation stages (June 2013, October 2013, and January 2014) was investigated in this study. Results revealed that TPC, TFC, and AA of olive leaf samples collected from Palestine are affected by harvesting time, and the geographical region. Highest TPC, TFC, and AA were obtained for samples collected in summer (June) compared to those collected in winter (January). TPC was found to be highest in north and lowest in south. The highest and lowest values of TFC and AA of the samples were alternating between north, middle, and south. It was found a positive correlation between AA and TPC as well as between AA and TFC. On the basis of these findings, it is concluded that olive leaves from Palestine is a rich source of phenolics, flavonoid compounds, and more rich compared to those from Iran, Greece, and Italy. Olive leaves therefore constitutes a natural source of potent antioxidants, and could potentially be used in food, pharmaceutical, cosmetic formulations as additives, preservatives, and antioxidants [Al-Rimawi, et al 2014].

Total phenolic content and antioxidant activities of olive leaf extracts were determined. Plant material was extracted with methanol and fractionated with solvents of increasing polarity, giving certain extracts. The qualitative changes in the composition of the extracts were determined after the storage of leaves for 22 h at 37°C, before the extraction. Total polyphenol contents in extracts were determined by the Folin-Ciocalteu procedure [Kontogianni, et al 2012].

There are many parameters affecting TPC, TFC, and AA of olive leaves, including, among others, geographical region, maturity stages or harvesting date, olive fruit cultivar, climate, type of soil..etc. [Al-Rimawi, et al 2014].

2.5.2. Dynamic Light Scattering

The value of the hydrodynamic radius for a system that contains a sugar based surfactant was determined with value as low as 11nm; using Dynamic light scattering; at aqueous phase volume fraction and temperature equal 90% and 25°C respectively. And the system was water + propylene glycol/sucrose Laurate/ethoxylated mono-Di-Glyceride/ peppermint Oil+ethanol [Fanun, 2010].

The solubilization behaviour of a number of essential oils (EOs) containing volatile phenolic constituents was investigated in five different micellar solutions. These oils include clove bud (*Eugenia caryophyllata*), thyme (*Thymus serpyllum*) and oregano (*Thymus capitatus*). Ternary and pseudo-ternary phase diagrams were constructed to assess the ability for microemulsion formation and dilutability of each system using non-ionic surfactants like Tween 80 and Tween 20. Particle sizes measured at different dilution lines ranged between 5.9 and 16.9 nm, using dynamic light scattering [Edris, et al 2012].

Chapter Three

Materials, Methods, and Experiments

3.1. Sample collection

Olive leaves samples were obtained from trees localized in the sunshine from different regions in Palestine, and most of the leaves were from grassland in Hebron\Halhol. Olive oil was obtained from Salfit.

3.2. Sample preparation

Fresh green leaves were dried at room temperature. The dried leaves were put into ethanol at 60 °C for 30 minutes to inactivate the enzymes. Then they were dried again at room temperature. The dried samples were grinded using a coffee blender until a fine powder was obtained. Mesh 30 was used to get fine powder of olive leaves. Then it was stored in a cool dry place until it was used for extraction.

3.3. Chemicals

- Ethanol 99.9 % was purchased from BIOTECH MEDICAL SUPPLLIERS GADOT.
- Hydrochloric Acid was purchased from SDFCI, sd fine-chem limited.
- Deionized water was supplied by a Milli-Q water purification system (Milli pore system, ALqudsUniversity) $\sigma < 3 \mu\text{S}$.
- Propylene Glycol was purchased from FINKELMAN LTD CHEMICALS.
- Isopropyl Myristate, Tween 80, Tween 20, Span 60, Folin–Ciocalteu, Sodium bicarbonate and Gallic acid were purchased from Sigma-Aldrich.

3.4. Instruments and Equipment

- Balance.
- Vortex.
- cross polarizers.
- Test tubes.
- Thermostatic water bath.
- Zetasizer Nano S (ZEN 1600) by Malvern Instruments Ltd. (Worcestershire, United Kingdom).
- UV-Visible spectrophotometer by analytikjena company.

3.5. Methodology

3.5.1. Extraction of Olive leaves

There are many methods and techniques used for Olive leaves extraction. Extraction depends on several parameters and on the matrix itself. In this research the focus will be on using Soxhlet for the extraction of Olive leaves.

3.5.1.1. Soxhlet extraction

3 grams of olive leaves sample placed in a thimble made of filter paper and inserted into the wide central tube of the extractor. Alternatively, the olive leaves, after imbibition with menstruum, may be packed in the extractor taking care that the bottom outlet for the extract is not blocked. 300 ml of the solvent (ethanol 99.9% / acidic water with pH 2.8) was placed in the flask. Then ethanol was heated to 50 °C / acidic water was heated to 70 °C. The process

is continued until the olive leaves are completely extracted. The extract was then cooled to room temperature. Then a 10 ml sample was taken to calculate the total extract. The extract was stored in a refrigerator at 2-5 °C. The advantage of this method is that large amounts of olive leaves can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale [Handa et al, 2008].

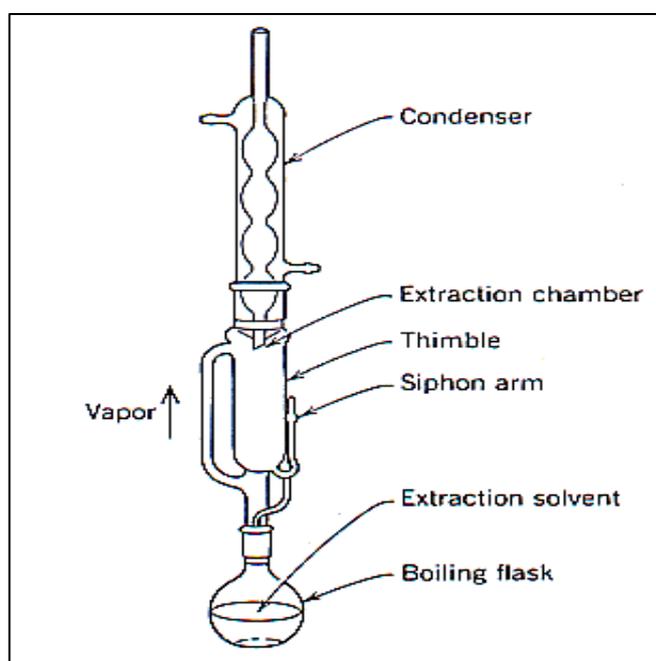


Fig.(3.1): Soxhlet apparatus for hot extraction [Handa ,et al 2008].

3.5.2. Constructing of Phase Diagram

The phase behavior of the systems consisting of OLE, oil (with or without co-oil), surfactant (or mixed surfactants) may be described on a ternary phase diagram whose apexes respectively represent the pure components. 1g of a mixture consisting of oil (with or without co-oil), surfactant (or mixed surfactants) at different weight ratios were prepared in culture tubes sealed with Viton lined screw caps and stirred at ambient temperature by vortex until clear

solution was obtained. Titrating these samples with OLE which was added dropwise until its solubilization limit was reached. Vigorous stirring followed all of the aqueous phase additions on a vortex mixer. The time for equilibration between each addition was typically, from a few minutes up to 24 h. Following equilibrium, the samples were checked for phase separation and birefringence. Polarized light was used to detect birefringence, since this distinguishes between anisotropic lamellar and hexagonal liquid crystal and the isotropic (nonbirefringent) micellar solution or cubic liquid crystal. Two or three-phase samples were either opaque or macroscopically phase separated. Only those transparent, isotropic, fluid, non-birefringent samples were considered as stabilized microemulsion. The boundaries of single phase were detected at the end; finally the phase diagram was drawn using Origin Pro 8 software.

3.5.3. Total phenolic compound test

Total phenolic compound test was done by Specord 40 UV-VIS spectrum, versatile single-beam spectrophotometer for the measurement of 190-1100 nm conforms to Ph.Eur. quality, manufactured by analytikjena company. Total phenolics were determined using Folin–Ciocalteu reagents. Forty microlitres of Olive Leaves extract or gallic acid standard was mixed with 1.8 ml of Folin–Ciocalteu reagent and allowed to stand at room temperature for 5 min, and then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After standing 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentration (100-500 ppm) were used for calibration curve [Index]. Results were expressed as mg gallic acid/100 g sample [Al-Rimawi, et al 2014 , Kontogianni, et al 2012 , Hajimahmoodi, et al 2008].

3.5.4. Dynamic light scattering test

Zetasizer Nano S (ZEN 1600) by Malvern Instruments Ltd. (Worcestershire, United Kingdom) was used for the measurements of the size of dispersed particles. Each liquid sample was inserted into a 10-mm diameter borosilicate test tube and centrifuged for 15 min at 3,000 rpm in order to remove dust. The test tube was then placed in a temperature-controlled vat of toluene as the index matching fluid. The light source was an argon ion laser (Spectra Physics-Lexel; $\lambda=514.5$ nm). Photons scattered by the sample were collected by an ITT PW130 photomultiplier tube mounted at 90° to the incident laser beam. The photoelectron count-time autocorrelation function was calculated with a BI 2030 AT (Brookhaven Instruments) digital autocorrelator and was analyzed using the constrained regularization algorithm, to give an intensity-weighted distribution of the translational diffusion coefficients D_z . Particle hydrodynamic size was calculated from the translational diffusion coefficient using the Stokes–Einstein relationship :

$$dH = kBT/6\pi\eta D_z$$

where dH is the hydrodynamic diameter, kB is Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity. The results are averages of three or four experiments. dH is obtained from the positions of the peaks of the intensity-weighted size distribution function [Fanun, 2009].

Chapter Four

Results and Discussion

Microemulsion formation of triglyceride oils like olive oil at ambient conditions (temperature and pressure) and without the addition of co-oil and/or alcohols is challenging at best. Olive oil microemulsion is elusive appears to be the unique structure of triglyceride molecules. Their long and bulky alkyl chains make triglycerides highly hydrophobic, while the ester region in the molecule causes high polarity; in combination, these lead to poor solubilization by the surfactant [Sabatini, et al. 2008].

So we wanted to formulate a microemulsion using olive oil as the main oil with sugar based surfactant, that is known for its good cutaneous tolerance, lower irritation potential and toxicity, and olive leaves extract as the aqueous phase. So for the first 5 systems ethanol was used as a solvent for the extraction of phenolic compounds from olive leaves, to prevent β -glucoside from breaking down oleuropein and converting it into other compounds that have high protein-denaturing like Oleuropein- and ligstroside-aglycones. Maybe this will play a major role in enhancing the role of polyphenols as a cosurfactant and help in the microemulsion formation. So we studied the work of three sugar-based surfactants (Span 60, Tween 20, Tween 80) to find out which one have the best emulsification properties, especially when we are dealing with a triglyceride oils like olive oil.

4.1. Phase diagram of 26% OLE / Span 60 / Olive Oil System.

In this phase diagram OLE of 26% total extract was extracted using 99.9% ethanol. Here the surfactant couldn't form any microemulsion region. This is because span 60 wasn't compatible with the system and couldn't emulsify the large triglycerides in olive oil.

4.2. Phase diagram of 26% OLE / Tween 20 / Olive Oil System.

In this phase diagram OLE of 26% total extract was extracted using 99.9% ethanol. Here the surfactant couldn't form any microemulsion region. All the phase diagram was a two-phase region. This is likely due to the fact that the surfactant film at the OLE-Olive oil interface found it difficult to penetrate the large triglyceride molecules.

4.3. Phase diagram of 26% OLE / Tween 80 / Olive oil System.

In this phase diagram OLE of 26% total extract was extracted using 99.9% ethanol. The result was a single clear isotropic region (L_2). The remainder of the phase diagram represents a two-phase region based on visual identification. Here Tween 80 managed to solubilize a small amount of the large triglyceride molecules at surfactant concentration equals~ 78%. Only around 8% of OLE was incorporated in the formation of microemulsion Fig.(4.1).

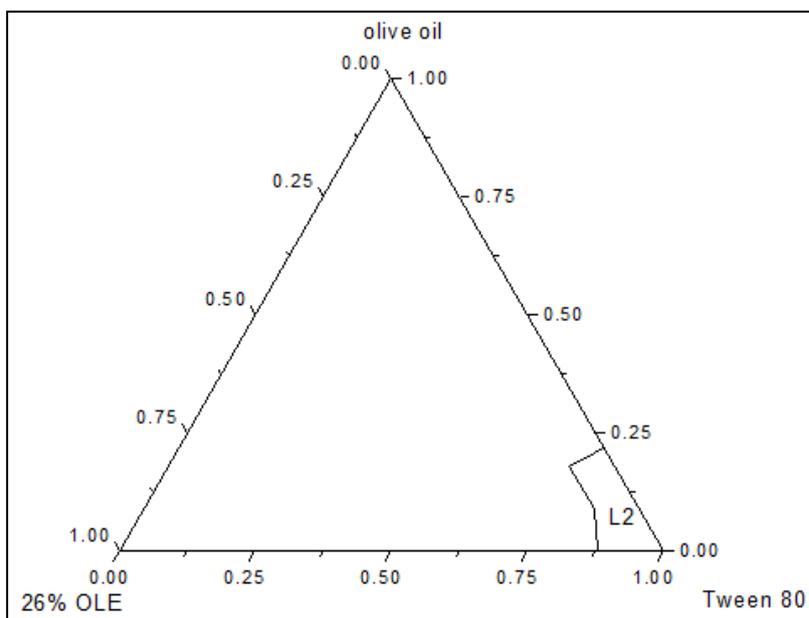


Fig.(4.1) Ternary Phase diagram of 26% OLE extracted using ethanol \ Tween 80 \ Olive Oil at ambient temperature.

4.4. Phase diagram of 26% OLE / Span 60:Tween 80 / Olive Oil System.

In this phase diagram OLE of 26% total extract was extracted using 99.9% ethanol. Here, mixture of Span 60 : Tween 80 (60% : 40%) made on purpose to attain some intermediate property or some synergetic effect. But we had the same result as Span 60 / OLE / Olive oil system, the mixture of surfactants couldn't form any microemulsion region.

4.5. Phase diagram of 26% OLE / Tween 80 / Olive Oil:Propylene glycol System.

In this phase diagram OLE of 26% total extract was extracted using 99.9% ethanol. The previous results indicate that Tween 80 has the most emulsification properties, so it was used as the main surfactant. In this system propylene glycol was used as a cosurfactant to enhance the work of Tween 80. So, Olive Oil : Propylene glycol (1:1) were added to the system. At surfactant concentration equals~ 90%, a single clear isotropic region (L_2) w/o microemulsion was formed, and the remainder of the phase diagram represents a two-phase region based on visual identification. 28% of OLE incorporated in (L_2) region while in OLE / Tween 80 / Olive Oil System only around 8% of OLE was incorporated in the formation of microemulsion. Using propylene glycol improved the miscibility of OLE with the olive oil Fig.(4.2).

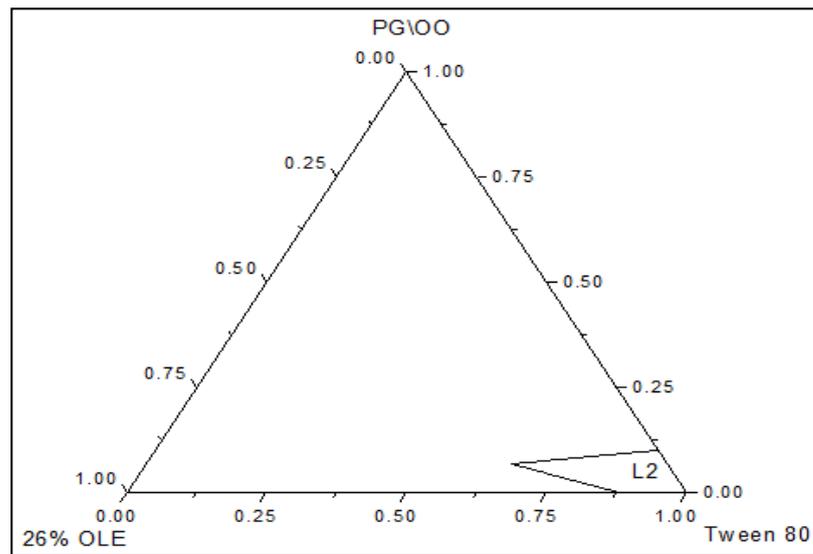


Fig.(4.2) Ternary Phase diagram of 26% OLE extracted using ethanol / Tween 80 / Olive Oil:Propylene glycol at ambient temperature.

From the results above it was found that we need to change using ethanol as a solvent to extract phenolic compounds from the olive leaves, since it didn't help in enhancing the microemulsion formation; this is because phenolic compounds mainly oleuropein are water soluble, addition of more lipophilic solvents like ethanol decreases the efficiency of extracting oleuropein and other phenolic compounds from the leaves [Ansaria, et al 2011, lou, 2011]. Scientists have shown that ethanol inactivate β -glucoside enzyme and prevent the breaking down of phenolic compounds [Pinnell, et al.2004, Fredrickson, 2000]. However it decreases the efficiency of extracting oleuropein and other phenolic compounds. At this point it was necessary to move toward using MQ water of pH=2.8, acidified with 0.1N HCl, as a solvent. pH=2.8 was chosen to prevent the ionization of the hydroxyl groups of oleuropein with the increase of pH which results in poor oleuropein recovery from the leaves [Ansari, et al 2011, Yateem, et al. 2014]. The work of (Span 60, Tween 20, Tween 80) was studied in order to find out which one has the most emulsification properties in this media.

4.6. Phase diagram of 32% OLE / Span 60 / Olive Oil System.

In this phase diagram OLE of 32% total extract was extracted using acidified water. The same result was obtained as OLE / Span 60 / Olive Oil System where OLE was extracted using ethanol; in which the surfactant couldn't form any microemulsion region. This is because span 60 wasn't compatible to the system and couldn't emulsify the large triglycerides molecules.

4.7. Phase diagram of 32%OLE / Tween 20 / Olive Oil System.

In this phase diagram OLE of 32% total extract was extracted using acidified water. The phase diagram indicated the presence of a clear isotropic regions (L_1) o/w microemulsion, and (L_2) w/o microemulsion region, the remainder of the phase diagram represents a two-phase region based on visual identification. However, (L_2) region was obtained at surfactant concentration equals~ 60% Fig.(4.3).

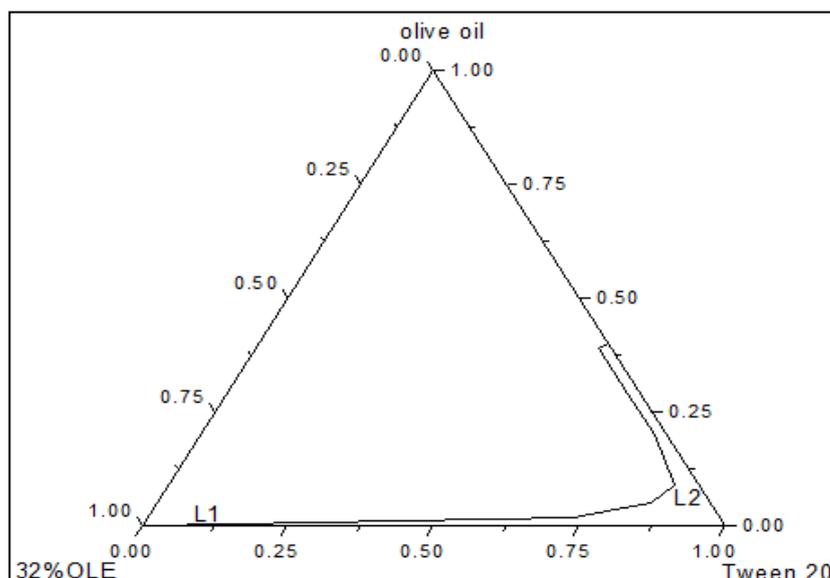


Fig.(4.3) Ternary Phase diagram of 32% OLE extracted using acidified water / Tween 20 / Olive Oil at ambient temperature.

Comparing the result of Fig.(4.3) with OLE / Tween 20 / Olive Oil system where OLE was extracted using ethanol, revealed a huge difference. In Fig.(4.3) (L₁) and (L₂) microemulsion regions were formed and (L₂) region was obtained at surfactant concentration equals~ 60% . While in the other one, there was no presence of microemulsion region, and all the phase diagram was a two-phase region.

This result indicates two things:-

- 1) Using acidic water as a solvent enhanced the extraction of oleuropein and other phenolic compounds.
- 2) Phenolic compounds helped in the formation of microemulsion and acted as a cosurfactant.

4.8. Phase diagram of 32% OLE / Tween 80 / Olive Oil System.

In this phase diagram OLE of 32% total extract was extracted using acidified water. The phase diagram indicated the presence of a clear isotropic regions (L₁) o/w microemulsion, (L₂) w/o microemulsion regions, the remainder of the phase diagram represents a two-phase region based on visual identification. However, (L₂) region was obtained at surfactant concentration equals~ 49% Fig.(4.4).

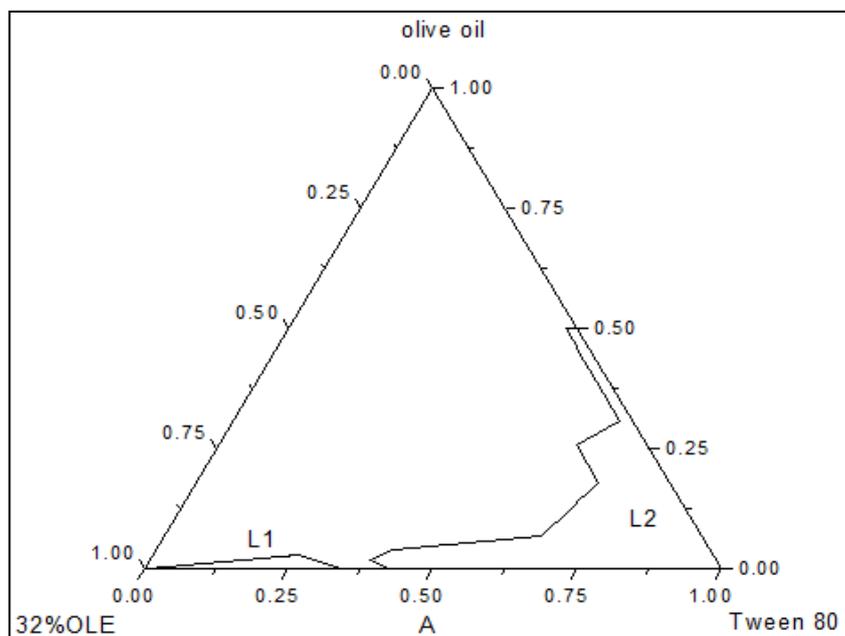


Fig.(4.4) Ternary Phase diagram of 32% OLE extracted using acidified water / Tween 80 / Olive Oil at ambient temperature.

Comparing the result of Fig.(4.4) with Fig.(4.1), reveals a big difference; in Fig.(4.4) (L_1) and (L_2) microemulsion regions were formed, and (L_2) region was obtained at surfactant concentration equals~ 49%. While in Fig.(4.1), only a single clear isotropic region (L_2) was formed and the remainder of the phase diagram represents a two-phase region based on visual identification, in which Tween 80 managed to solubilize a small amount of the large triglyceride molecules at high percentage equals~ 78%.

This result indicates two things:-

- 1) Using acidic water as a solvent enhanced the extraction of oleuropein and other phenolic compounds.
- 2) Phenolic compounds helped in the formation of microemulsion and acted as a cosurfactant.

Since Tween 80 has the most emulsification properties of olive oil between the three used surfactants, it was used as the main surfactant in the following systems. In order to have a complete comparison, the phase behavior of MQ water / Tween 80 / Olive oil was studied.

4.9. Phase diagram of MQ water / Tween 80 / Olive Oil System.

Here (L₁) and (L₂) microemulsion regions were formed, and (L₂) region was obtained at surfactant concentration equals~ 90%. The remainder of the phase diagram represents a two-phase region based on visual identification Fig.(4.5).

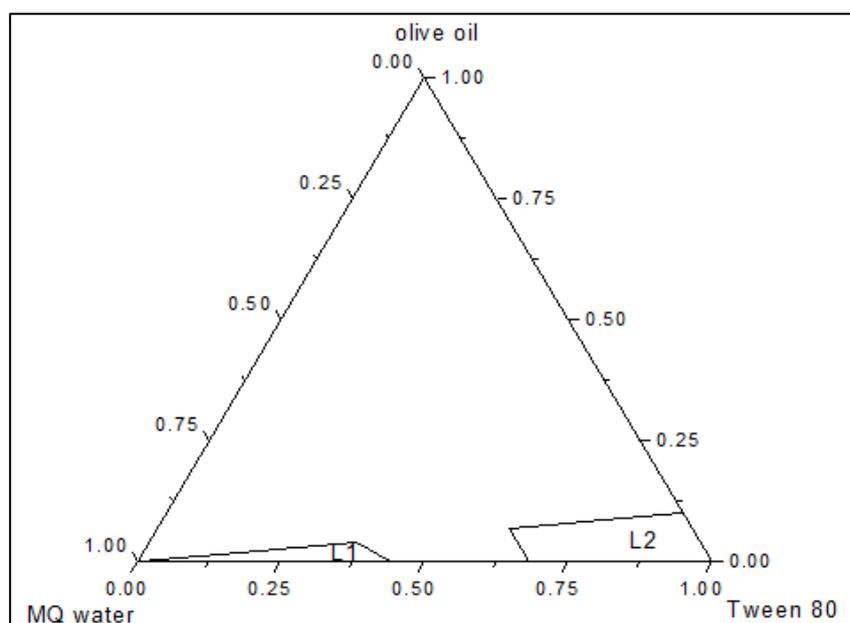


Fig.(4.5) Ternary Phase diagram of MQ water / Tween 80 / Olive Oil at ambient temperature.

From Fig.(4.5) it is obvious that the phenolic compounds played a significant role as a cosurfactant. Here (L₂) region was obtained at a surfactant concentration equals~ 90% while in Fig.(4.4) (L₂) region was obtained at a surfactant concentration equals~ 49%.

Another OLE batch of 22% total extract was prepared using MQ water with pH=2.8, acidified with 0.1N HCl, as a solvent. This extract was used in the titration of the following two systems.

4.10. Phase diagram of 22% OLE / Tween 80 / Olive Oil:Propylene glycol System.

In this phase diagram OLE of 22% total extract was extracted using acidified water. Here propylene glycol was used as a cosurfactant, to enhance the work of Tween 80. So, olive oil : propylene glycol (1:1) were added to the system. At surfactant concentration equals~ 80% a single clear isotropic region (L_2) w/o microemulsion was formed, the remainder of the phase diagram represents a two-phase region based on visual identification Fig.(4.6).

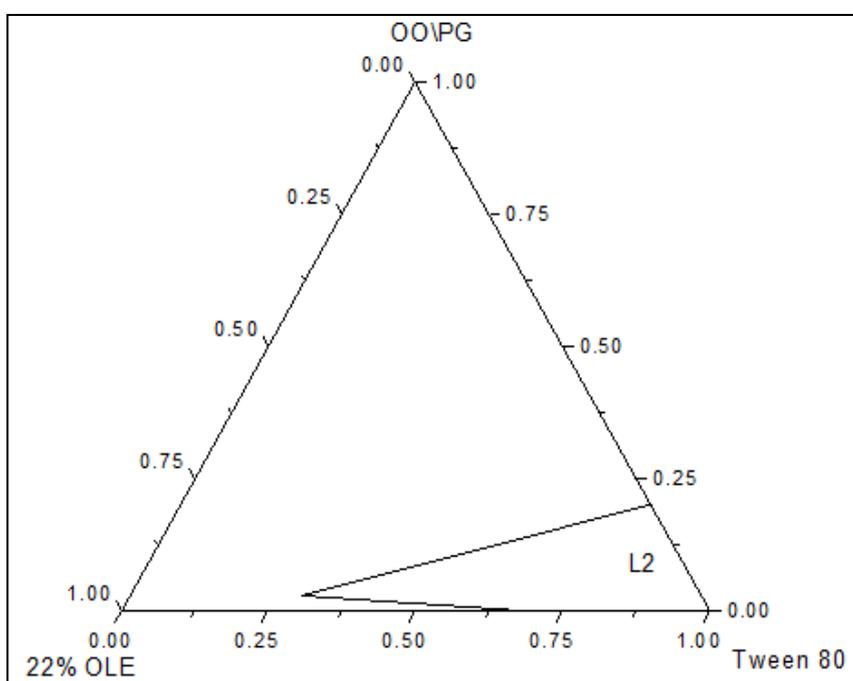


Fig.(4.6) Ternary Phase diagram of 22% OLE extracted using acidified water / Tween 80 / Olive Oil:Propylene glycol at ambient temperature.

When comparing the result of Fig.(4.6) with Fig.(4.2), it was found that in Fig.(4.6) a single clear isotropic region (L_2) w/o microemulsion was formed, at a percentage of surfactant equals~ 80%. With approximately 68% of the OLE was incorporated in the (L_2) region. While in Fig.(4.2) a single clear isotropic region (L_2) w/o microemulsion was formed, at surfactant concentration equals~ 90%. Around 28% of the OLE was incorporated in the (L_2)

region. Using propylene glycol as a cosurfactant improved the miscibility of OLE with the olive oil.

4.11. Phase diagram of 22% OLE / Tween 80 / Olive Oil:IPM System.

In this phase diagram OLE of 22% total extract was extracted using acidified water. Isopropyl myristate (IPM) was used as a co-oil since it can easily form microemulsions with conventional surfactants; therefore, it is often mixed with triglyceride oils like olive oil to enhance microemulsion formation [Sabatini, et al. 2008]. So, Olive Oil : IPM (1:1) were added to the system. The phase diagram indicated the presence of a clear isotropic (L_2) w/o microemulsion region, the remainder of the phase diagram represents a two-phase region based on visual identification. However, (L_2) region was obtained at surfactant concentration equals~ 68% Fig.(4.7).

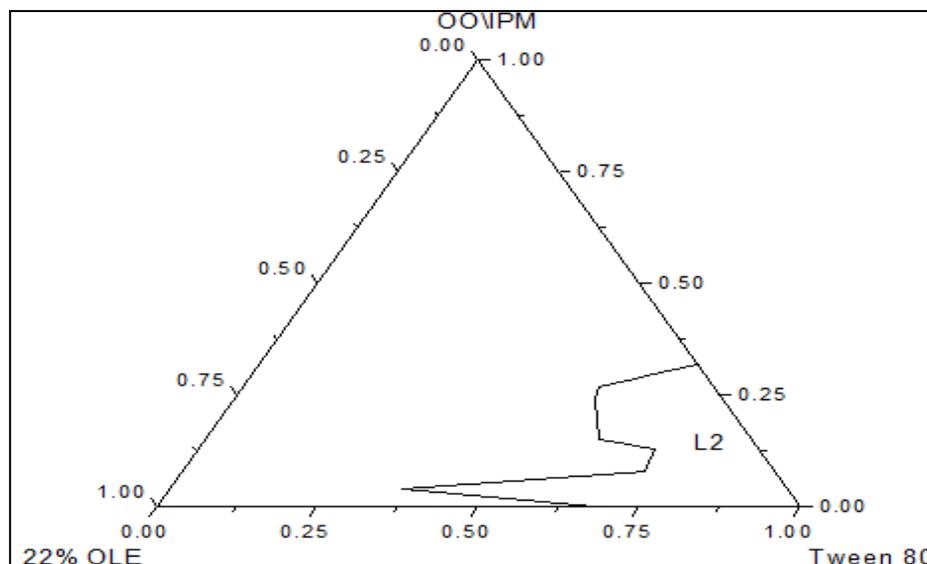


Fig.(4.7) Ternary Phase diagram of 22% OLE extracted using acidified water / Tween 80 / Olive Oil:IPM at ambient temperature.

In Fig.(4.7) presence of IPM as a co-oil enhanced the microemulsion formation. Tween 80 managed to solubilize the large triglyceride molecules at surfactant concentration equals~ 68%, and around 20% OLE was incorporated at this this concentration.

Another OLE batch of 34% total extract was prepared using MQ water with pH=2.8, acidified with 0.1N HCl, as a solvent. This extract was used for the titration of the last two systems. Here we wanted to study the effect of increasing the percentage of total extract on enhancing the microemulsion formation.

4.12. Phase diagram of 34% OLE / Tween 80 / Olive Oil:IPM System.

In this phase diagram OLE of 34% total extract was extracted using acidified water. The phase diagram indicated the presence of a clear isotropic (L_1), (L_2) and a solid anisotropic LC region. The remainder of the phase diagram represents a two-phase region based on visual identification. However, w/o microemulsion (L_2) region was obtained at surfactant concentration equals~ 50% Fig.(4.8).

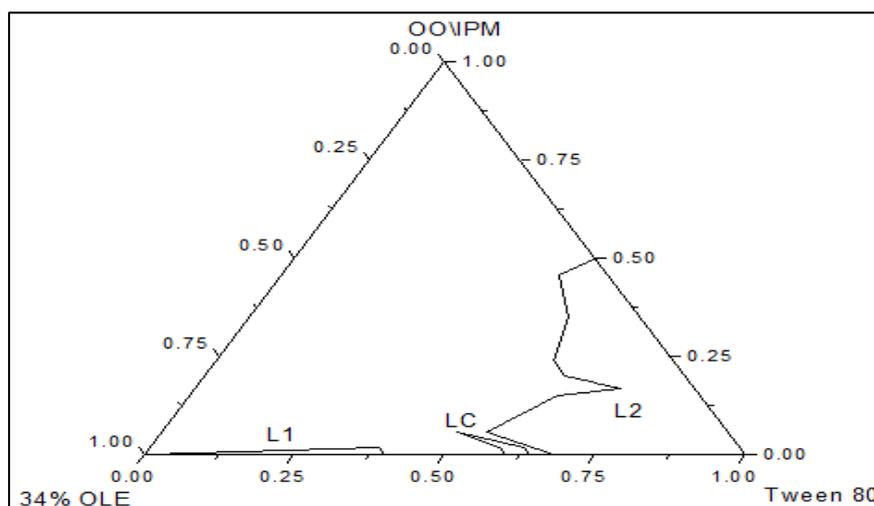


Fig.(4.8) Ternary Phase diagram of 34% OLE extracted using acidified water / Tween 80 / Olive Oil:IPM at ambient temperature.

Comparing Fig.(4.8) with Fig.(4.7), it was found that in Fig.(4.8) two new regions were formed, (L_1) o/w microemulsion and a solid anisotropic LC region. Also (L_2) region was formed at surfactant concentration equals~ 50%. While in Fig.(4.7), only (L_2) region was formed at surfactant concentration equals~ 68%. This result indicates that increasing the

percentage of total extract enhances microemulsion formation, which indicates that the phenolic compounds in the OLE act as a cosurfactant, and increasing the amount of the total extract will increase the total phenolic content.

4.13. Phase diagram of 34% OLE / Tween 80 / IPM System.

In this phase diagram OLE of 34% total extract was extracted using acidified water. Here we wanted to study the effect of using IPM as the main oil on the emulsification process. The phase diagram indicated the presence of a clear isotropic (L_1), (L_2) regions, anisotropic liquid LC region, anisotropic very viscous LC region and anisotropic solid LC region. The remainder of the phase diagram represents a two-phase region based on visual identification. However, w/o microemulsion (L_2) region was obtained at relatively low surfactant concentration equals~ 30% Fig.(4.9). And The different phases are shown in Fig(4.10). This result ensures that microemulsion formation of triglyceride oils like olive oil at ambient conditions (temperature and pressure) and without the addition of co-oil and/or alcohols is challenging at best. Olive oil is considered to be more difficult to solubilize in microemulsions than IPM. This is attributed to the presence of the large triglyceride molecules. IPM can easily form microemulsion even at low surfactant concentration.

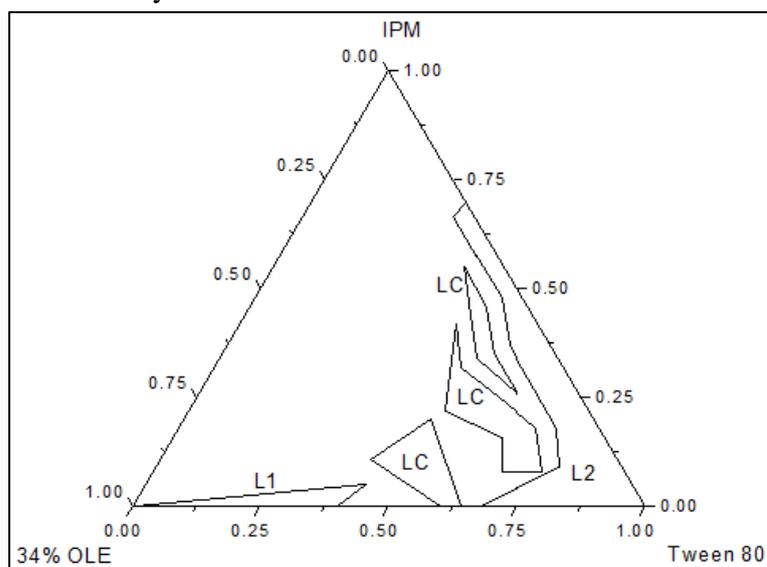


Fig.(4.9) Ternary Phase diagram of 34% OLE extracted using acidified water / Tween 80 / IPM at ambient temperature.

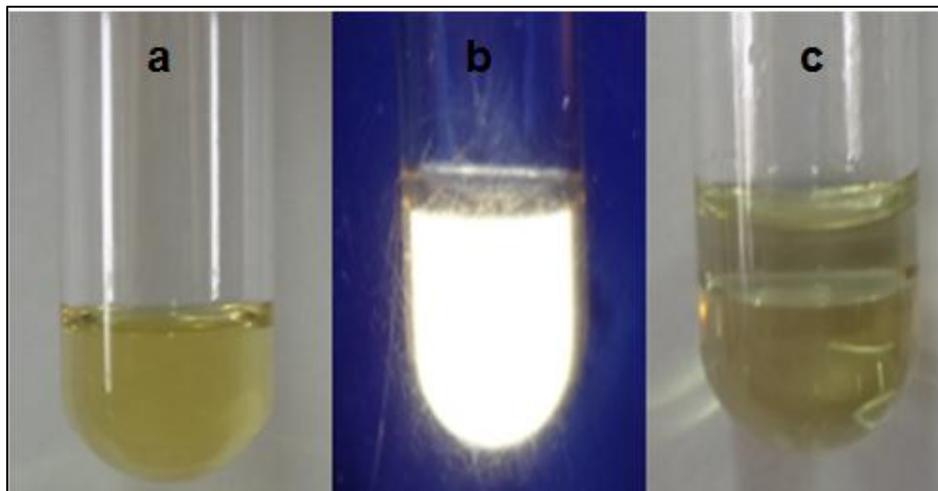


Fig.(4.10) Photos for samples representing 34% OLE/Tween 80/IPM microemulsion (a), anisotropic LC (b), and a two phase mixture (c).

4.14. Total Phenolic Content

Total phenolic content (as mg Gallic acid/g of dry olive leaf) was measured for OLE batch of 34% total extract that was prepared using MQ water with pH=2.8, acidified with 0.1N HCl, as a solvent. It was 23.49 mg/g.

Also Total phenolic content (as mg Gallic acid/g of dry olive leaf) was measured for OLE batch of 22% total extract that was prepared using MQ water with pH=2.8, acidified with 0.1N HCl, as a solvent. It was 13.53 mg/g.

This result indicates that there is a proportional relationship between OLE total extract and total phenolic content.

4.15. Dynamic Light Scattering

Dynamic light scattering (DLS) can be applied to obtain the hydrodynamic radius of the micelle, by measuring the intensity fluctuation of scattered light by the micelles when these undergo Brownian diffusion. In this part, we used the *DLS* technique to investigate the microemulsion system, as a function of temperature at aqueous phase volume fraction. The

values of the average hydrodynamic diameter (dH) as function of temperature in the aqueous phase rich region are presented in Fig.(4.11) and Fig(4.12) increases with temperature this increment indicates that the tails of the surfactants molecules residing on different droplets or fusion of droplets which lowers the curvature energy makes the micelles grow in size as the temperature increases the microemulsion droplets deform by thermal fluctuations. The droplets may undergo attractive interactions that lead to aggregation between the droplets. Similar results of the behavior of the hydrodynamic diameter as functions of temperature were reported in previous study [Fanun, 2010].

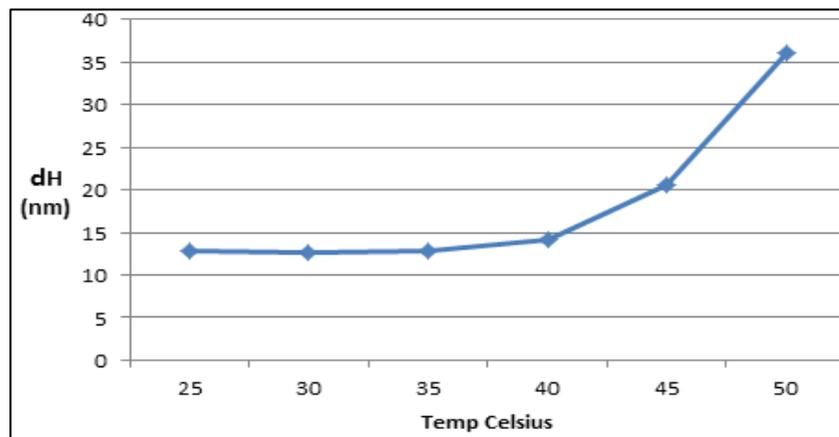


Fig.4.11 The values of the average hydrodynamic diameter (dH) as function of temperature in the aqueous phase rich region for **Fig.(4.9)** of (L1) sample with 95% surfactant concentration and diluted till 92% .

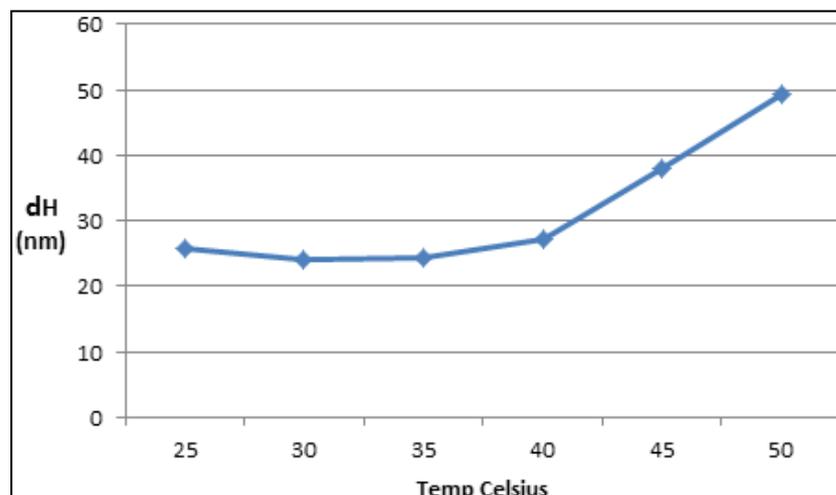


Fig.4.12 The values of the average hydrodynamic diameter (dH) as function of temperature in the aqueous phase rich region for **Fig.(4.8)** of (L1) sample with 90% surfactant concentration and diluted till 92%.

4.16. Conclusion

1. Using ethanol as a solvent to extract phenolic compounds from the olive leaves didn't help in enhancing the microemulsion. OLE that was extracted using acidified water gave better results.
2. Tween 80 has the most emulsification properties of olive oil among the three sugar based surfactants that were used.
3. Phenolic compounds helped in the formation of microemulsion and acted as a cosurfactant.
4. A stable, biocompatible microemulsion was formed that uses the benefits of OLE and extra virgin olive oil components and emulsifying these two with a biocompatible surfactant, that can be used in numerous applications, in foods, pharmaceuticals cosmetics and much more.

4.17. Future work

1. It is very interesting to accomplish this study by other interventions to determine optimum method for extraction phenolic compounds from olive leaves, and to study the effect of the OLE with the highest phenolic content on the phase behavior of OLE/Tween 80/olive oil.
2. To study the stability of oleuropein in the OLE and in the L1, L2 and LC regions over time.

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السلوك الطوري لمركبات الفينول الموجودة في مستخلص أوراق الزيتون

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مشرف ثاني: البروفيسور صالح أبو لافي

الملخص

تهدف هذه الرسالة الى البحث في استحلاب زيت ثلاثي الدهون وهو زيت الزيتون، و صياغة لمستحلب حيوي صديق للبيئة ذو جزيئات بالغة الصغر (microemulsion)، ويمكن استخدامه في الاطعمة، والأدوية، ومستحضرات التجميل. وهذا تم باستخدام مستخلص أوراق الزيتون و منشطات السطوح (surfactants) غير الأيونية مثل سوربيتان استر و الايثوكسل سوربيتان استر، اللواتي تمتزّن بقدرة جيدة من التسامح الجلدي، وانخفاض في مستوى التهيج والسمية. منشطات السطوح السكرية المستخدمة في هذه الدراسة هي (polyoxyethylene sorbitan monooleate) المعروف باسم Tween (80)، (polyoxyethylene sorbitan monolaurate) المعروف باسم Tween (20) و (sorbitan monostearate) المعروف باسم (Span 60).

أما بالنسبة للزيوت فتم استخدام زيت الزيتون كزيت أساسي، و أيزوبروبيل ميرستات المعروف باسم (IPM). بروبلين جليكول استخدم كمساعد للسطوح المنشطة (cosurfactant). وتم استخدام مستخلص أوراق الزيتون. تهدف هذه الرسالة أيضا إلى البحث في دور المركبات الفينولية كمساعدات للسطوح المنشطة (cosurfactant) في تعزيز تكوين مستحلب ذو جزيئات بالغة الصغر (microemulsion).

لقد تم في البداية دراسة تأثير استخدام مستخلص أوراق الزيتون الذي تم استخلاصه باستخدام الايثانول كمذيب على السلوك الطوري ل زيت الزيتون Span 60 \مستخلص أوراق الزيتون، و زيت الزيتون/Tween 20 /مستخلص أوراق الزيتون، و زيت الزيتون/Tween 80 /مستخلص أوراق الزيتون، و زيت الزيتون/ خليط من منشطات السطوح/مستخلص أوراق الزيتون، و زيت الزيتون/ بروبلين جليكول: Tween 80/مستخلص أوراق الزيتون.

ثم تم دراسة تأثير استخدام مستخلص أوراق الزيتون بنسبة 32%، و الذي تم استخلاصه باستخدام الماء الحمضي كمذيب على السلوك الطوري ل زيت الزيتون/ Span 60 /مستخلص أوراق الزيتون، و زيت الزيتون/Tween 20 /مستخلص أوراق الزيتون، و زيت الزيتون/Tween 80 /مستخلص أوراق الزيتون. و بعد أن تم دراسة السلوكيات الطورية السابقة تم اختيار منشط السطوح السكري الأفضل (Tween 80) من بين منشطات السطوح التي تم استخدامها لامتيازته بأفضل صفات استحلاب، واستخدامه في السلوكيات الطورية القادمة.

ثم تم البحث في تأثير استخدام مستخلص أوراق الزيتون بنسبة 22% الذي تم استخلاصه باستخدام الماء الحمضي على السلوك الطوري ل زيت الزيتون Tween 80 / IPM: /مستخلص أوراق الزيتون، ودراسة تأثير استخدام IPM كزيت مساعد (co-oil) على عملية الاستحلاب. وبنفس المستخلص تم دراسة تأثير استخدام بروبلين جليكول كمساعد للسطوح المنشطة (cosurfactant) على السلوك الطوري ل زيت الزيتون: بروبلين جليكول/ Tween 80 /مستخلص أوراق الزيتون. ثم تم البحث في تأثير استخدام مستخلص أوراق الزيتون بنسبة 34% الذي تم استخلاصه باستخدام الماء الحمضي كمذيب على السلوك الطوري ل زيت الزيتون Tween 80 / IPM: /مستخلص أوراق الزيتون و Tween / IPM /مستخلص أوراق الزيتون. تم تعيين المناطق المتباينة الخواص باستخدام المستقطب المتقاطع (cross polarizers). المحتوى الكلي من المركبات الفينولية تم تحديده لمستخلص أوراق الزيتون ذو نسبة 22% و 34% باستخدام جهاز يسمى (UV-Visible spectrophotometer). تم قياس القطر الهيدروديناميكي للمستحلب باستخدام جهاز يسمى (Dynamic light scattering).

لقد وجد بأنه استخدام الكحول كمذيب في عملية استخلاص ورق الزيتون لم يساعد في تعزيز تكوين مستحلب ذو جزيئات بالغة الصغر (microemulsion). مستخلص أوراق الزيتون الذي تم استخلاصه باستخدام الماء الحمضي أعطى نتائج أفضل. منشط السطوح الذي يعرف باسم Tween 80) يمتلك أفضل صفات استحلاب لزيت الزيتون من بين منشطات السطوح التي تم استخدامها. المركبات الفينولية ساعدت في تكوين مستحلب ذو جزيئات بالغة الصغر (microemulsion)

ولعبت دور مساعد للسطوح المنشطة (cosurfactant). المحتوى الكلي من المركبات الفينولية (ملغم حمض الجاليك/غم من ورق الزيتون الجاف) لمستخلص أوراق الزيتون بنسبة 34% هو 23.49 ملغم/غم, و 13.53 ملغم/غم لمستخلص أوراق الزيتون بنسبة 22%. يبلغ القطر الهيدروديناميكي للمستحلب لنظام زيت الزيتون: Tween 80/ IPM /مستخلص أوراق الزيتون 25.88 نانوميتر على درجة حرارة 25 °س و 12.78 نانوميتر لنظام Tween 80/ IPM /مستخلص أوراق الزيتون.

6. Index

Calibration Curve

Concentration of gallic acid (ppm)	Absorbance (765 nm)
100	0.1583
250	0.4901
300	0.5931
400	0.7477
500	1.0065

