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**Phase Behaviour, Antibacterial and Antioxidant activity
of Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*)
Extracts**

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of Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*)
Extracts**

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Thesis Approval

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Jerusalem-Palestine

1439-2017

Dedication

To my family especially my father and my mother who supported me all the way since the beginning of my life

To my sisters and brothers who have been a great source of motivation and inspiration

Asma Abu Makhu

Declaration:

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

Signed. ....

Asma Shukri Salman Abu Makhu

Date:9/12/2017

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Asma Abu Makhu

Abstract

The solubilisation behaviour of two Palestinian medicinal plants extracts which are Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) was investigated in five different micellar solutions. The extract of these medicinal plants were prepared using soxhlet method for alcoholic extract. A ternary phase diagram was constructed to assess the ability for microemulsion formulation and dilutability of each system using non-ionic surfactants, and presence of solubilisation enhancers were also studied. The antibacterial activities of these extracts by determining the ability to kill or inhibit the growth of living bacterial cells were evaluated using the well diffusion method, and the inhibitory zones were recorded in millimetres. The antioxidant potential was performed by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method.

Results of phase behaviour study showed that Tween 80 was more suitable to solubilize each of Sage and Thyme leaves extracts compared with Tween 20. It was obvious that all systems containing Propylene glycol (PG) and short chain alcohol (ethanol) showed an increase in the total microemulsion region, which is attributed to their actions as a co-solvent and co-surfactant. The antibacterial evaluation study of both Sage and Thyme extracts showed a high inhibition zone against *Staphylococcus aureus* with 22.5 mm and 17.5 mm respectively as gram positive bacteria, and against *Candida albicans* with 20.0 mm and 29.0 mm for Sage and Thyme respectively. On the other hand, no activities for both extracts were found against *Escherichia coli*. The antibacterial activity was also tested on the non-ionic microemulsion formulation (22% plant extract+ 54% Tween 80+ 24% water: PG) for Sage and Thyme. They showed significant activity against each of *S.aureus*, *E.coli* and *C.albicans* with much lower extract concentration compared to the surfactant free formulation. Both of Sage and Thyme ethanolic extracts act as a natural antioxidant agents with a relatively high inhibition percentage (84.72% and 86.26%), respectively. On

the other hand the non-ionic microemulsion formulation (26% plant extract+ 62% Tween 80+ 12% water: PG) showed a very high antioxidant scavenging activity compared to the pure Sage and Thyme ethanolic extracts which have higher extract concentration.

Results of this thesis shed the light on the solubilisation capacity and phase behaviour of Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) in non-ionic microemulsion, and the potential of using it in antibacterial and antioxidant applications.

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Chapter one

Introduction

1.1. Microemulsion

The microemulsion concept was first scientifically reported by Schulman and Hoar as early as 1940s, they observed that isotropic and optically transparent dispersions of oil in water (o/w) or water in oil (w/o) formed spontaneously in the presence of a surfactant and a co-surfactant such as aliphatic alcohol [1]. Later in 1981 Danielsson and Lindman define the microemulsion as a system of water, oil and an amphiphile which is a single optically isotropic and thermodynamically stable liquid solution [2].

Microemulsions are macroscopically homogenous mixture but microscopically they are heterogeneous and form a multitude of structures. There are differences between emulsions, microemulsions and nanoemulsions in terms of stability and structure. In contrast to the microemulsions the emulsions and nanoemulsions are thermodynamically unstable systems and their formation requires input of energy through high shear conditions. The other difference is that the average drop size of the emulsions and nanoemulsions grows continuously with time so the phase separation ultimately occurs under gravitational force, while in microemulsions the size of micelles are in the range of 10-100 nm depending on some parameters such as surfactant type, concentration and the extent of other dispersed phase [3,4,5].

A well-known classification of microemulsions is that of Winsor [6], who studied the phase behaviour of water-oil-surfactant mixtures and classified four types of phase equilibria as in figure (1.1), where type (I) indicates surfactant-rich water phase (lower phase) that coexists with surfactant-poor oil phase. Type (II) is surfactant-rich oil phase

(the upper phase) that coexists with surfactant-poor water phase. Type (III) represents the surfactant rich middle-phase which coexists with both water (lower) and oil (upper) surfactant-poor phases called bicontinuous. Type (IV) is a single phase homogeneous mixture. When surfactant (and co-surfactant), water and oil are combined in correct proportions a single phase microemulsion is formed (type IV).

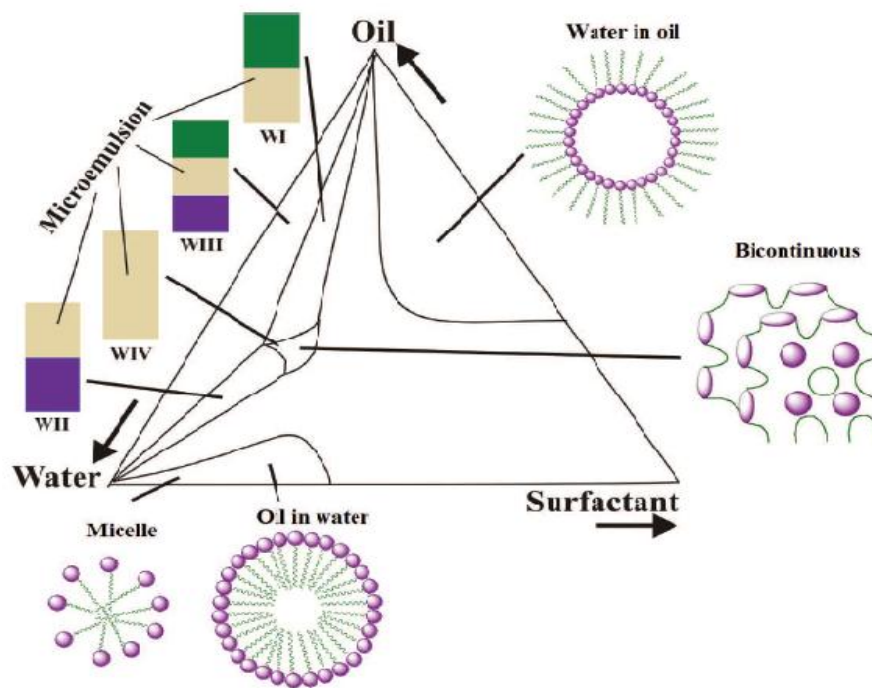


Figure 1.1: Schematic Ternary phase diagram of various types of microemulsion systems as classified by Winsor.

Solubilisation and interfacial properties of the microemulsions depend upon pressure, temperature and also on the nature and concentration of the oil and surfactant [5,7]. Microemulsions are usually characterized by ternary phase diagram, composed of three

edges which are namely oil, water, and surfactant. The co-surfactant that acts synergistically with the surfactant to alter the interfacial curvature and therefore lowers interfacial tension, co-surfactant are usually grouped together with the surfactant at a fixed ratio, and the co-solvent are grouped with the water edge [8].

Microemulsions have found applications in a wide variety of chemical and industrial processes; their wide use in both research and industry is in part due to their unique properties that are low interfacial tension, high thermodynamic stability, high interfacial area, and the ability to dissolve immiscible liquids.

Beside their main and interested applications in pharmaceutical industry to enhance drug delivery [9], they are used in enhanced oil recovery [10], and as lubricant, corrosion inhibitors, coating and textile finishing and it is also used in cosmetics, detergency, agrochemical, food, environmental remediation and detoxification, analytical applications as liquid membranes and biotechnology [11].

1.2.Surfactants

Surfactants (or surface active agent) are organic compounds that interact with an interface. Surfactants have a characteristic molecular structure consisting of a structural group that has strong attraction for the water (solvent), called hydrophilic group, together with a group that has very little attraction for the water, known as hydrophobic group, which is usually a long-chain hydrocarbon [12], as shown in figure (1.2).

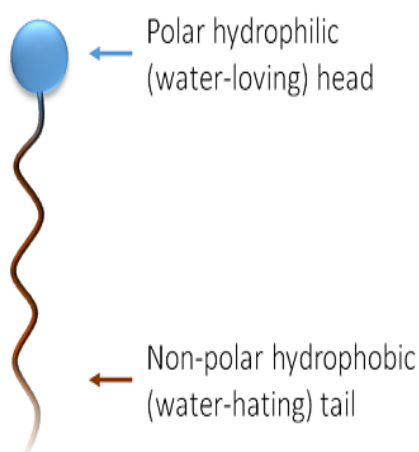


Figure 1.2: General structure of a surfactant molecule.

Generally, based on the nature and the type of the surface active moiety group present in the molecule, surfactants are classified as anionic in which the hydrophilic part is a negatively charged group, cationic the hydrophilic part is a positive charge, non-ionic surfactants the hydrophilic has no charge, but derives its water solubility from highly polar groups, and in case both cationic and anionic centres are present in the same molecules, they are termed as Zwitterionic (amphoteric) surfactants [13].

Co-surfactants are usually used in conjugation with surfactants being incapable of reducing the interfacial tension of oil and water to form a microemulsion. The most common

co-surfactants are short chain alcohols (C_1 - C_{10}), which reduce the tension and increase the fluidity of the oil-water interface, thereby increasing the entropy of the system. These short chain alcohols also increase the motility of the surfactants nonpolar tail region, allowing greater penetration by oil molecules and therefore stabilizing the system and facilitating the formation of microemulsion [14,15].

1.3.1 Non-ionic surfactants

Surfactants that carry no electrical charge, as their water solubility is derived from the presence of polar functionalities capable of significant hydrogen bonding interaction with water [16].

Surfactants from this group are commonly used to formulate microemulsions due to their low toxicity, lack irritation and not affected by water hardness [17]. The water soluble surfactants Tween 80 and Tween 20 are used in this study.

Tween 80 and Tween 20 are common non-ionic surfactants, emulsifiers, wetting agents and solubilizers that are used in a wide variety of industrial applications; used in food products, medications, and cosmetics. Tween 20 is a clear, yellow to yellow-green viscous liquid derived from polyethoxylated sorbitan and lauric acid showed in figure (1.3). Tween 80 is a viscose, yellow to amber liquid, derived from polyethoxylated sorbitan and oleic showed in acid figure (1.4). They are non-toxic, environmental friendly, biocompatible and commercially inexpensive surfactants. [18,19,20].

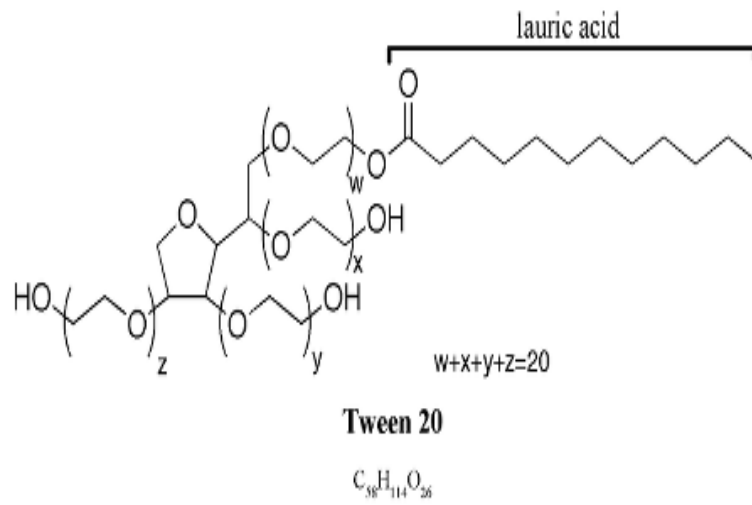


Figure 1.3: Chemical structure of Tween 20 (Polyoxyethelene (20) sorbitan monolaurate)

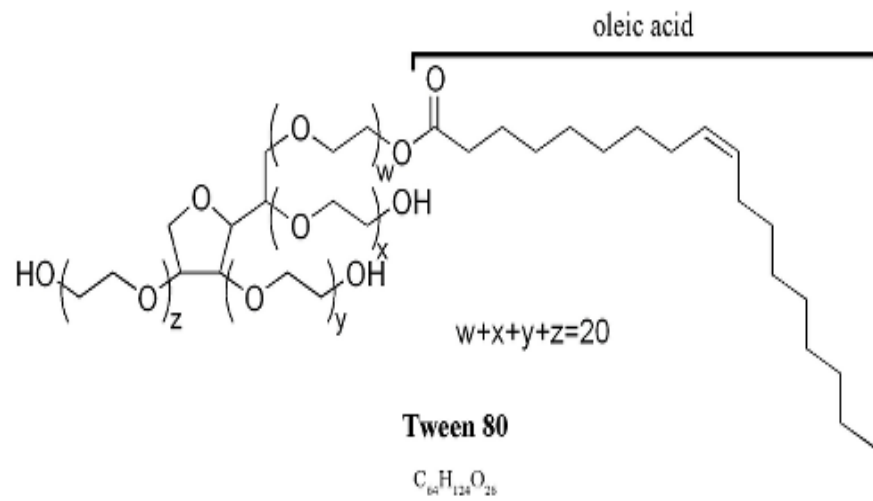


Figure 1.4: Chemical structure of Tween 80 (Polyoxyethelene (20) sorbitan monooleate)

1.3.1 Sage (*Salvia Officinalis*)

One of the most popular herbs in the Palestinian territories, its scientific name is *Salvia Officinalis* derived from the Latin word *salvere*, which means “to be saved”. It belongs to the Lamiaceae family, native to the Mediterranean area and Asia Minor and now cultivated widely.

It is an evergreen perennial subshrub grows up to 75 cm in high with features woody, branching stems, and pebble-like patterned, aromatic, grayish leaves. Soft surface with fine hair like filaments growing on either side. During summer it bears violet-blue flowers in bunch attract bees [21].



Figure 1.5: leaves of Sage (*Salvia Officinalis*).

There are several types of sage grown either for medicinal or culinary purposes, which are pineapple sage, clary sage, three lobed sage and azure sage. The herb leaves are used in traditional medicine as herbal tea to treat digestive and circulation disturbances, bronchitis,

cough, asthma, angina, mouth and throat inflammations, depression, excessive sweating, skin disease and many other diseases [22].

The essential oil of *salvia* species provides several benefits from a wide range of properties. It functions as an effective antibacterial, antioxidant, anti-inflammatory, free radical scavenging, antimalarial and antitumor agent. It has been found to be very effective in the development of novel natural drugs to prevent, treat, and control many minor health problems as well as more serious and complicated diseases such as Alzheimer's, diabetes, and cancer [23,24].

The chemical composition of *salvia officinalis* essential oil composes mainly of α and β -thujone, camphor, and borneol showed in figure (1.6).

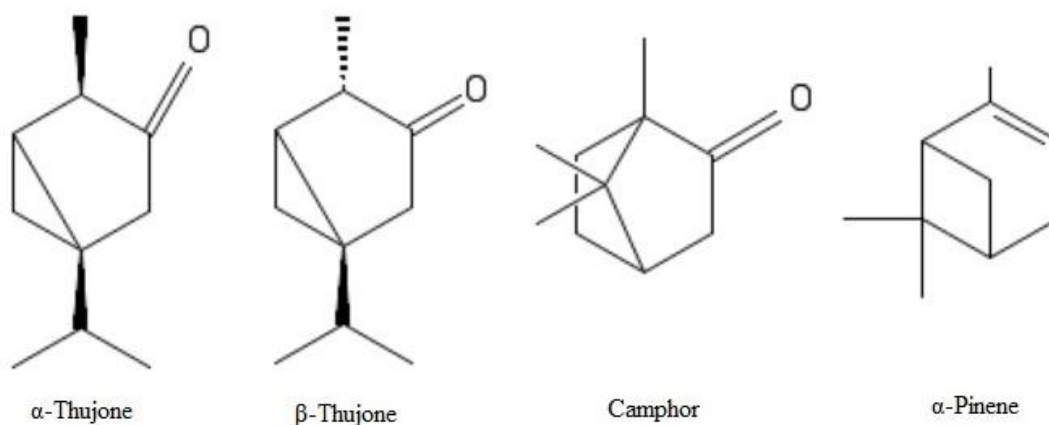


Figure 1.6: The main chemical compounds of Sage (*Salvia Officinalis*) [23].

1.3.2. Thyme (*Thymus Vulgaris*)

Are aromatic plants of the Mediterranean region and common herb in North Africa, and is commercially cultivated in large scale in many countries. Thyme plant is an easy to grow on rocky soil and hot, dry condition, which requires little or no care. The scientific name of thyme is *Thymus Vulgaris*; it belongs to the Mint family, *labiatae*. It is herbaceous perennials subshrubs with a bushy, woody-based evergreen plant, rarely grows more than 40 cm tall, with highly aromatic, tiny, gray-green leaves which is responsible for its characteristic flavour and fragrance. A beautiful purple flowers bloom in the early summer. [25]



Figure 1.7: Thyme (*Thymus Vulgaris*)

Thyme species and extracts are used as a traditional medicine as herbal tea to treat cold, flu and cough. It has been reported that thyme in general contains various medicinal benefits and is used as carminative, digestive, antispasmodic, anti-inflammatory and expectorant as well as powerful disinfectant in oral pharmaceutical preparation and flavoring agent of many food products. [26]

The essential oils of the thyme species contain large amounts of thymol, which is strong antibacterial agent [27], as well as a strong antiseptic and antioxidant [28].

Thymus vulgaris essential oil is a mixture of monoterpenes. The two main compounds of this oil are the natural terpenoid thymol and its phenol isomer carvacrol that are shown in figure (1.8).

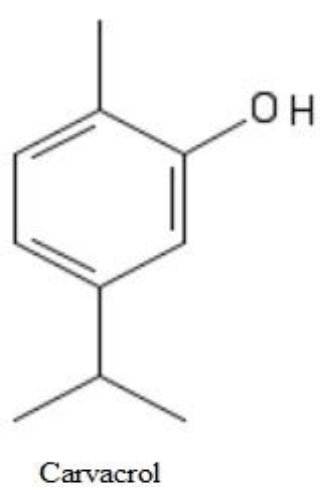
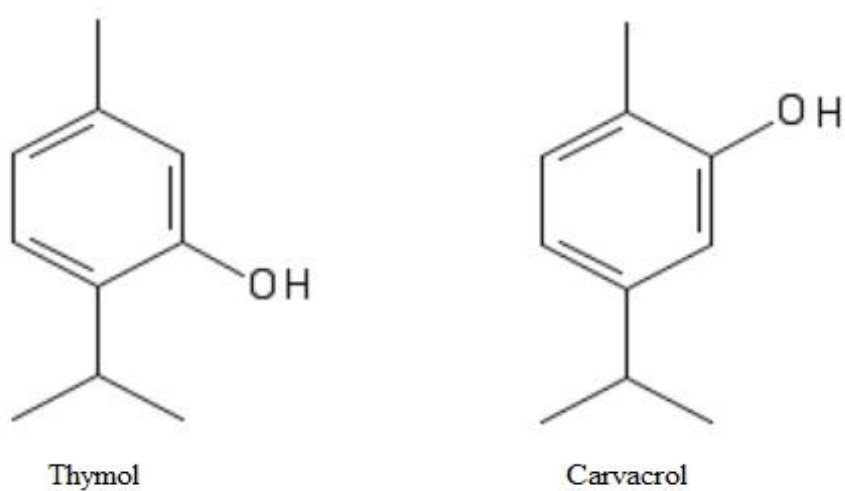


Figure 1.8: The main chemical compounds of *thymus vulgaris* [29].

1.4. Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) extraction method

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedure. The products so obtained from plant are relatively impure liquids, semisolid or powders. There are many extraction methods that have been reported for the plants extractions, such as infusion, soxhlet extraction, super critical fluid, ultrasound extraction and microwave assisted extraction [30].

Soxhlet extraction is one of the oldest method and most widely used approaches for conventional extraction of solid sample. The advantage of this method is that large amounts of plants can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs. It consists of a simple distillation process repeated a number of times [31].

In this method the finely grinded leaves is placed in thimble made of strong filter paper, which is placed in a chamber of Soxhlet apparatus as in figure (1.9). The extracting solvent in the round bottom flask is heated using heat mantel to reflux, and its vapors are condensed in a condenser. The solvent vapour travels up to distillation arm and drips into the reservoir containing the thimble, and the condenser ensure that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent, some of the desired compound will then dissolve in the warm solvent. When the soxhlet chamber is almost fill the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle is repeated many times, over hours or days.

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compounds is concentrated in the distillation flask, the advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.

After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound, the non-solvent portion of the extracted solid remains in the thimble, and is usually discarded [32].

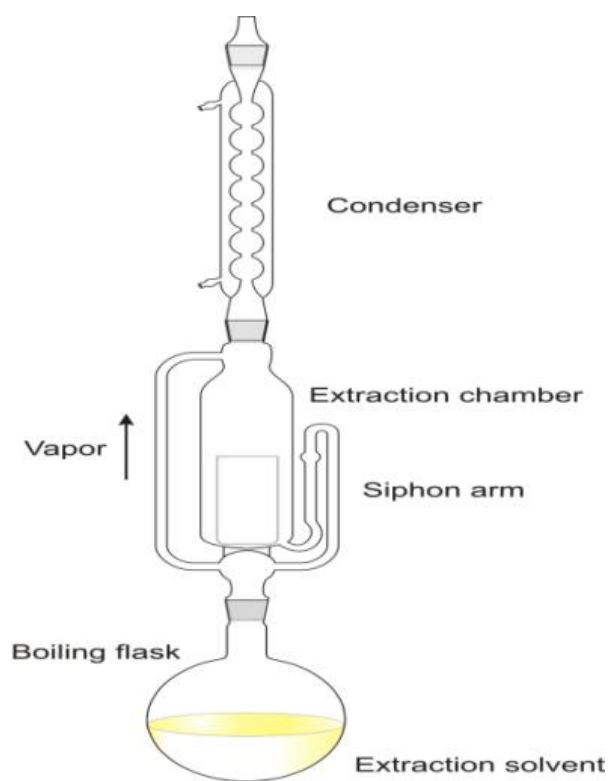


Figure 1.9: Soxhlet Extractor scheme

Chapter Two

2. Literature review

H. Shaaban and A. Edris studied factors affecting the phase behaviour of carvacrol microemulsions and its antimicrobial activity. The factors affecting the phase behaviour were the type of surfactants and presence of solubilisation enhancers. The results showed that the phase behaviour of carvacrol is a challenging phenolic compound which did not solubilize easily in a fully dilutable non-ionic microemulsion. Incorporation of propylene glycol (PG) as a solubilisation enhancers and short chain alcohol like ethanol was solved this problem. The antimicrobial activity of the non-ionic carvacrol microemulsion formulated with Tween 20 and a solubilisation enhancers did not exhibit better antimicrobial activity than the surfactant free carvacrol. On the other hand, the cationic cetylpyridinium chloride (CPC) formulated carvacrol microemulsion showed significantly higher antibacterial activity than Tween 20 formulated microemulsion [33].

N. Garti *et. al.* Studied the improvement of oil solubilization in oil/water food grade microemulsion in the presence of polyols and ethanol. The compositions included water, oil, short chain alcohols (ethanol), polyols (propylene glycol) and several surfactants including tween 80 and tween 20. The solubilization capacity was dramatically improved in the presence of ethanol and propylene glycol, by increasing the fluidity of the interface, and the liquid crystalline phase area was diminished drastically. Tween 80 showed a larger microemulsion area than tween 20, since tween 80 being more hydrophobic surfactant that solubilized the maximum oil in the aqueous surfactant phase [34].

D. Smit *et. al.* Studied the effects of chain length of surfactants on the interfacial tension. They presented simple oil/ water/ surfactant model, the experimental results on various

model of surfactants indicate that increasing the tail length of the surfactant makes it more effective in decreasing the interfacial tension [35].

S. Abah and L. Egwar compared between two methods of extracting to test the antimicrobial susceptibility of plant extract. They found that the Soxhlet extraction yielded more extract than the other method which is cold extraction, indicating that it is a better method to obtaining more extractable component from leaves. They found that Soxhlet extracts were more oilier than the cold one and suggesting that Soxhlet extraction could be one of the novel methods of extracting oil from leaves. On the other hand, they found that cold method extraction produced wider zone of inhibition and more activity because the action of heat in Soxhlet extraction had inactivated some active components of the extraction [36].

H. N. Qaralleh *et. al.* Studied the antibacterial activity in vitro of thyme from Jordan. A dried ground powder leaves and stems were extracted with number of solvents which are water, ethanol, dichloromethane and hexane using Soxhlet method. The antibacterial activity of these extract was evaluated against bacteria using disk diffusion method. The result showed that the leaves had stronger antibacterial activity than stems extracts, and the ethanolic extract had the highest yield products than all other solvents. They also distinguished between the antimicrobial activity of leaves ethanol extracts (LEE) and essential oils leaves extracts (LEO) of thyme leaves, the (LEO) showed greater antibacterial activity than (LEE) [37].

A. Ashour and Z. ElAstal studied the antimicrobial activity of some Palestinian medical extracts which contain sage and thyme. They used the disk diffusion method to evaluate the antimicrobial activities of the extracts against ten different pathogenic microorganisms. The aqueous extracts of sage and thyme showed a broad antimicrobial action against most of the tested microorganisms, and no antimicrobial effect on E.coli as gram negative bacteria. They recommended that sage and thyme extracts may be used for food preservation, as well as pharmaceutical and natural plant based products [38].

M. M. Abdelfadel *et. al.* studied the effect of extraction methods on total phenolic compounds, antioxidant and antimicrobial activities of some spices and herbs extracts including *Thymus vulgaris*. They compared between cold and hot extracts methods. The results showed that hot extract led to increase the total phenolic compounds in thyme, and its antioxidant activity was increased from 82.3% to 91.9% in comparison with cold extraction method. On the other hand, the antibacterial effect of thyme extracts was decreased by hot extraction methods and that is due to the decrease in some of thyme phenolic compounds which has good antibacterial effects [39].

M. Tofana *et. al.* evaluated the antioxidant activity and phenolic content in different *Salvia Officinalis L.* extracts. They used three different techniques and five solvents including methanol and ethanol, for extraction of bioactive compounds from *Salvia Officinalis L.*, the total phenolic content and the antioxidant activity of plant extracts were determined by Folin-Ciocalteu method and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, respectively. Methanolic extract exhibited the highest content in phenolic compound and antioxidant capacity (85.12%). The ethanolic extract showed very good antioxidant capacity (78.43%) as well as high phenolic content [40].

Chapter Three

3. Objectives:

To extract the Sage and Thyme leaves by using Soxhlet Ethanol Extraction Method.

To investigate the best ternary Phase Diagram for both extracts based on non-ionic surfactant.

To study the effect of using short chain alcohol and using propylene glycol as solubilisation enhancers.

To study the efficacy of Sage and Thyme extracts as antibacterial and antioxidant.

To study the efficacy of Sage and Thyme extracts contained microemulsion formulations and solubilisation enhancers as antibacterial and antioxidant.

Chapter Four

4. Methods and Experiments

4.1. Materials

Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) leaves were collected from Ramallah-Palestine, and were certified to approve the botanical identification by Dr. Khaled Sawalha (Botanist & Associate professor at Biology department, faculty of science & technology. Al-Quds University- Palestine). Ethanol 95% (EtOH), propylene glycol (PG), Tween 80, Tween 20, distilled water was used in all microemulsion formulation.

Mueller Hinton Agar, sterile cotton swab, micropipette, *Staphylococcus aureus* as gram-positive bacteria, *Escherichia coli* as gram-negative bacteria and *candida albicans* yeast, DPPH (1, 1-diphenyl-2-picrylhydrazine), ascorbic acid.

4.2. Instrument and Equipment

Soxhlet equipment (round bottom flask, extraction chamber, condenser, thimble, heat mantel), four digit analytical balance, vortex, cross polarizer, 10ml glass test tubes with screw cap, syringes, thermostatic water bath, plastic petridishes, Buchner funnel, Sterilizer, Incubator, UV-Vis Spectrophotometer.

4.3.Methods

4.3.1. Sage and Thyme leaves sample preparation

The fresh sage and thyme leaves were washed with water to remove dust, and then the leaves were exposed to air drying at room temperature for 2 weeks. The dried leaves were crushed in order to decrease the particle size and increase the surface area, and sample were then stored in dark and cool place until used for extraction.

4.3.2. Soxhlet ethanol extraction of sage and thyme leaves

About 18 g of grinded air dried leaves material were extracted with 300 ml ethanol 95% added to 1000 ml round bottom flask. The process runs for a total 12 h, and the extracts were filtered through Whatman filter paper using buncher funnel to make sure there are no impurities, then the ethanol were evaporated and recollected using rotary evaporator, the extracts were then stored at 4°C until use.

4.3.3. Constructing of ternary phase diagram

The pseudo ternary phase diagrams consisting of oil, water, surfactant and co-surfactant mixture were constructing using water titration method.

For the microemulsion formulation each of Sage and Thyme extracts were dissolve in 95% ethanol at two different ratios (1:2) and (1:3) by weight, and were mixed with the surfactant (T80 or T20) at different weight ratios as in table (4.1), and were inserted in 10ml glass test tubes with screw caps.

Table 4.1: Surfactant/ Oil weight ratio.

Weight ratio	1:9	2:8	3:7	4:6	5:5	6:4	7:3	8:2	9:1	10
Surfactant	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Oil	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0

The mixture was then vortexed for 2-3 minutes, due to the high viscosity of the surfactant and extracts in order to guarantee a homogenous dispersion. After 48h a drop by drop titration of water phase (water or water: PG) with specific weights was injected as in table (4.2). The tubes then were left at rest for 24h to reach equilibrium before the next addition of water phase and analysing.

The tubes temperature was controlled by placing it within the thermostatic water path at (25 ± 1) °C if necessary.

Table 4. 2: Water phase percentage and Weight.

<u>%Water phase</u>	4%	8%	12%	16%	20%	24%	28%	32%	36%
<u>Weight</u>	0.0417	0.0453	0.0494	0.0541	0.0595	0.0658	0.0732	0.0817	0.0919
<u>%Water phase</u>	40%	44%	48%	52%	56%	60%	64%	68%	72%
<u>Weight</u>	0.1042	0.1191	0.1374	0.1603	0.1894	0.2273	0.2773	0.3473	0.4466
<u>%Water phase</u>	76%	80%	84%	88%	92%	96%			
<u>Weight</u>	0.5955	0.8337	1.2070	2.0833	4.1667	12.5			

Five microemulsion systems for each of sage and thyme extract were prepared at room temperature; the composition of each system is detailed in table (4.3).

Table 4. 3: Microemulsion composition of five different systems.

<u>System #</u>	<u>Composition</u>
System(1)	Water + Tween80 + extract/ethanol (1:2)
System(2)	Water + Tween20 + extract/ethanol (1:2)
System(3)	Water: PG + Tween80 + extract/ethanol (1:2)
System(4)	Water: PG + Tween20 + extract/ethanol (1:2)
System(5)	Water: PG + Tween80 + extract/ethanol (1:4)

Microemulsion was identified by visual inspection after each addition of water phase as transparent, single phase and low viscous mixture. The anisotropy was detected by cross polarizers, and finally the phase diagrams were drawn using Origin 2017.

4.3.4. Antibacterial activity

Antibacterial activities of Sage and Thyme extracts and their microemulsion formation were evaluated using well diffusion method on Mueller- Hinton agar.

Mueller-Hinton agar was prepared by adding 19g to 1 L of distilled water, and then boiled to dissolve the medium completely, then it was sterilised by autoclaving at 121°C for 30 minutes. After that it was cooled to about 45°C, and an amount of 20-30 ml of Mueller-Hinton agar was poured on plastic petridishes of the same size and allowed to solidify. Agar plates were streaked with the reference bacterial strains which are: *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*, under aseptic conditions to prevent contamination. Wells (diameter =9 mm) were filled with 50µl of the test samples (sage and thyme extracts and their microemulsions formulations, ethanol 95% as negative solvent), and incubated at 37°C for 24h. After the incubation period, the diameter of the growth inhibition zones was measured from edge to edge of the clear area around the wells containing the samples. No measurement was taken if no clear zone of inhibition was observed. Measuring rule in millimetre (mm) was used to take the measurement from edge of the well to the end of the clear zone of inhibition.

4.3.5. Antioxidant capacity

The antioxidant capacity was assessed using the DPPH free radical scavenging assay according to Odriozola-Serrano *et al.* [41]. DPPH is the easiest, simple and reasonably costly method and most commonly used method for the evaluation of the antioxidant activity of a sample [42].

A volume of 3.9 ml from 0.1mM ethanolic DPPH solution was added to 0.1 ml of sample solution of different concentration, and the mixture was vigorously shaken, after 30 minutes of incubation in darkness, the absorbance of each sample was measured at 517 nm against a blank of ethanol using UV-VIS Spectrophotometer. Inhibition percentage was measured by comparing with control solution, which had only reagent DPPH with no extract in it, ascorbic acid was used as positive control, the percentage of DPPH radical scavenging capacity of each plant extract was calculated using equation (1).

$$\text{RSA\%} = [A_0 - A_1 / A_0] \times 100 \quad [1]$$

Where: RSA is the radical scavenging activity, A_0 is the control absorbance, A_1 is the sample absorbance.

Chapter Five

5. Results and Discussion

4.2.Ethanol Soxhlet extraction of Sage and Thyme

Solvent extraction is one of the most widely employed methods for preparation of leaves extraction. Solvent extraction (solid-liquid extraction) involves the process of leaching which is a separation technique that involves removal of soluble solids from a solid mixture by employing a suitable solvent [44]. In this study ethanol 95% was used as a solvent, which is the second most important solvent after water and the least toxic of alcohols, which make it more suitable for using in industry and consumer products [45].

The extracts of Sage and Thyme leaves were extracted by Soxhlet method using ethanol 95% as an organic solvent, for total of 12h. A dark green solution was obtained after many cycles of the solvent, and then the solvent was removed using the rotary evaporator technique, yielding a dark green paste extracted compound. The extraction yield percentage of the extracted leaves of Sage was about 43.2% and about 46.35% for Thyme based on air dried leaves. High ethanolic extracts yield was also found in previous published results [37], they showed that the ethanolic extract using Soxhlet method had the highest yield products, compared to dichloromethane and hexane, and found that the leaves yield crude extracts were higher than stems crud extracts.

5.2. Formation of microemulsion

5.2.1. Phase behaviour of Tween 20 or 80/ Water / Plant Extract: Ethanol (1:2)

Ternary phase behaviour of the sage and thyme ethanolic extracts were obtained a different microemulsion regions under the same formulation conditions, using two different non-ionic surfactants which are Tween 20 (polyoxyethylene (20) sorbitane monolaurate) and Tween 80 (polyoxyethylene (20) sorbitane monooleate) at 25°C.

Figure (5.1) presents the ternary phase diagram of the system water/ Thyme+ ethanol/ Tween 20 at 25°C, made of (1:2) ratio of Thyme extracts and 95% ethanol. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% water from the point containing 60% Thyme+ ethanol extract and 40% Tween 20, and extend to up to the water apex (100%).

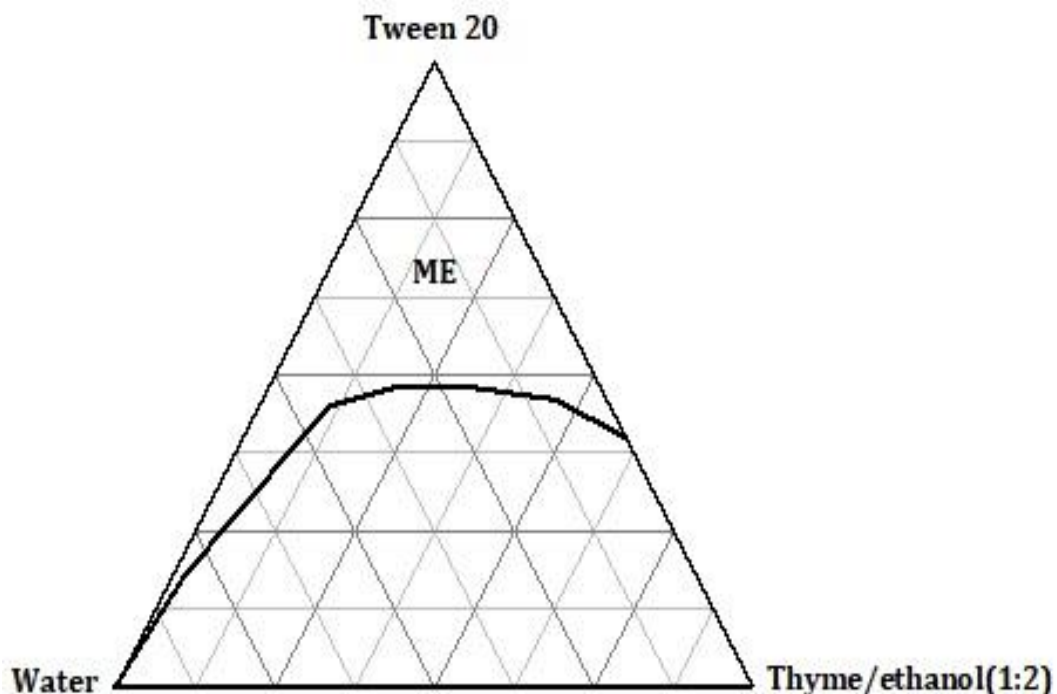


Figure 5.1: Ternary Phase diagram of system: Water/ Thyme: ethanol (1:2)/ Tween 20 at 25°C. The one phase region is represented by ME.

Figure (5.2) presents the ternary phase diagram of the system water/ Thyme+ ethanol/ Tween 80 at 25°C, made of (1:2) ratio of Thyme extracts and ethanol. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% water from the point containing 65% Thyme+ ethanol extract and 35% Tween 80 point, and extend to up to the water apex 100%.

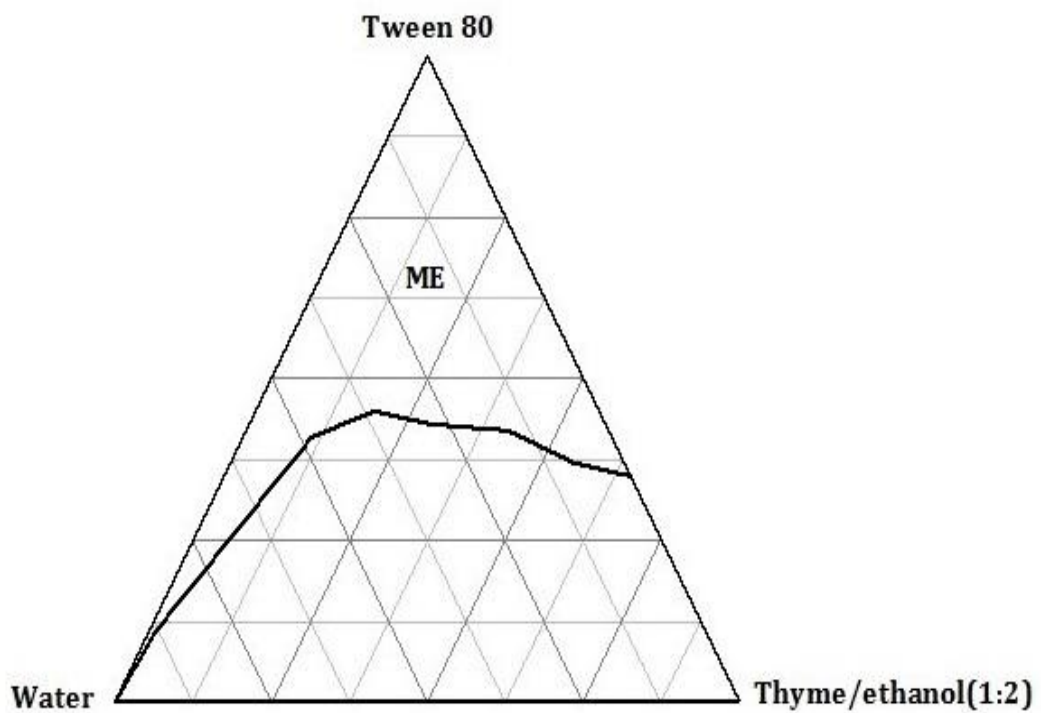


Figure 5.2: Ternary Phase diagram of system: Water/ Thyme: ethanol (1:2)/ Tween 80 at 25°C. The one phase region is represented by ME.

Figure (5.3) presents the ternary phase diagram of the system water/ Sage+ ethanol/ Tween 20 at 25°C, made of (1:2) ratio of Sage extracts and ethanol. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% water from the point containing 60% Sage+ ethanol extract and 40% Tween 20 point, and extend to up to the water apex 100%.

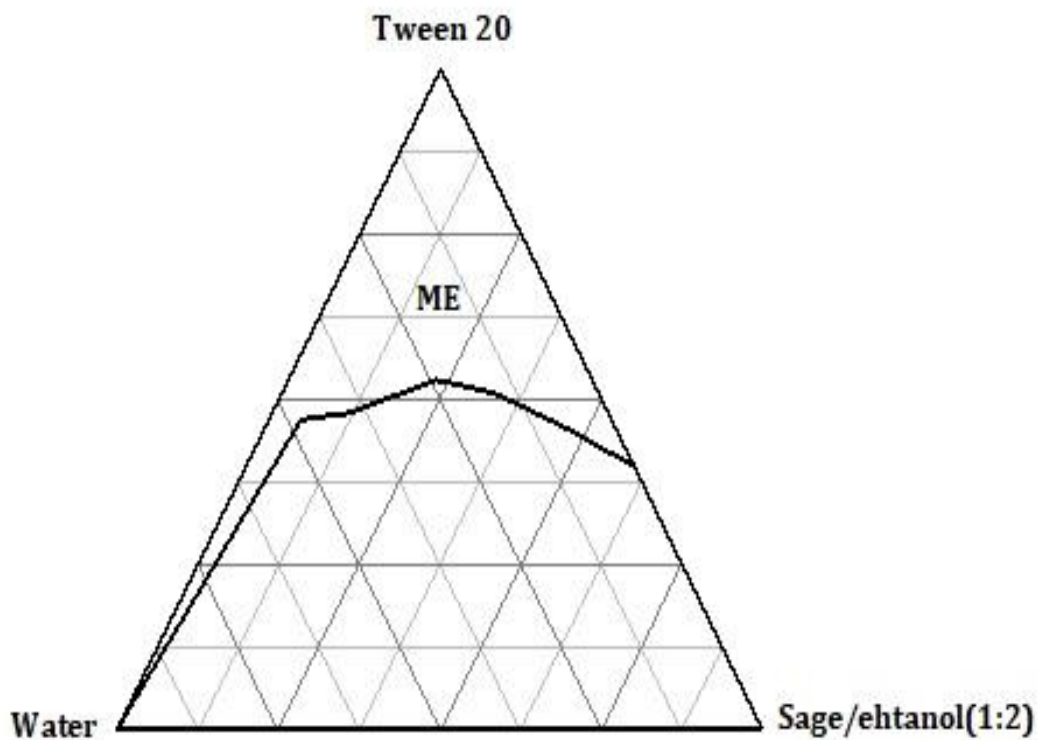


Figure 5.3: Ternary Phase diagram of system: Water/ Sage: ethanol (1:2)/ Tween 20 at 25°C. The one phase region is represented by ME.

Figure (5.4) presents the ternary phase diagram of the system water/ Sage+ ethanol/ Tween 80 at 25°C, made of (1:2) ratio of Sage extracts and ethanol. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% water from the point containing 65% Sage+ ethanol extract and 35% Tween 80 point, and extend to up to the water apex 100%.

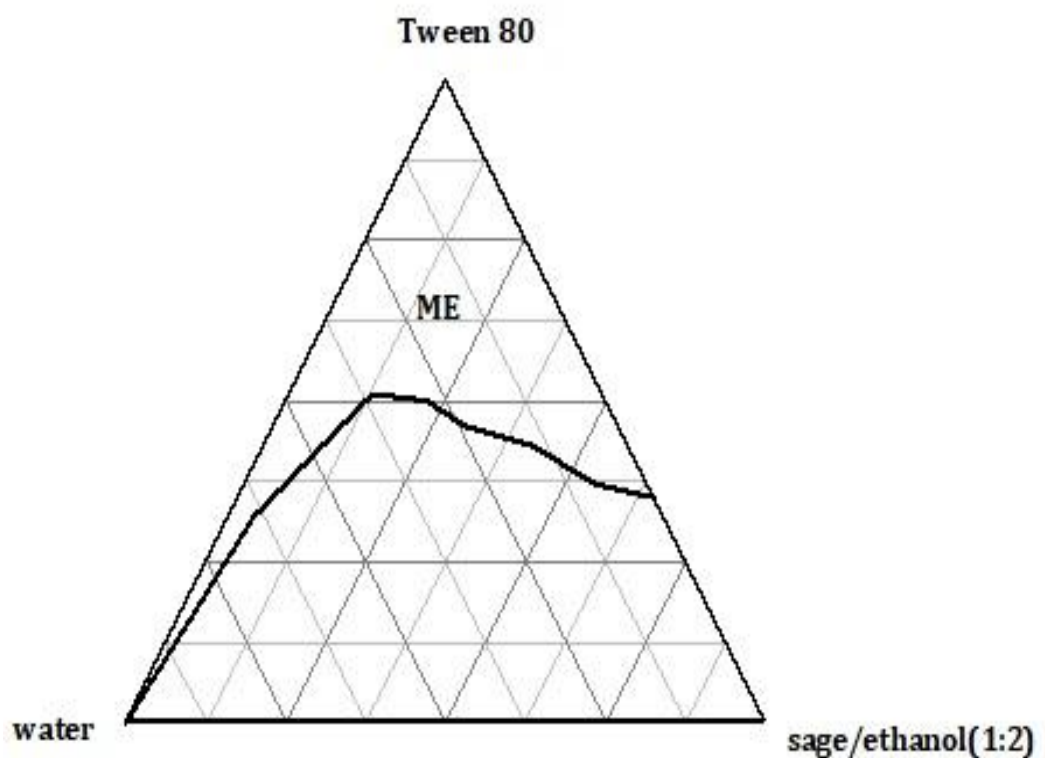


Figure 5.4: Ternary Phase diagram of system: Water/ Sage: ethanol (1:2)/ Tween 80 at 25°C. The one phase region is represented by ME.

The above figures showed that each of sage and thyme extracts formulated in microemulsions using Tween 80 exhibited larger solubilisation regions compared with those formulated with Tween 20. As Tween 20 (C₁₂) being more hydrophilic surfactant (HLB = 16.7), it solubilized the minimum oil phase (sage and thyme), while Tween 80 (C₁₈) is more hydrophobic surfactant (HLB = 15.0), it solubilized the maximum oil in the aqueous surfactant phase. That's mean that the Solubilization of each of sage and thyme extracts is sensitive to the hydrocarbon chain length of the surfactant, and it is favoured with the longer carbon chain length which is Tween 80. This is reflected by the fact that interaction between the interface and oil decreased as the hydrocarbon chain length of the surfactant decreased [35].

5.2.3. Phase behaviour of Tween 20 or Tween 80/ Water: PG (1:1)/
Plant Extract: Ethanol (1:2)

Ternary phase behaviour of Sage and Thyme ethanolic extracts was studied upon addition of Propylene glycol as solubilisation enhancer, which was grouped together with the water phase at a fixed ratio.

Figure (5.5) presents the ternary phase diagram of the system water+ PG/ Thyme+ ethanol/ Tween 20 at 25°C, made of (1:2) ratio of Thyme extracts and ethanol, and (1:1) ratio of aqueous solution of water and PG. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% (water: PG) from the point containing 70% Thyme+ ethanol extract and 30% Tween 20 point, and extend to up to the water apex 100%.

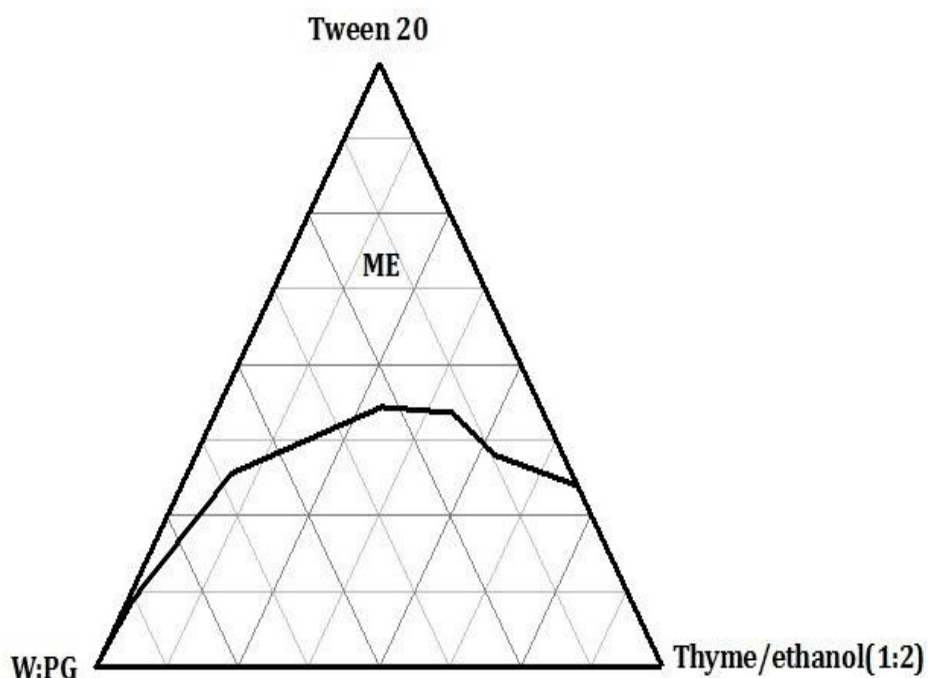


Figure 5.5: Ternary Phase diagram of system: Water: PG/ Thyme: ethanol (1:2)/ Tween 20 at 25°C. The one phase region is represented by ME.

Figure (5.6) presents the ternary phase diagram of the system water+ PG/ Thyme+ ethanol/ Tween 80 at 25°C, made of (1:2) ratio of Thyme extracts and ethanol, and (1:1) ratio of aqueous solution of water and PG. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4%(water: PG) from the point containing 75% Thyme+ ethanol extract and 25% Tween 80 point, and extend to up to the water apex 100%.

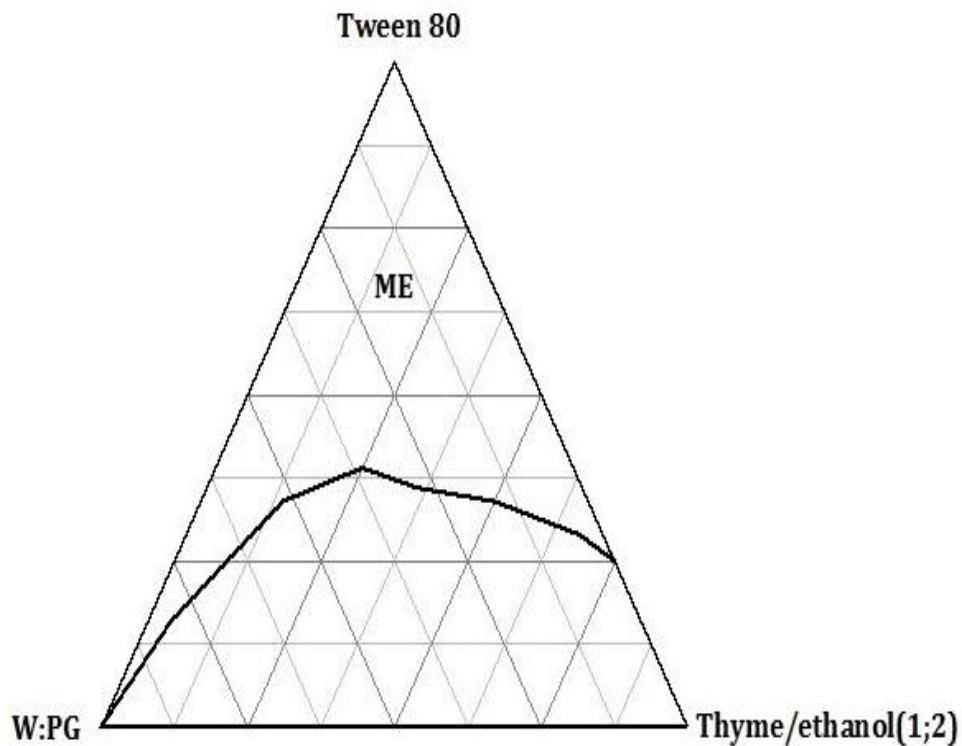


Figure 5.6: Ternary Phase diagram of system: Water: PG/ Thyme: ethanol (1:2)/ Tween 80 at 25°C. The one phase region is represented by ME.

Figure (5.7) presents the ternary phase diagram of the system water+ PG/ Sage+ ethanol/ Tween 20 at 25°C, made of (1:2) ratio of Sage extracts and ethanol, and (1:1) ratio of aqueous solution of water and PG. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4%(water: PG) from the point containing 70% Sage+ ethanol extract and 30% Tween 20 point, and extend to up to the water apex 100%.

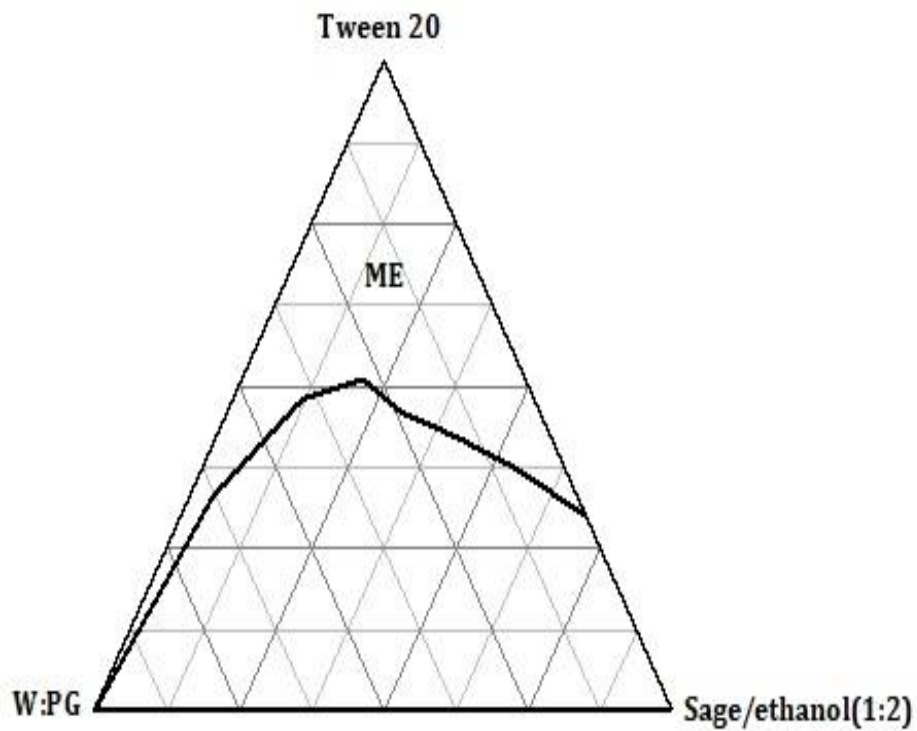


Figure 5.7: Ternary Phase diagram of system: Water: PG/ Sage: ethanol (1:2)/ Tween 20 at 25°C. The one phase region is represented by ME.

Figure (5.8) presents the ternary phase diagram of the system water+ PG/ Sage+ ethanol/ Tween 80 at 25°C, made of (1:2) ratio of Sage extracts and ethanol, and (1:1) ratio of aqueous solution of water and PG. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% (water: PG) from the point containing 75% Sage+ ethanol extract and 25% Tween 80 point, and extend to up to the water apex 100%.

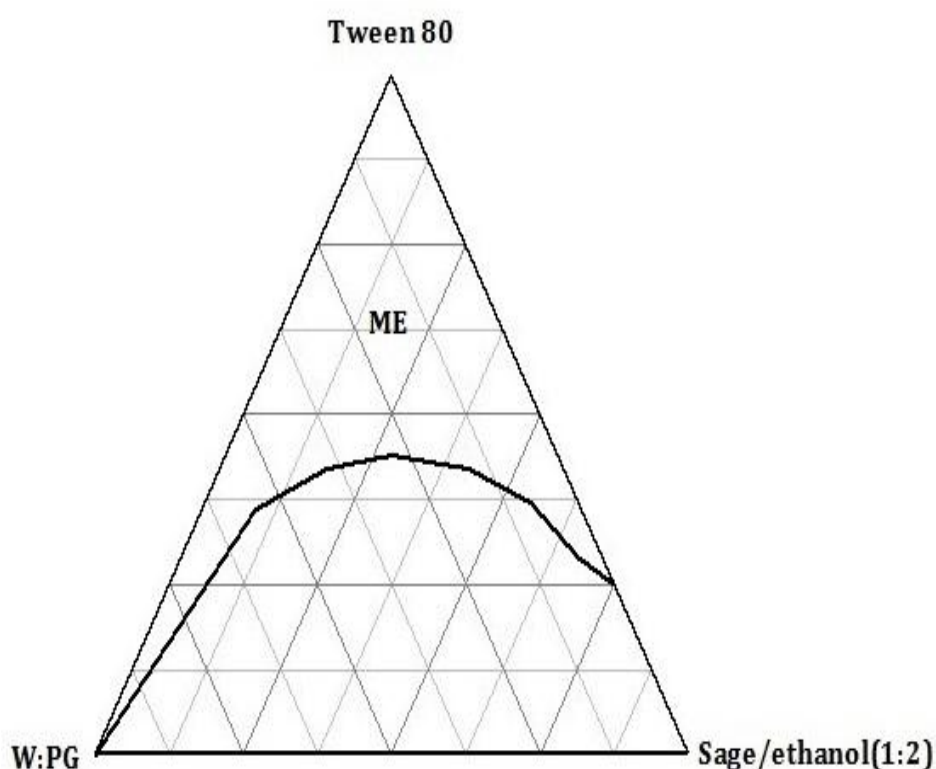


Figure 5.8: Ternary Phase diagram of system: Water: PG/ Sage: ethanol (1:2)/ Tween 80 at 25°C. The one phase region is represented by ME.

The change of the phase behaviour in the presence of propylene glycol (PG) was clearly observed in the above figures, where PG was used as co-surfactant, which shares the non-ionic surfactant to decrease the droplet size and increase the interfacial fluidity forming this wide microemulsion regions. [34].

5.2.3. Phase behaviour of Tween 80, Water: PG (1:1) and Plant Extract: Ethanol
(1:4)

Ternary phase behaviour of Sage and Thyme ethanolic extracts was studied upon increase the weight ratio of ethanol which acts as a solubilisation enhancer in addition to Propylene glycol.

Figure (5.9) presents the ternary phase diagram of the system water+ PG/ Thyme+ ethanol/ Tween 80 at 25°C, made of (1:4) ratio of Thyme extracts and ethanol, and (1:1) ratio of aqueous solution of water and PG. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4% (water: PG) from the point containing 80% Thyme+ ethanol extract and 20% Tween 80 point, and extend to up to the water apex 100%.

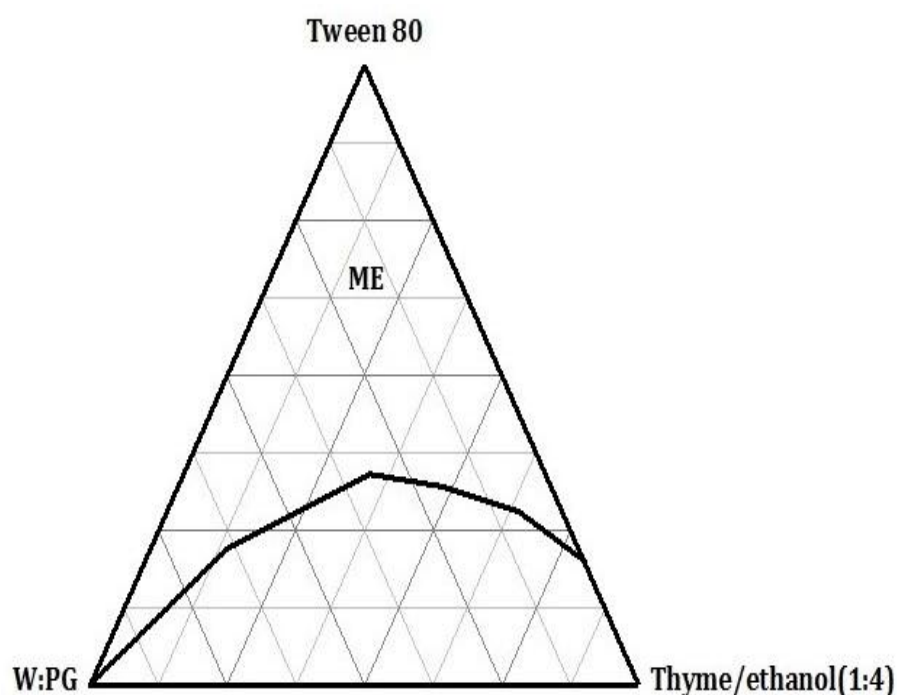


Figure 5.9: Ternary Phase diagram of system: Water: PG/ Thyme: ethanol (1:4)/ Tween 80 at 25°C. The one phase region is represented by ME.

Figure (5.10) presents the ternary phase diagram of the system water+ PG/ Sage+ ethanol/ Tween 80 at 25°C, made of (1:4) ratio of Sage extracts and ethanol, and (1:1) ratio of aqueous solution of water and PG. The microemulsion region starts as a single clear isotropic and not shiny solution upon the addition of the first 4%(water: PG) from the point containing 85% Sage+ ethanol extract and 15% Tween 80 point, and extend to up to the water apex 100%.

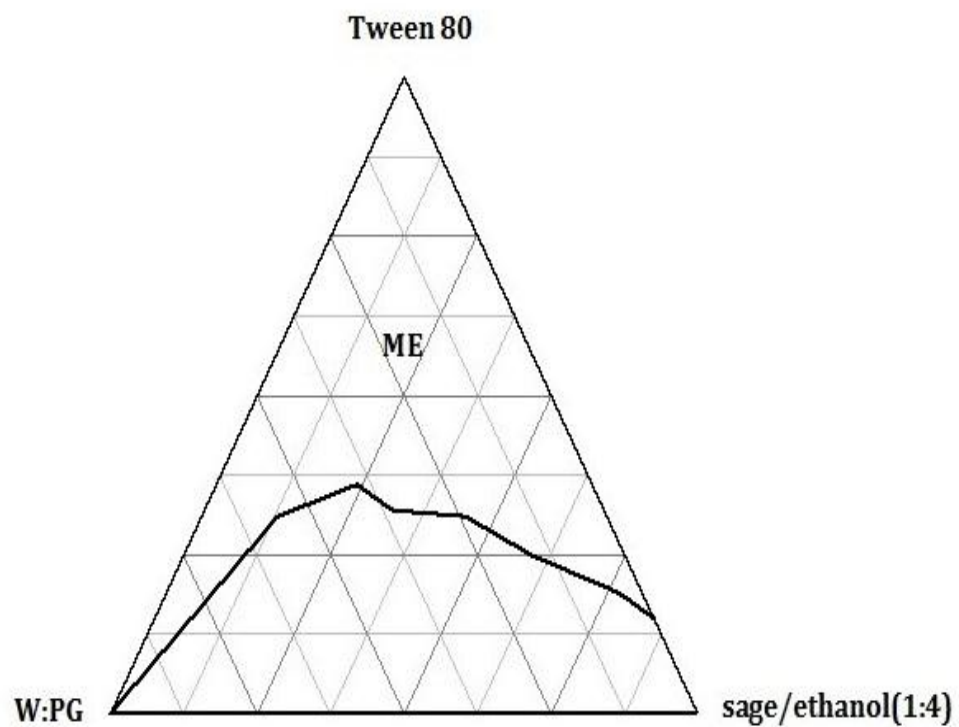


Figure 5.10: Ternary Phase diagram of system: Water: PG/ Sage: ethanol (1:4)/ Tween 80 at 25°C. The one phase region is represented by ME.

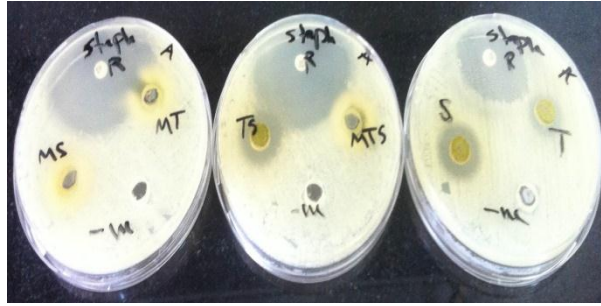
Figures 9.5 and 10.5 showed a significantly increase in the one phase (ME) microemulsion regions for both of Sage and Thyme extracts, by increasing the weight ratio of ethanol from (1:2) grouped with the extract to (1:4) . This is attributed to the ethanol action as both co-solvent and co-surfactant. It has been reported that ethanol can modify the polarity of molecules and improve the interface flexibility of microemulsion [34], it is also reported that the addition of ethanol improves the solution properties of surfactants [48].

5.4. Antibacterial activity test.

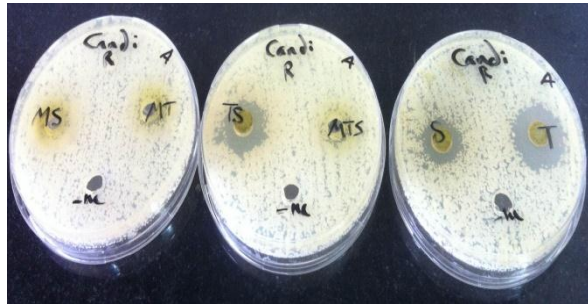
Antibacterial activity was measured for 6 samples as described in table (4), each of Sage and Thyme extracts at (1:4) weight ratio to ethanol, and mixture of both of extracts Sage and Thyme to investigate any synergistic effect. In addition to the microemulsion formulation of sage microemulsion (22% Sage + 54% Tween 80 + 24% Water: PG) and Thyme microemulsion (22% Thyme + 54% Tween 80 + 24% Water: PG).

These samples were tested against three different bacteria which are *Staphylococcus aureus* as gram positive, *Escherichia coli* as gram negative and *Candida albicans* yeast, using the well diffusion method. Penicillin, Ampicillin and Gentamicin were used as positive controls for *S. aureus*, *E. coli* and *C. albicans*, respectively. 95% ethanol and Tween 80 were used as negative controls. The zone of inhibition for each sample was measured using a ruler in millimetre (mm); the data in table (5.1) are average diameter for the duplicate repeated tests.

The inhibition action of Sage extracts were more pronounced against *S. aureus* with 22.5mm, than *C. albicans* yeast with 20.0 mm, whereas, it showed no activity against *E. coli*. The Thyme extracts showed high significant action on *C. albicans* 29 mm, when compared to action on *S. aureus* 17.5mm, and no activity against *E. coli*. With regard to the mixture of both Sage and Thyme extracts a high activity has been shown against *C. albicans* yeast 26.5 mm, and lowers one against *S. aureus* 19.5 mm. This indicates the existence of a synergistic effect between both Sage and Thyme extracts mixture. Taking into account that the concentration of each extract decreased when mixing.



(a)

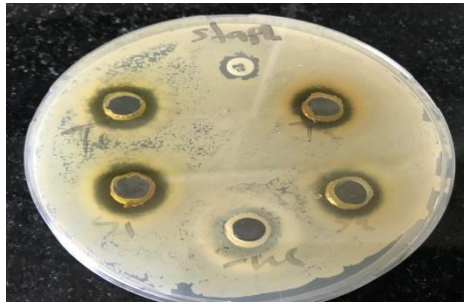


(b)

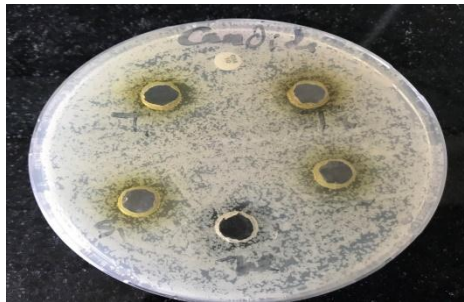


(c)

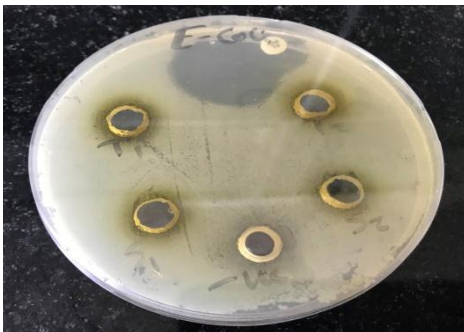
Figure 5.11: Zone of inhibition for Sage extract and microemulsion, Thyme extract and microemulsin, Sage and Thyme extract mixture, Sage and Thyme microemulsin mixture against (a) *Staphylococcus aureus*, (b) *Candida albicans*, (c) *Escherichia coli*.



(a)



(b)



(c)

Figure 5.12: zone of inhibition of Sage microemulsion and Thyme microemulsion on (a) *Staphylococcus aureus*, (b) *Candida albicans*, (c) *Escherichia coli*.

The antibacterial activity of the microemulsion formulation for Sage (22% Sage + 54% Tween 80 + 24% Water: PG) against *S. aureus* showed a higher inhibition zone (15 mm), than its extract formulation (22.5 mm), (by keeping in mind that the concentration percentage of Sage in the extract formulation was about 25%, and was about 6% in the microemulsion formulation). Microemulsion formulation of Sage also showed a higher zone of inhibition against *E. coli* (10.5 mm) and *C. albicans* (13 mm) in comparison with its extract formulation.

The antibacterial activity of the microemulsion formulation for Thyme (22% Thyme + 54% Tween 80 + 24% Water: PG) also showed higher activity against *S. aureus* (16 mm), *E. coli* (12 mm) and *C. albicans* (16 mm) than their extract formulation.

A higher antibacterial activity was indicated by Sage and thyme microemulsion formulation mixture against *S. aureus* (12 mm) than Sage and Thyme extract mixture. On the other hand, there was no detected activity against *E. coli* and *C. albicans*.

The findings that *C. albicans* yeast and *S. aureus* are susceptible to extracts obtained from Sage and Thyme leaves agreed with the susceptibility of that microbes to different plant extracts reported by A. Ashor, Z. Elastal [38], who studied the antimicrobial activity of Sage and Thyme crude extracts and found that no antimicrobial effect on *E. coli*, but a high activity was reported against *S. aureus* and *C. albicans*.

These results are consistent with previous reports regarding Gram positive bacteria [46]. Gram positive bacteria (*Staphylococcus aureus*) were found to be more susceptible than Gram negative bacteria (*Escherichia coli*), this could be explained according to the cell wall of Gram positive bacteria is less complex and lack the natural sieve effect against large molecule due to the small pores in their cell envelope which may leads to easier penetration through Gram positive bacteria cell wall.

Table 5. 1: Antibacterial activity of Sage and Thyme extracts and their microemulsion formulation.

System #	Composition	Symbol	Bacteria	Inhibition zone (mm)
1	Sage extract: Ethanol (1:4)	S	<i>Staphylococcus aureus</i>	22.5
			<i>Escherichia coli</i>	0.0
			<i>Candida</i>	20.0
2	Thyme extract: Ethanol (1:4)	T	<i>Staphylococcus aureus</i>	17.5
			<i>Escherichia coli</i>	0.0
			<i>Candida</i>	29.0
3	Sage & Thyme extracts (1:1)	ST	<i>Staphylococcus aureus</i>	19.5
			<i>Escherichia coli</i>	0.0
			<i>Candida</i>	26.5
4	Sage Microemulsion (22% Thyme + 54% Tween 80 + 24% Water: PG).	MS	<i>Staphylococcus aureus</i>	15
			<i>Escherichia coli</i>	10.5
			<i>Candida</i>	0.0
5	Thyme Microemulsion (22% Thyme + 54% Tween 80 +24% Water: PG).	MT	<i>Staphylococcus aureus</i>	16
			<i>Escherichia coli</i>	12
			<i>Candida</i>	0.0
6	Sage Thyme Microemulsion	MST	<i>Staphylococcus aureus</i>	12.0
			<i>Escherichia coli</i>	0.0
			<i>Candida</i>	0.0

5.4. Antioxidant activity test:

DPPH radical scavenging activity is one of the most widely used methods for screening the antioxidant activity of plant extracts, because this method is simple and sensitive. The DPPH antioxidant assay is based on the ability of (a stable free radical) to discolour in the presence of antioxidants.

Antioxidant activity was measured for each of Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) ethanolic extracts, the results in figure (22) show that Sage ethanolic extract exhibit a significant inhibition percentage with 84.72%, which agreed with M. Tofana *et. al.* [40], who determined the DPPH scavenging activity of Sage (*Salvia Officinalis*) methanolic and ethanolic extract, and reported the result as (85.12%) and (78.43%), respectively. Thyme ethanolic extracts shows higher inhibition percentage than Sage extract with 86.28%, which is consistent with previous study of G. Ruberto and M. Barratta [47], who studied the antioxidant activity of about 100 pure components of essential oils, and they observed that the phenolic compound such as thymol and carvacrol showed the highest antioxidant activity.

The antioxidant activity was also studied for the microemulsion formulation of each of Sage (26% Sage+ 62% Tween 80+ 12% water: PG) and Thyme (26 %Thyme+ 62% Tween 80+ 12% water: PG). The inhibition percentage was much higher than their surfactant free extracts formulation, if we consider the concentration of the plant extract in each formulation, which are (25%) in the ethanolic extracts, and about (5.28%) in the microemulsion formulation.

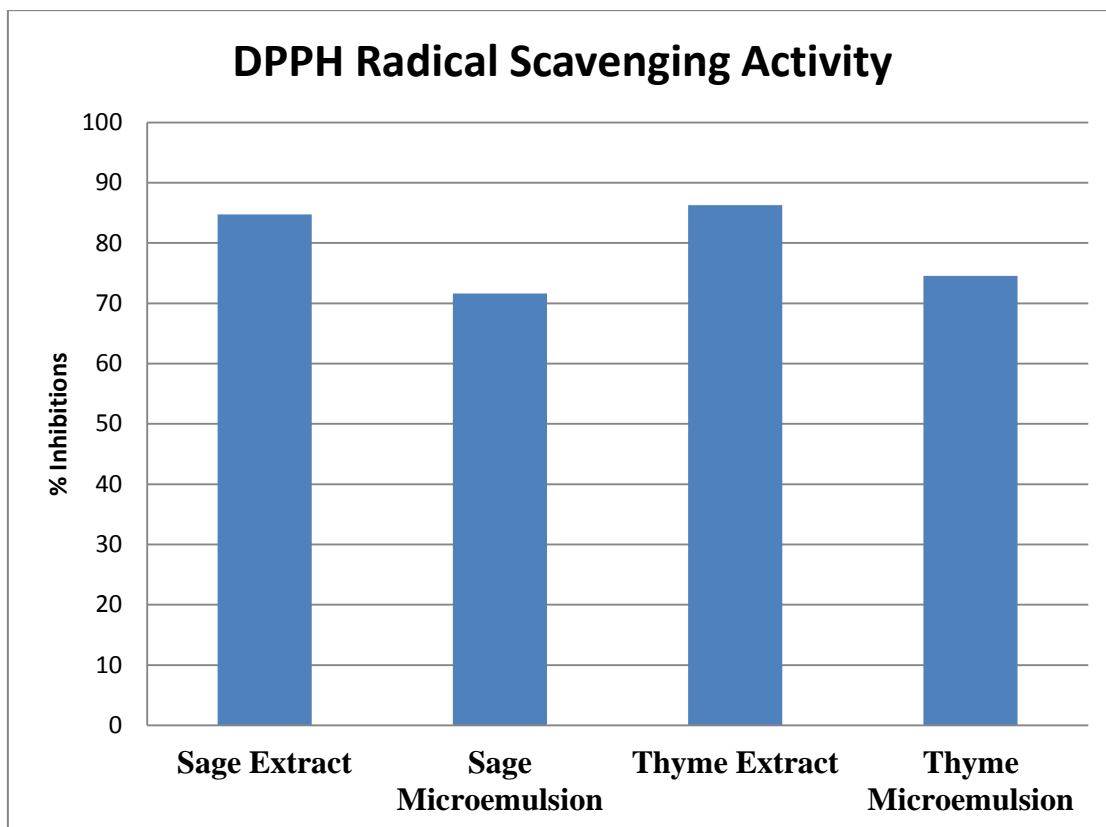


Figure 5.13: Antioxidant capacity using DPPH scavenging activity of Sage Extract (84.72%), Sage microemulsion (71.65%), Thyme Extract (86.26%), Thyme microemulsion (74.55%), error bars indicate mean \pm SD of duplicated experiments.

6. Conclusion

Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) plant leaves have a long history of medicinal and culinary uses in Palestine in particular. Soxhlet method was chosen for the extraction process using 95% ethanol as solvent. The current work illustrates the solubilisation capacity of each of Sage and Thyme ethanolic extracts in dilutable microemulsion using a commonly applied non-ionic surfactant Tween 80, in the addition to short chain alcohol and propylene glycol as co-surfactant.

Antibacterial activity was examined for both Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) leaves extract against three microorganisms namely: *staphylococcus aureus*, *Escherichia coli* and *Candida albicans*, and exhibits high activity against *S. aureus* and *C. albicans* for leaves extracts. A higher antibacterial activity was indicated for their non-ionic microemulsions formulations against *S. aureus*, *C. albicans* and *E. coli*. It was also indicated that the microemulsion formulation of Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) leaves extract exhibit a higher inhibition percentage using the DPPH scavenging activity test than their free surfactant formulation.

It was concluded that the Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) microemulsions are stable, self-preserving antibacterial and antioxidant agents, rather than the chemical activity of their individual components.

7. Future works

Probing deeper, the results in this thesis provide a strong foundation for future studies on the biomedical application of the Sage (*Salvia Officinalis*) and Thyme (*Thymus Vulgaris*) medical leaves microemulsion, and also on the formation of nanoemulsion for the same systems.

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سلوك مراحل المستحلبات الدقيقة وفاعلية مضادات الجراثيم ومضادات الاكسدة لمستخلص الميرمية والزعرتر.

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ملخص:

يهدف هذا البحث لدراسة سلوك مراحل المستحلبات الدقيقة لمستخلصات نباتية طبية في فلسطين وهي الميرمية والزعرتر من خلال دراسة خمسة أنظمة مختلفة في التركيب ونسب المواد المكونة منها باستخدام نوعين من المؤثرات السطحية غير الأيونية وهي توين 20 وتوين 80 (Tween 20 & Tween 80).

تم دراسة فاعلية مضادات الجراثيم على المستخلصات الكحولية للميرمية والزعرتر وايضاً على المستحلبات الدقيقة المكونة منها باستخدام طريقة انتشار حفرة عمودية عميقة (Well diffusion method) ، وايضاً تمت دراسة فاعلية مضادات الاكسدة باستخدام طريقة (DPPH radical scavenging activity).

نتائج سلوك مراحل المستحلبات الدقيقة اظهرت ان توين 80 ملائم اكثر لإذابة كل من مستخلص الميرمية والزعرتر مقارنةً بتوين 20. واطهرت ايضاً ان الذائبية ازدادت عند استخدام البروبولين جلايكول (PG) وسلسلة قصيرة من الكحول (ethanol) كمذيبات مساعدة.

تقييم مذيبات الجراثيم لكل من الميرمية والزعرتر اظهرت فاعلية عالية للمستخلصين ضد كل من *Escherichia coli* . من ناحية اخرى اظهرت النتائج فعالية عالية للمستحلبات الدقيقة المكونة من الميرمية والزعرتر ضد كل من مضادات الجراثيم التي تمت دراستها وبتركيز قليل جداً مقارنة مع تركيز تركيبية

المستخلصات منفردة. كما وبينت الدراسة ان مستخلصات الميرمية والزعتر عبارة عن مضادات للأكسدة بطبيعتها ولكن تزداد فاعليتها عند استخدامها كمستحلبات دقيقة.

نتائج هذه الأطروحة تسلط الضوء على سعة الذائبية للمستحلبات الدقيقة المكونة من مستخلصات الميرمية والزعتر بالإضافة الى المستحلبات غير الأيونية ، وامكانية استخدامها في تطبيقات مضادات الجراثيم ومضادات الأكسدة.