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Multi extraction methodologies and analysis of some polyphenols from olive leaves extract and investigating the phase behavior of OLE/Olive Oil/Tween 80

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Thesis Approval

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Jerusalem – Palestine

1434/3013

Dedication:

I would like to dedicate this scientific work to the soil of my father, the first teacher and friend.

I would like to dedicate this scientific work to the greatest woman in the world my Mother.

I would like to dedicate this scientific work to my wife which motivates me so much.

Mustafa Lubada

Declaration:

I certify that this thesis submitted for the degree of master is my own research, expect 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.

Signed: ……..

Mustafa A.M. Lubada

Date: 23/ 10 /2013

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Abstract:

This is scientific approach to extract the polyphenols compounds from olive leaves , analyzing them using liquid chromatography HPLC and investigating the phase behavior of OLE/olive oil/tween 80 .

Microemultion is one of the pharmaceutical delivery dosage forms which can combine hydrophilic compounds with hydrophobic compounds using surfactants. Preparing the olive leaves by cleaning, grinding, sieving with mesh 30 and using suitable solvents for extraction in order to optimize the total amount of biophenols extraction.

Decoction (boiling), soxhlet , ultrasound extraction (Sonication) and reflux distillation are the most effective extraction methods can be used for olive leaves extraction in a short time. Methanol 100% is the best solvent for extraction Oleuropein beside Ethanol 100% which is less toxic solvent. Water/Ethanol 75/25 % is the suitable solvent for extraction the total biophenol compounds. Using temperature up to 100 ^{0}C reduce the amount of biophenol compounds. Using acidic PH solvents are the suitable PH for extracting the biophenol compounds. Acidic media is the suitable media for elongate the stability of OLE. Preserving OLE in a low temperature also elongate the stability life of OLE. Increasing the amount of OLE increase the region of water/oil at low amount of surfactant. Analysing some biophenol compounds with good range of linearity and LOD and LOQ.

This research recommends the farmers not to throw the olive leaves in the field which cause bad effect on the fertility on the soil. Analyze the poliphenols compounds during the four seasons of the year to find the maximum concentrations of these compounds. Investigate the phase behavior of the OLE with different oils and surfactants.

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Abbreviations:

OLE	Olive Leaves Extract
HPLC	High Pressure Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
HIV-1	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
HSV	Herpes simplex virus
Hba1c	Hemogloben Blood Concentration Analysis Level 1
SFE	Super Fluid Extraction
OBPs	Olive polyphenols
EtOH	Ethanol
UV-Vis	Ultra violet-Visible
DAD	Diode Array Detector
RP	Reverse Phase
MeCN	Acetonitrile
TFA	Trifloroacetic Acid
UAE	Ultra Assisted Extraction
DC	Decoction
TLC	Thin Layer Chromatography
LOD	limit of detection
LOQ	Limit of quantification
W/O	Water in oil
O/W	Oil in water
LC	Liquid Chrystal

Chapter 1

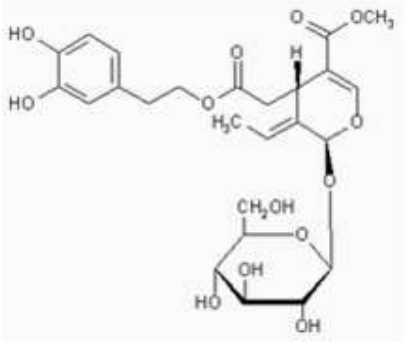
1.1 Introduction

Palestine is one of the Mediterranean countries with a large amount of olive trees (*Olea europaea)*. Olive oil is the most important oil used, is extracted from olive fruit. Recently, it has been revealed that health benefits are only attributed to olive oil but also includes its leaves [Lee-Huang et.al, 2003].

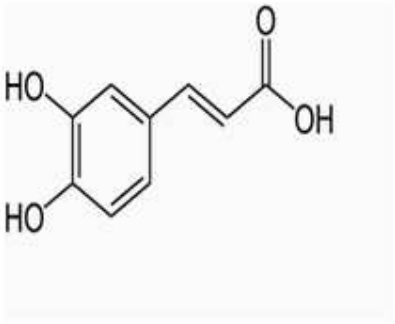
1.2 Olive leave extract bio-phenols compound structures

Separation and analysis of olive leaf extract (OLE) by using HPLC wavelength 280 nm. revealed the following phenolics, hydroxytyrosol; tyrosol; luteolin-7-O-glucoside; verbascoside; apigenin-7-O-glucoside; oleuropein [Hayes et.al, 2011].

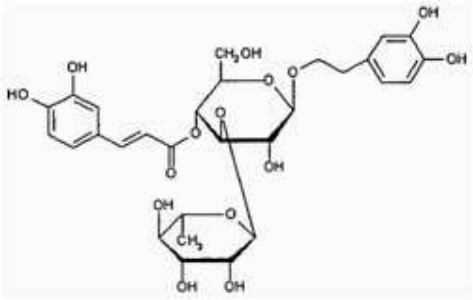
Olive leaf extract is rich of phenolic compounds also contains, caffeic acid quercetin and chryseriol [Dekanski et.al, 2009].Figure (1.1)shows the OLE chemical structures.



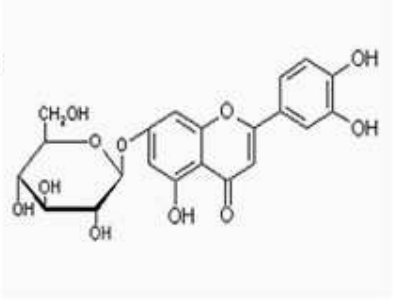
Oleuropein



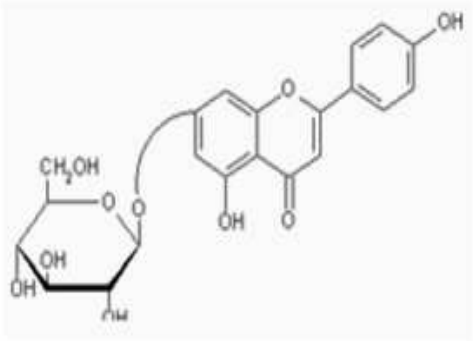
Hydroxytyrosol



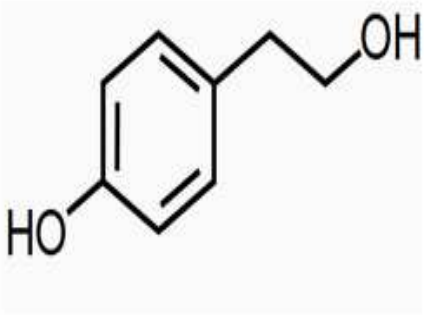
Verbascoside



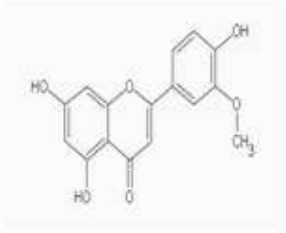
luteolin7-*O*-glucoside



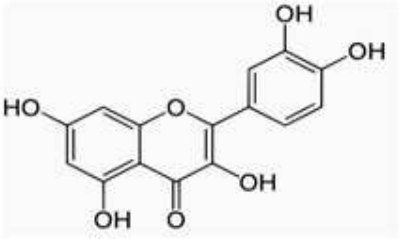
Apigenine-7-*O*-glucoside



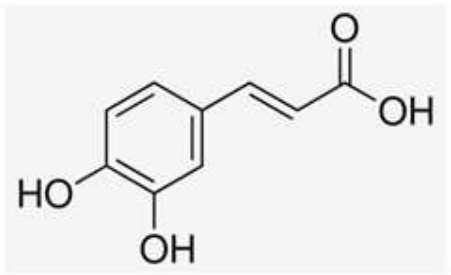
Tyrosole



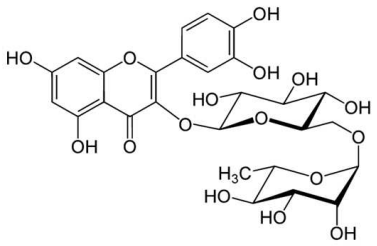
Chryseriol



Quercetin



Caffeic Acid



Rutin

Figure 1.1: OLE polyphenol compounds structure

1.3 Health benefits of olive leaves extract

Due to the high content of high polyphenolics content,olive leaves extract (OLE) found to be useful for many acute and chronic diseases. Many recent investigations had been done on therapeutic effect of olive and its leaves on diseases like hypertension microbial, viral, inflammatory, antioxidant activity and other diseases [Zari et.al, 2011, Susalit et.al, 2011, Hansen et.al 2002, Yamada at.al 2009, Soni et.al, 2006].

1.3.1 Antiviral Activity

The antiviral activity of (OLE) preparations analysed by liquid chromatography-coupled to mass spectrometry (LC-MS) against HIV-1 infection and replication had been investigated. It was found that OLE inhibits acute infection and cell-to cell transmission of HIV-1 as assayed by syncytia formation. OLE also inhibits HIV-1 replication [Lee-Huang et.al, 2003].

AIDS patients have begun to use OLE for a variety of indications, among them to strengthen the immune system, to relieve chronic fatigue, to boost the effects of anti-HIV medications, and to treat HIV-associated Kaposi's sarcoma and HSV infections [Carusoet.al, 1999].

1.3.2 Antioxidant

It has been found that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds. [Skeget et.al, 2005]. Leaves without copper residues also proved to be a good natural source of antioxidants Copper remaining in the leaves can elicit the formation of reactive oxygen species, which are neutralized by the polyphenolic compounds, resulting in the lowering of their antioxidant capacities [Ferreira et.al, 2007], as the amount of phenol compounds increase the antioxidant activity increase.

1.3.3 Antibacterial

Two of the oleuropeien hydrolysis products have antibacterial action against certain species, the aglycone and elonolic acid inhibited growth of the four species of lactic acid bacteria. The aglycone of oleuropein and elenolic acid were much more inhibitory when the groth medium contains 5%NaCl and 150 µg of either compound per ml prevented growth of lactobacillus plantarum [Fleming et.al, 1973].

1.3.4 Antifungal activity

The antifungal activity of phenolic compounds against alternaria solani, Botrytis cinerea and Fusarium culmorum were investigated. Olive pomace, a by-product of olive oil production and olive leaves were a rich source of phenolic compounds. The extracted

phenolics were added to a medium to reach phenol concentration of 0.1 and 0.2 % (w/v) and tested for their antifungal activity against the three fungi. In addition, the fungi were cultivated in a medium containing a commercial fungicide in the concentration of 0.2 % (w/v). Both of the phenolic concentrations inhibited the growth of all three fungi with the higher being more effective. It resulted in a four-fold reduction of the mycelial growth rate of A. solani and F. culmorum. Phenolic compounds derived from olive leaves and pomace are a natural fungicide against common pathogens [Winkelkusen et.al, 2005].

1.3.5 Anti-inflammatory

Inflammation is considered as a critical factor in many human diseases. phenolic compounds, have shown anti-inflammatory activity in-vitro and in-vivo. These natural compounds express anti-inflammatory activity by modulation of pro-inflammatory gene expression such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several pivotal cytokines, mainly by acting through nuclear factor-kappa B and mitogen-activated protein kinase signaling [Santangelo et.al, 2007].

1.3.6 Hypoglycemic effect

When a 500 mg OLE tablet taken orally once daily. The glucose homeostasis including Hba1c and plasma insulin was measured. To determine the mechanism through which OLE affected starch digestion and absorption. The rats treated with OLE exhibited significantly lower HbA1c and fasting plasma insulin levels .OLE is associated with improved glucose homeostasis in humans also. [Wainstein et.al, 2012].

OLE may be helpful in inhibiting hyperglycemia and oxidative stress caused by diabetes, and that it may be able to help diminish the complications of diabetes. OLE also indicated

a significant antidiabetic activity with the olive leaf decoction and supports the traditional usage to control of diabetes [Abu-Zaiton et.al, 2012].

The administration, for 4 weeks, of oleuropein and hydroxytyrosol rich in OLE, significantly decreased the serum glucose and cholesterol levels and restored the antioxidant perturbations. These results suggested that the antidiabetic effect of oleuropein and hydroxytyrosol might be due to their antioxidant activities restraining the oxidative stress which is widely associated with diabetes pathologies and complications [Jemai et.al, 2009].

1.3.7 Hypertension and Lipid profile

Olive (Olea europaea L.) leaf extract at the dosage regimen of 500mg twice daily (1000mg daily) effectively lowered systolic and diastolic blood pressures in human with stage-1 hypertension. The anti-hypertensive activity of the extract was comparable to that of captopril, given at its effective dose of 12.5–25mg twice daily. The study also demonstrated the safety and tolerability of the extract, administered orally at a dose of 500mg twice daily. Additionally, the beneficial effects of the extract on lipid profile, particularly in reducing plasma LDL, total-cholesterol and triglyceride levels were strongly indicated by this trial. Further studies, involving particularly subjects with dyslipidemia, are needed to confirm the extract's effect on lipid profile [Susalit at.al ,2011].

1.3.8 Anti-atherogenic activity

Some researches speak about the importance of olive and its phenol compounds like oleuropein as anti-atherogenic activity [Visioli et.al, 2001].

Nutritional benefit of olive oil and its biophenolic compounds in the prevention of coronary heart disease can be also related to the high content of oleuropein and its derivatives. Moreover, the well documented antithrombotic and antiatherogenic activity of olive oil polyphenols, indicate these antioxidants as possible therapeutic tools for the pharmacological treatment of coronary heart disease as well as in the case of cardiac surgery, including transplantation [Manna et.al, 2004].

1.4 Microemulsion

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 [Microemulsion, 2009].

1.4.1 Applications of microemulsion

There has been a revolution in the last two decades in the utilization of microemulsion systems in a variety of chemical and industrial processes [Paul B.K et.al, 2001]. For example ,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.

1.4.2 Drug Delivery

Microemulsions also can be used as drug delivery systems in many ways [Microemulsion, 2009]. For example, Applications in oral drug delivery, transdermal and dermal delivery ,parenteral drug delivery ,ocular drug delivery and in mucosal drug delivery

1.4.3 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 the formulation 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’ [Microemulsion, 2009]. Investigations the phase behavior of OLE with olive oil using surfactants introduce to use OLE in a different formulations with exist of olive oil or other oils.

Chapter 2

2.1 Literature review

Recently (OLE) becomes an important subject, and that is not strange because of the huge health benefits as it mentioned in the introduction. Many extraction methods had been developed to optimize the amount of (OLE) phenol compounds extract.

2.1.1 Extraction Methods

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 CO_2 modified with 20% methanol at 300 bars and 100 C^0 was followed by that of CO_2 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].

Jap´on-Luj´ and Luque de Castro used superheated liquid extraction to extract oleuropein and related biophenols from olive leaves like verbacoside, apigenin-7-glucoside and luteolin-7-glucoside. Complete yield of extraction had achieved without degradation of the target analytes in 13 min using low-toxic ethanol–water mixtures as extractant . pH 7.5 shows to be the best pH and provides better extraction efficiencies for all OBPs. 70% ethanol and 1ml/min flow-rate were selected at 140 C^0. The amount of oleuropein extracted was 23 mg/g of olive leaves in a short time beside the other biophenoles [Ja on-luj an et.al, 2006].[a]

Pre-treatments on extraction of oleuropein and related biophenols from olive leaves can also increase the amount of yield extraction . Using steam, hot water blanching and UV-C irradiation as pre-treatments can significantly increase oleuropein yield from 25 to 35 times compared to non-steam blanched sample. No significant UV-C effect was observed in oleuropein content, while hot water blanched samples showed significantly higher oleuropein yields compared to untreated samples. Also losses of oleuropein content were higher in hot water blanching than in steam blanching [Stamatopoulos et.al 2012].

In order to get faster and more efficient extraction method for olive leaves than traditional methods such as maceration/stirring (25 min are required versus 24 h of the conventional method), a dynamic ultrasound assisted approach had been used by R. Jap´on-Luj´an et al. to extract OBPs from olive leaves. The influence of the pH on the extracts obtained under

the optimal conditions was checked. Different pH's used to achieve extractant, pH's of 2 and 12, respectively. The comparison of the extracts thus obtained with that from the extractant without pH change (pH 8) showed that at pH 12, the amount of OBPs extracted decreased 27% for apigenin- 7-glucoside, 35% for oleuropein and luteolin-7-glucoside and 40% for verbacoside, probably owing to ester bond hydrolysis. However, the concentrations of the target analytes at pH 2 were similar to those in the extract at pH 8 [Ja on-luj an et.al, 2006] .[b]

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

Stefania Mylonaki et.al, found that Extractions under magnetic stirring at 400 rpm and at room temperature (22±2 ℃) with different factors had been investigated to optimize the yield of extraction, pH, Time of extraction and composition of ethanol with water adjusted the pH with acetic acid. (60% Ethanol, pH 2 and 5 h) was found to be the optimal conditions to get the high amount of biophenol compounds. The optimisation of polyphenol extraction from olive leaves using water/ethanol/citric acid mixtures showed that duration of the extraction and the ethanol concentration are the primary factors that affect the yield. pH was less significant in this regard [Mylonaki et.al, 2008].

Bilek used response surface methodology to optimize the extraction conditions for total phenolic compounds from dried olive leaves. They found that 43% ethanol in water (v/v) at 50 ºC for 15 hours of extraction and 7 times solvent/solid ratio are the recommended

optimal conditions for the total phenolic compounds from olive leaves samples were placed in a volumetric flask and subjected to stirring [Bilek , 2010].

2.1.2 Analyzing Method

After the extraction, the compounds of the OLE have to be separated, identified and analyzed.

Phenolic compounds show high absorption in the UV region; so the most commonly used detector for HPLC is a multiwavelength UV or UV–vis detector [Ja on-luj an et.al, 2006][b]. HPLC analyses can be performed with a diode array detector. The suitable stationary phase is C18 column (5 µm particle size; 250 mm 4.6mm). The mobile phases were formic acid (19:1) (A) and methanol with different composition. The flow rate about 0.9 ml.min−1 with elution at room temperature. The injection volume is 10 µl and chromatograms were recorded at 280 nm for oleuropein [Aouidi et.al,2012].

Dragana Dekanski et.al used another method for analyzing the biophenol compounds in olive leaves extract. They used a diode array detector (DAD) and the chromatographs were recorded at λ= 260 nm (for flavonoids and oleuropein) and at 325 nm (for caffeic acid).

The spectra recorded at 360 nm were used to identify luteolin and chryseriol. HPLC separation of components was achieved using a Li- Chrospher 100 RP 18e (5 µm), 250 mm×4 mm i.d. column with a gradient mobile phase flow rate of 1.0 ml/min. The mobile phase A consisted of 500 ml of H_2O plus 9.8 ml of 85 % H_3PO_4 (w/w), while B was MeCN [Dekanski et.al, 2009].

J.E. Hayes et.al, [Hayes et.al, 2011] used another method for the quantification of phenolics in the olive leaf extract. The column of separation was conducted on a Zorbax SB C18, 5mm, 150 _ 4.6 mm column. The gradient profile was based on a method of Tsao and Yang [Tsao et.al 2003]. Acetic acid in 2 mM sodium acetate (final pH 2.55, v/v) was used as eluent A and 100% acetonitrile was used as eluent B. The column temperature was set at 37 C^0 and the flow rate was 1 ml/min. Chromatograms were recorded at a wavelength of 280, 320, 360, and 520 nm.

Ansaria .M, et.al, used also RP-HPLC. Analyzing method for quantification the amount of oleuropein which detected wit UV detector at wave length 280 nm using water (adjusted to pH 3):acetonitrile (80:20) at flow rate 1 ml/min. Linear dynamic range and limit of detection were found to be 50-900 μg /ml and 9.5 μg/ ml, respectively. Intra- and inter-day precision of the method were calculated as RSD% of 1.2 and 5.7 respectively [Ansaria et.al, 2011].

Mylonaki.S et.al, [Mylonaki et.al, 2008] used another method for analyzing the biophenol compounds in olive leaves extract (luteolin 3′, 7-O-diglucoside; quercetin ,O-rutinoside (rutin);, luteolin 7-O-glucoside;, luteolin 7-O-rutinoside;, apigenin 7-O-rutinoside, luteolin 3′-O-glucoside;, oleuropein) . The equipment was an HP (Hewlett Packard, Palo Alto, CA, USA) 1090 series II liquid chromatography coupled to an HP 1100 diode array detector. The column was a Superspher 100 RP18, 4 μm, 125×2 mm (Merck), protected by a guard volume packed with the same material. Both columns were maintained at 40 °C. Eluent (A) and eluent (B) were 0.05% aqueous trifluoroacetic acid (TFA) and MeCN containing 0.05% TFA, respectively. The flow rate was 0.3 mL/ min, and the elution programme used

was as follows: 5 min, 7% B; 50 min, 35% B; 60 min, 80% B. The eluate was monitored at 270, 290, 320, and 360 nm.

2.2 Problems

Each year, the farmers take care of their olive trees, cut the branches (pruning) ,and then burn them or throw them away and that could cause environmental problems. By exposure of these olive leaves to the rain the phenol compounds transfer to the soil which increasedits toxicity to be about 1000 times more than toxicity of municipal waste water [Morillo et.al 2009]. Due to their instability, the polymerization of phenols compounds during staying in the soil into condensed high-molecular-weight polymers that are particularly difficult to degrade [Ayed et.al, 2005]. The antimicrobial activity of the olive leaves phenol compounds may results in more damage than beneficial effects on soil fertility.

The percentage of olives leaves which reach with the olives fruit to the mills contains about from 5% to 10% depending on practices applied [Lafka et.al, 2013]. This big amount of olive leaves goes to the waste.

Beside the environmental problem we also have an economic problem in Palestine, so gathering these branches and get using them in a beneficial way especiall in pharmaceutical products will contribute to economic benefits.

2.3 Aims of the study

The aims from this study are

1- Performing a good extraction method and optimizing it in order to extract the maximum amount from olive leave extract and its bio-active phenol compounds using it in the different fields.

2- Developing an analytical method for analyzing the main polyphenols compounds in olive leaves extract.

3- Investigating the phase behavior of olive leaves extract in order to help the formulator in finding the proper oily dosage form in order to preserve and increase the stability of OLE polyphenols compounds.

Chapter 3

3 - Experimental work

3.1 Extraction Techniques

There are many methods and techniques used for extraction. Extraction depends on several parameters and on the matrix itself. In this research the focus will be on the extraction of medicinal plants and specifically on olive leaves extracts.

3.1.1 General Methods of Extraction of Medicinal Plants

Handa et.al in their book speak in details about extraction of herbal drugs [Handa et.al ,2008]. Among these extraction methods are:

1- Maceration 2- Infusion 3-Digestion 4- Decoction 5- Percolation 6- Hot Continuous Extraction (Soxhlet) 7- Aqueous Alcoholic Extraction by Fermentation 8- Counter-current Extraction 9- Ultrasound Extraction (Sonication) 10- Supercritical Fluid Extraction 11- Phytonics Process.

3.1.2 Methods of extraction used to extract olive leaves.

3.1.2.1 Ultrasound Extraction (Sonication)

The application of ultrasonic assisted extraction (UAE) in food processing technology is of interest for enhancing and increasing extraction of components from plant and animal materials [Vilkhu et.al.]. Extraction enhancement by ultrasound with frequencies ranging from 20 kHz to 2000 kHz has been attributed to the propagation of ultrasound pressure

waves, and resulting cavitation phenomena. High shear forces cause increased mass transfer of extractants. [Jian-Bing et.al. 2006].

They highlight that while it is relatively easy to achieve extraction on the laboratory bench and scale but it is very challenging to attempt extraction on an industrial scale. Several key issues and observations relating to (UAE) have been identified, as follows, (1) the kind and tincture of the tissue being extracted and the location of the components to be extracted with respect to tissue structures, (2) the preparation and pretreatment of the tissue before the extraction, (3) the nature of the biophenol component being extracted (4) increasing surface mass transfer [Balachandran et.al, 2006], also (5) intraparticle diffusion (6) loading of the extraction chamber with suitable amount of substrate (7) increased yield of extracted components and (8) increased rate and efficiency of extraction, particularly early in the extraction cycle enabling major reduction in extraction time and higher processing throughput [Moulton et.al 1987].

3.1.2.2 Maceration

In this method of extraction, the whole of powdered crude drug (plant ,leaves) is placed in a stoppered container with the solvent or in a beaker and allowed to stand at room temperature or at certain temperature for a period of time (at least 3 days) with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed and squeezed, and the combined liquids are clarified by filtration or decantation after standing [Handa et.al, 2008].

Figure 3.1: Maceration extraction with water bath with temperature controller

3.1.2.3 Decoction (boiling)

Decoction (DC) is the traditional extraction method for oral administration of Chinese medicines [Xie et.al, 2009]. In this process, the crude drug or leaves are boiled in a certain volume of water or solvent for a period of time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. The starting ratio of crude drug or leaves to water is fixed, e.g. 1:4 or 1:16; the volume is then brought down to one-fourth its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further [Handa et.al, 2008].

3.1.2.4 Soxhlet

In this method, the finely ground crude drug or grind leaves are placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber E of the Soxhlet

apparatus as illustrated in Figure (3.2).The extracting solvent in flask A is heated, and its vapors condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug 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].

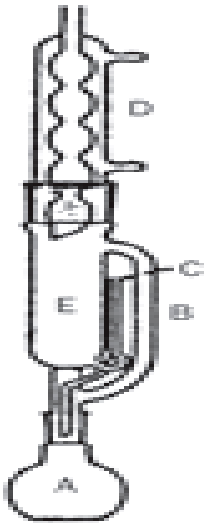


Figure 3.2: Soxlet apparatus

In figure (3.3) another type of soxhlet apparatus using for extraction the fats and oils in some certain food products. The disadvantage of this type of soxlest instrument that we cannot use water as co-solvent in the extraction, and also the sample size used is small.

Figure 3.3: Soxhlet apparatus with temperature controller (Soxtest).

3.1.2.5 Reflux Distillation

Reflux distillation is a good method for extraction. The sample put in a round bottom flask with a solvent and boiled for a certain time. There is a water condenser to condense the solvent which can save it in a circulation so in this case we cannot lose the solvent and increase the efficiency of extraction.

Figure 3.4: Reflux distillation

In this picture we can see the round bottom flask where the sample can be put with the solvent, and the round bottom flask was surrounded with a heat mantle with a temperature controller .Above the flask there is a water condenser to condense the solvent and return it back to the flask.

3.1.3 Parameters for Selecting an Appropriate Extraction Method.

3.1.3.1 Preparation of olive leave sample

- Any foreign matter (branches, stones,etc ….) was completely removed.
- Using the right plant part (leaves). The olive leaves were collected at the march of 2013 from Ramallah region.

- Conditions used for drying the plant material largely depend on the nature of its chemical constituents. Hot or cold blowing air flow for drying is generally preferred. In This research the drying of leaves were done at room temperature. [Ansari et.al. 2011]
- Grinding methods wre specified and techniques that generate heat should be avoided as much as possible to prevent the degradation of components.
- Powdered plant material was passed through suitable sieves to get the required particles of uniform size. Olive leaves were dried and milled to mesh 30.

3.1.3.2 Nature of constituents:

- If the therapeutic value and the biophenol compounds lie in non-polar constituents, a non-polar solvent may be used. Polar solvents are preferable for biophenol compounds.
- -If the constituents and the extracts are thermolabile, extraction methods like cold maceration and percolation are preferred .For thermos table constituents, Soxhlet extraction (if nonaqueous solvents are used) and decoction (if water is the menstruum) are useful.
- Suitable precautions must be taken when dealing with constituents and components that degrade while being kept in organic solvents.
- In case of hot extraction, higher than required temperature must avoided.
- Standardization of time of extraction is important, as:
  • Insufficient time means incomplete extraction.
  • If the extraction time is longer, unwanted constituents may also be extracted.
- The number of extractions required for complete extraction is as important as the duration of each extraction.
- The quality of water or menstruum used should be specified and controlled.

- Concentration and drying procedures must ensure the safety and stability of the active constituents. Drying under reduced pressure (e.g. using a Rotavapor) is widely used. Lyophilization figure (3.5), although expensive, is increasingly employed.

Figure 3.5: Lyophilizer instrument

- The design and material of fabrication of the extractor are also to be taken into consideration.
- Analytical parameters of the final extract, such as TLC and HPLC fingerprints, should be documented to monitor the quality of different batches of the extracts.

3.2 Reagents and chemicals

- Ethanol 99.8 % and Methanol 99% were purchased from Sun Pharm LTD.
- Acetonitrile 99.9 % Chromasolv gradient grade for HPLC was purchased from Sigma Aldrich.
- Sodium acetate 99 % and KH2PO4 were purchased Sigma USA.
- Acetic Acid was purchased from LOBA CHEMI PVT.LTD, India

- Hexane was purchased from BIO-LAB LTD, Jerusalem
- Petroleum ether was purchased from J.T.Baker ,USA
- Hydrochloric Acid and Phosphoric acid was purchased from Merck KGaA,Germany.
- Ultra Pure water was achieved from ELGA Water system.
- HPLC Vials was purchased from Waters.
- Luteolin 7-glucoside >98% , Oleuropein > 98% , Rutin Hydrate > 94% and Hydroxytyrosol 98% Referance standards were purchased from Sigma Aldreich ,USA.
- Verbascoside 86.87 % was purchased from HWI ANALYTIK GmbH, Germany.

3.3 High performance Liquid chromatography (HPLC)

The quantitative amounts of phenolic compounds of olive leaves extract were determined by using HPLC. The basic principle of HPLC is using the chromatography concept to separate and detecting the separated compounds using UV detector.

HPLC contains reservoir for mobile phase solvents to elute the components through the column using a pump. The stationary phase (column) is a non polar silica gel in general for separate the olive leaves biophenol components. The UV detector detect the components and send a signals to be presented as chromatogram.

Agilent Technologies, Inc [http://polymer.ustc.edu.cn] is one of the famous companies in chromatography and instrumental analysis speaks in details about HPLC. HPLC is an abbreviation for high performance or high pressure liquid chromatography. HPLC has been used for about 35 years and is the largest separations technique used.

HPLC is a separation technique that involves the injection of a small volume of liquid sample into a tube packed (columne) with tiny particles (3 to 5 micron (μm) in diameter called the stationary phase) where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. An output from this detector is called a "liquid chromatogram".

HPLC is used for optimum the separation of chemical and biological compounds that are non-volatile.

Typical non-volatile compounds are:

- Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol)
- Salts like sodium chloride and potassium phosphate
- Proteins like egg white or blood protein
- Organic chemicals like polymers (e.g. polystyrene, polyethylene)
- Heavy hydrocarbons like asphalt or motor oil
- Many natural products such as ginseng, herbal medicines, plant extracts
- Thermally unstable compounds such as trinitrotoluene (TNT), enzymes.

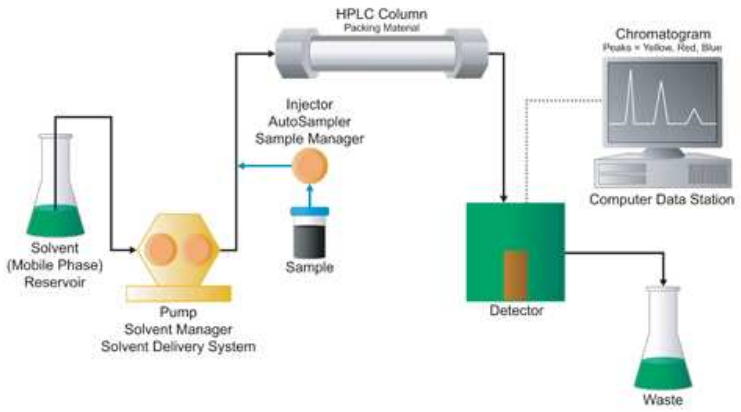


Figure 3.6: Schematic of an HPLC. [www.comsol.com]

3.3.1 Method of analyzing for Rutin ,Verbascoside and Luteolin 7-glucoside .

For analyzing Rutin ,Verbascoside and Luteolin 7-glucoside ,the following parameters used for this purpose.

HPLC Aparatus	Autosampler Merck HITACHI, Elite LaChrom
Column	XBridge ^{TM} C18 4.6*250 mm ,Waters
Column Temp.	40 C^0
Flow Rate	1.5 ml/min
Wavelength (λ)	350 nm
Mobile Phase	potassium phosphate Buffer pH 3 (Gradient)
0-15 Min.	14% Acetonitrile 86% Buffer
15-30 Min.	30% Acetonitrile 80% Buffer
30-35 Min.	14% Acetonitrile 86% Buffer

Table 3.1: HPLC conditions for analyzing Rutin ,Verbascoside and Luteolin 7-glucoside.

3.3.2 Method of analyzing for Hydroxytyrosl

The HPLC conditions for analyzing Hydroxytyrosol as in the next table.

HPLC Aparatus	**Autosampler Merck HITACHI ,Elite LaChrom**
Column	**XBridge TM C18 4.6*250 mm ,Waters**
Column Temp.	**40 C0**
Flow Rate	**1.5 ml/min**
Wavelength (λ)	**277 nm**
Mobile Phase.	**Potassium phosphate Buffer pH 3 (Gradient)**
0-7 min.	**7% Acetonitrile 93% Buffer**
7-10	**50% Acetonitrile 50% Buffer**
10-13	**7% Acetonitrile 93% Buffer**

Table 3.2: HPLC conditions for analyzing Hydroxytyrosol

3.3.3 Method of Analyzing for Oleuropein.

HPLC Aparatus	**Autosampler Merck HITACHI ,Elite LaChrom**
Column	**XBridge TM C18 4.6*250 mm ,Waters**
Column Temp.	**40 C0**
Flow Rate	**1.5 ml/min**
Wave Length (λ)	**248 nm**
Mobile Phase	**Potassium phosphate Buffer pH 3**
0-16 min.	**19% Acetonitrile 93% Buffer**

Table 3.3: HPLC conditions for analyzing oleuropein.

3.4 Phase diagram

The phase behavior of the OLE consisting of OLE, oil (olive oil), surfactant (Tween 80) may be described on a phase tetrahedron whose apexes respectively represent the pure components.1g of a mixture consisting of olive oil, tween 80 surfactant at different weight ratios were prepared in culture tubes sealed with Viton lined screw caps and stirred at room tempareture by vortex until clear solution was obtained. Titrating these samples with OLE which was added drop wise until its solubilization limit was reached. Vigorous stirring followed all of the aqueous phase (OLE) additions on a vortex mixer.

The anisotropy by cross polarizers and polarizing microscope. The single isotropic sample which will be dark under cross polarizers will be regarded to either cubic or micelle; which can be distinguished by viscosity. The anisotropic lamellar liquid crystal and hexagonal liquid crystal are determined by the cross polarizers and polarizing microscope. Detect the boundary of single phase; finally draw the phase diagram using Origin Pro 8.1 software [Salem, S 2012]. By Drawing this phase diagram the regions of W/O and O/W will be clear at certain composition of OLE /olive oil/tween 80 which will help the formulator design a delivery system aqueous or oily based.

Chapter 4

4- Results and Discussion

4.1 Best Extraction Method

Different methods of extraction had been investigated in this research, Maceration, decoction (boiling), soxhlet, ultrasound extraction (sonication), stirring and reflux distillation.

Figure (4.1) shows the amount of oleuropein extracted using different methods of extraction. Different samples were prepared and extracted for one hour using methanol 99% as a solvent and the extract analyzed by HPLC for quantification of oleuropein using 75% sodium acetate buffer pH 3.6, and acetonitrile 25%, C18 column and the oleuropien was detected at 260 nm with UV-Vis detector as clear in the chromatograms Figures. (4.3), (4.4).

Figure (4.1) shows the efficiency of different methods for extraction of olueropein from olive leaves. Reflux distillation method of extraction gives the highest amount of oleuropein extracted, at the same time maceration extraction method shows the minimum amount.

Figure (4.2) shows the total amount of biophenols compounds extracted from OLE. Sonication method was the best method used with a yield amount of 35.2% and maceration method with showed a minimum yield percent.

The concept of reflux distillation and soxhlet methods of extraction are to condense the solvent (methanol) and bring it back to use again to increase the efficiency of solvent in extraction. The boiling point of methanol is 64.7 ^{0}C which helps to increase the amount of

extraction which has an advantage over soxhlet .The other methods also showed good amount of extraction, but we need to consider the expensive cost as sonication beside the long time of maceration. Decoction is also a satisfactory method of extraction but it should be followed with a solvent collector like condenser to condense and collect the solvent.

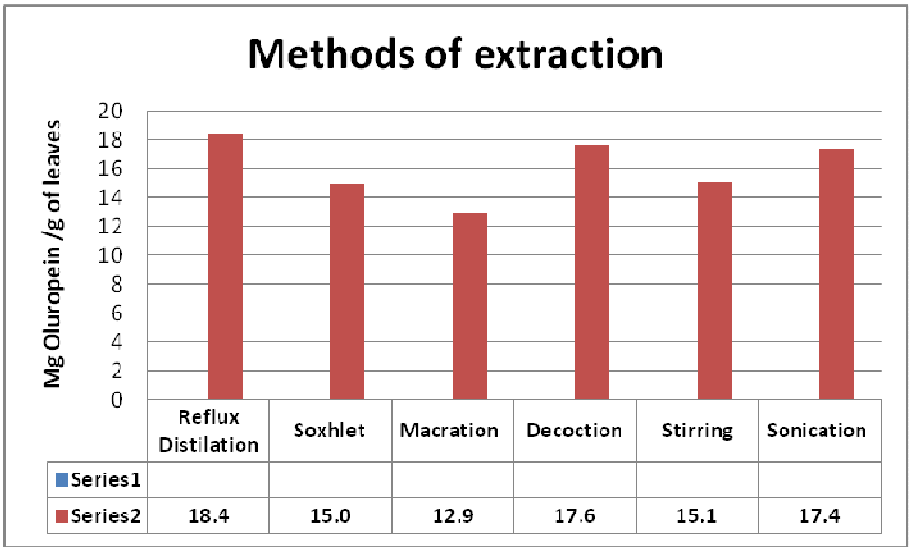
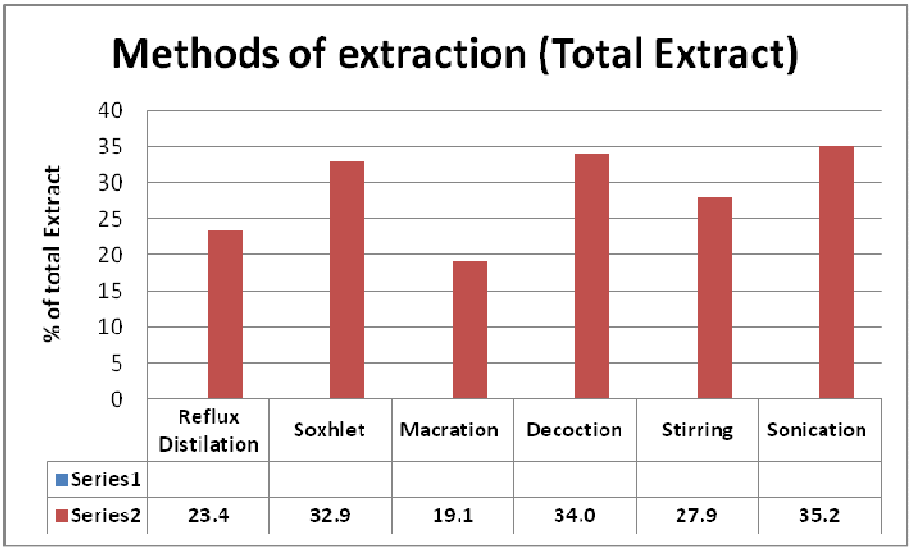


Figure 4.1: Amount of oleuropein extracted using different methods of extraction.



Figuer 4.2: Total amount of OLE compound extracted using different methods of extraction.

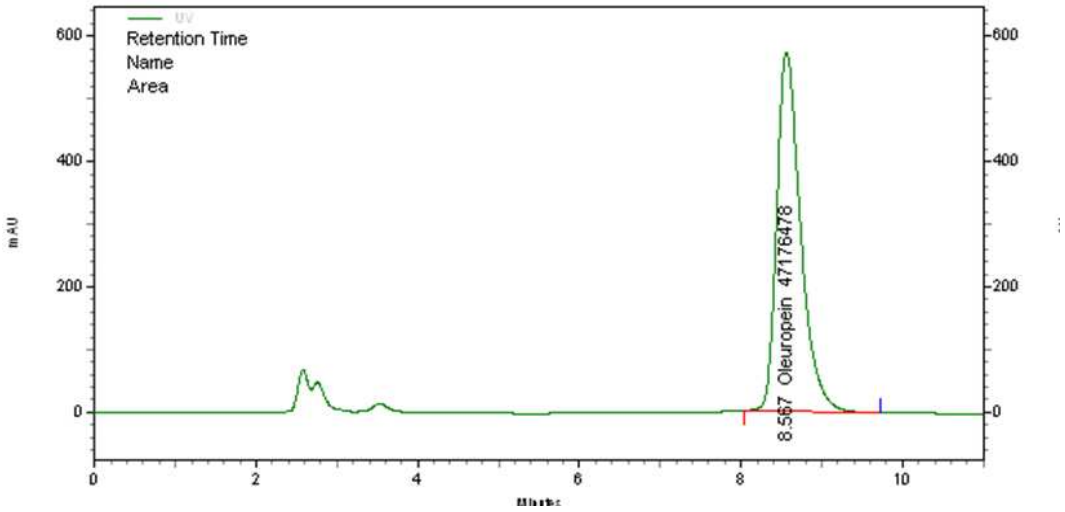


Figure 4.3: HPLC Chromatogram of oleuropein reference standard for the extraction analysis

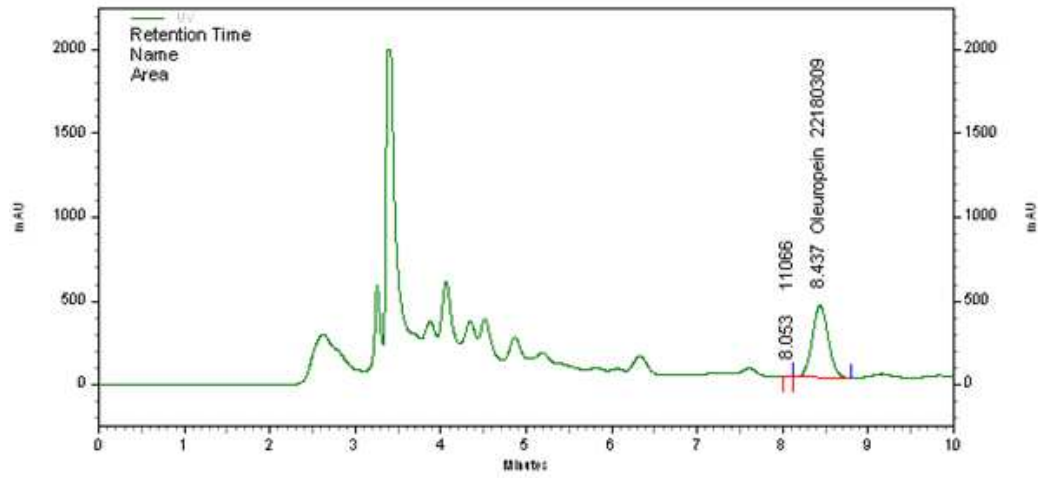


Figure 4.4: HPLC Chromatogram of olive leaves extract for the extraction analysis

4.2 Best solvents for extraction

Different solvents were used for extracting the biophenol compounds form olive leaves. Hexane and petroleum ether turned out to be inadequate as extraction solvents. As the biophenol compounds of olive leaves are polar compounds therefore using non polar solvents like hexane and petroleum ether would not be appropriate. As shown in figure (4.5), the amount of oleuropein extracted by hexane was 0.05 mg /g and for petroleum ether was 0.005 mg/g of olive leaves.

Different compositions of water /ethanol/methanol mixtures were used to investigate the best solvent to increase the yield of leaves extract. 10 g of olive leaves were put in 500 ml round bottom flask, 250 ml of different pure solvents were added (Water 100% adjusted to pH=1with hydrochloric acid 35%. ethanol 99.8 %, methanol 99%) beside of different compositions of water /ethanol/methanol from 25% to 75%.

From figure (4.6), we can see that the amount of oleuropein extracted using methanol 100% is about 18.4mg/g of olive leaves which is greater than ethanol 100% which is 17.8 mg/g and for water 100% is the minimum amount which is 0.1 mg /g of olive leaves. For the total biophenol compounds extract (total extract) the situation is opposite, from figure (4.1) we can see that the percentage of total biophenol compound extracted by methanol 100% is 23.4% and for ethanol 100% is 25% but For water 100% is the greatest which is 33.8%.

Using water as co-solvent with both ethanol and methanol reduced the amount of oleuropein as the amount of water decreesed, but the amount of total extract increase at 25% of water and reduce at amount of 50% but come back to increase at 75% to get the maximum amount of total extract for water/ethanol 75/25 is 34.3% and water /methanol 75/25 is 27.4%.

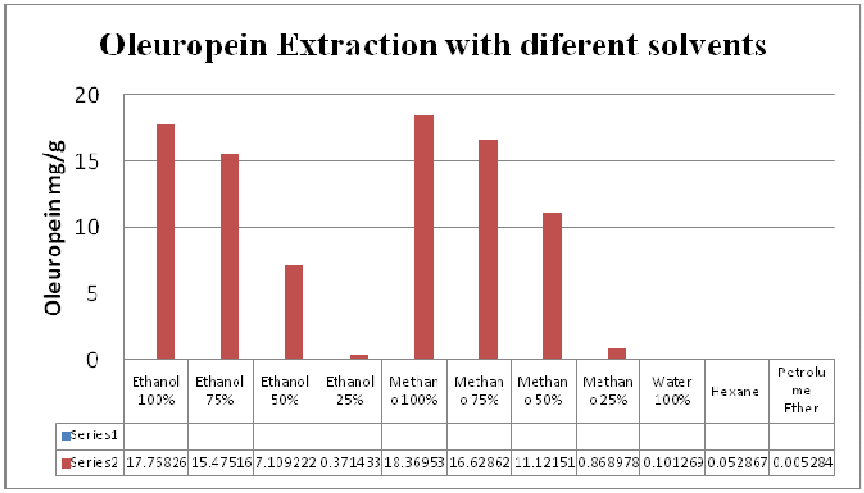


Figure 4.5: Amount of oleuropein extraction.

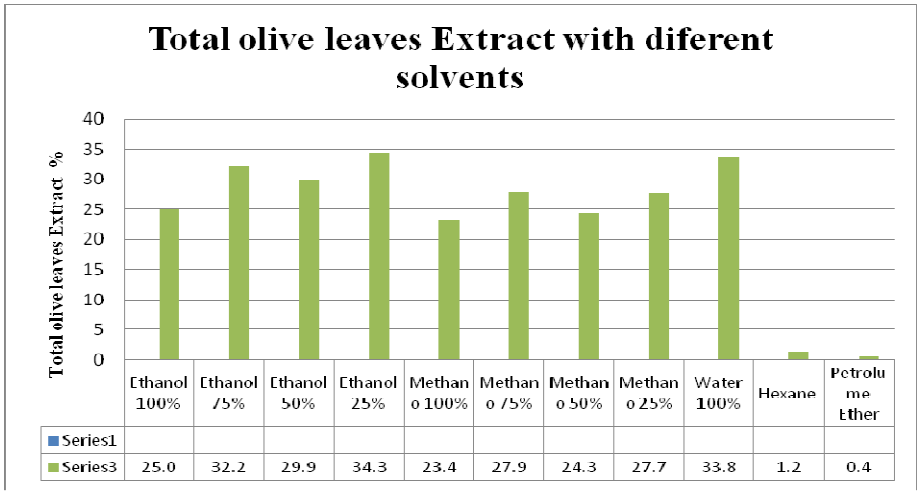


Figure 4.6: Total OLE compounds extraction.

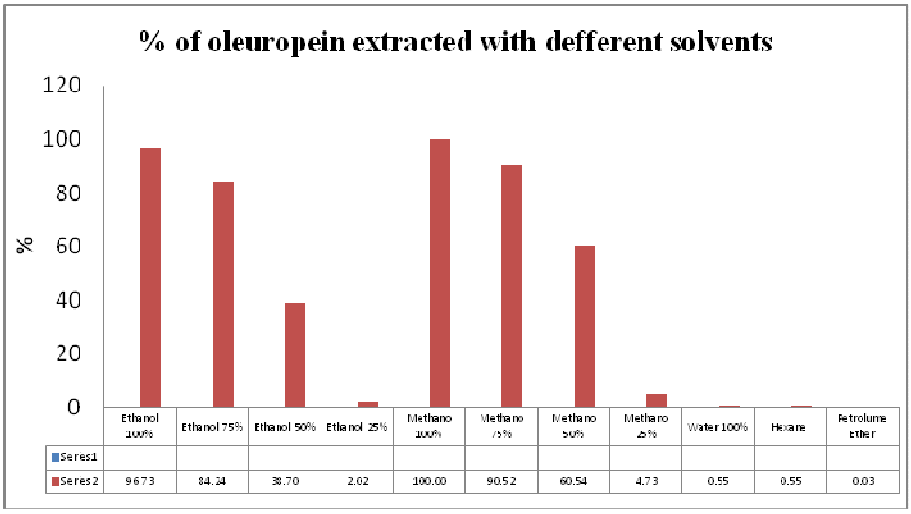


Figure 4.7: Percentage of oleuropein extracted with different solvents.

From these results we can see that the polar solvents is the suitable solvents for increasing the amount of extraction as the polarity increases the amount of extract increase .Not only the polarity is the main factor for increasing the yield of extraction but ,also the temperature is an important factor . When using water 100% we see that the amount of oleuropein decreases, which means that at the boiling point of water at 100 C^0 degradation and decomposition,happenes to the oleuropein.

4.3 Effect of pH on extraction

Different samples of 1g in weight of olive leaves were sonicated at different pHs and the oleuropen compound analyzed by HPLC as an indication for the amount of extract yield.

From figure (4.8) we can see that the highest yield of extraction occurs at pH 1 and the lowest at pH 13.

From figure (4.9) we can see the percentage of each pH assuming that pH 1 which is the highest is 100%.

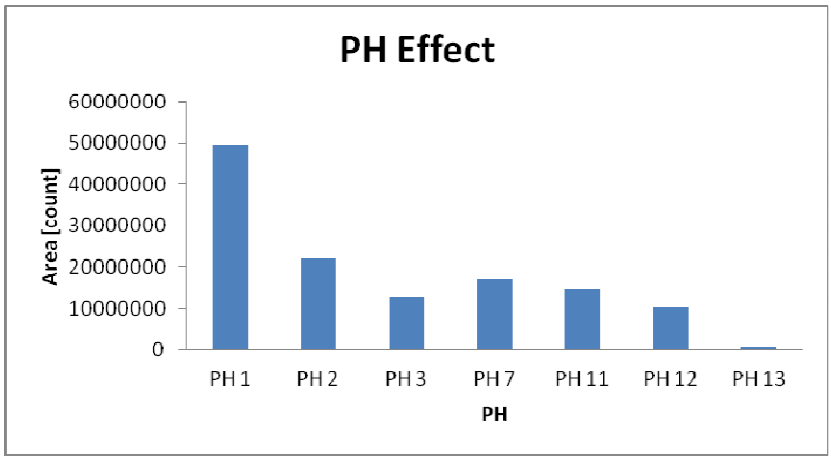


Figure 4.8: Effect of pH on amount of Oleurpein Extraction.

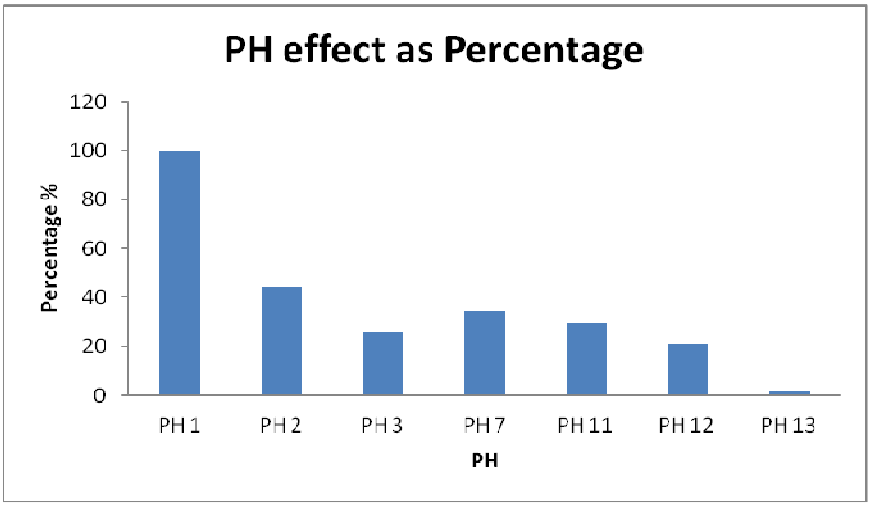


Figure 4.9: pH Effect as Percentage.

Using maceration as another method of extraction to investigate the effect of pH on the amount of oleuropein extraction .Ten grams of olive leaves sample were macerated in 250 ml ultra pure water at different pHs 1 and 3 at 60 C^0 for 4 hours as the conditions of Ansaria .M et.al. It is found that the amount of oleuropein at pH 3 is 0.9 mg/g of olive leaves is higher than pH 1 which is 0.1 mg/g. Jap´on-Luj´an .R, et.al studied the influence of pH on the extraction efficiency on some Biophenol compounds from olive leaves pH 7.5 which showed to be optimal for all OBPs as seen in figure (4.10).

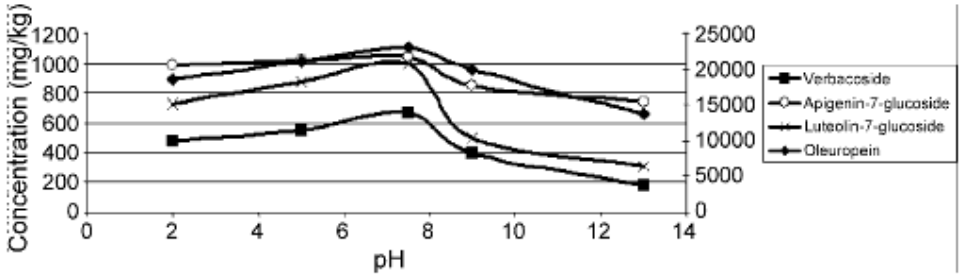


Figure 4.10: Concentration-pH relationship for oleuropein and verbacoside, apigenin-7-glucoside and luteolin-7-glucoside [Japon-Lyan .R. at.al, 2006].

From the above we can see that pH factor is not the main parameter which has the main effect of biophenol extraction. Using different methods of extraction can change the effect of pH as we see at sonication method we get the maximum amount of extraction at pH 1 and for another method was at pH 3 and for third method was at pH 7.5.

4.4 Optimizing time of extraction.

As we see, the boiling method is one of the most effective methods to yield large amounts of olive leaves extract. For optimizing the time of extraction, a sample weight of one gram boiled in 100 ml of ultra pure water and within the first hour samples have been analyzed

each five minutes by HPLC ,and the next second hour samples have been analyzed each 15 minutes and the third hour each 30 minutes.

From figure (4.11), as the time increased the amount of extraction increased until about 60 min. but after that the concentration of oleuropein slightly decreased by the degradation of heat.

From figure (4.12) we can see that from the first 5 min., the amount of extraction is very big and then increased approximately in a moderate way. After 45 min., the amount of extraction becomes stable. As shown in the figure, 45 minutes is the optimum time to have the maximum extract from olive leaves .if we see the interval from 20 min. to 45 min the increase of the amount of extraction is about 12.5% in 25 min so from the economical side this will increase the running cost of extraction from energy and time so it is suitable to chose 20 min. for the time of extraction.

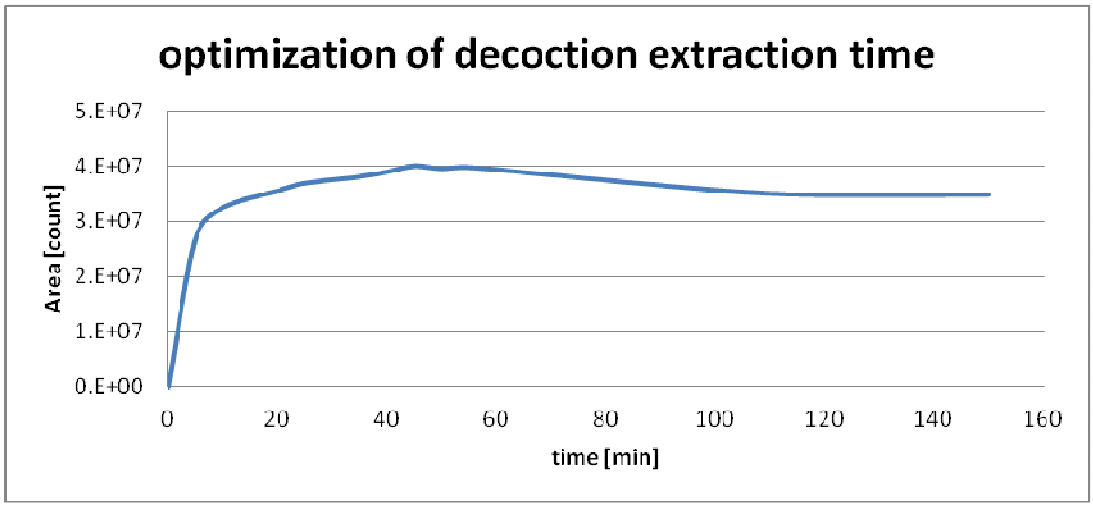


Figure 4.11: Optimization of decoction extraction time.

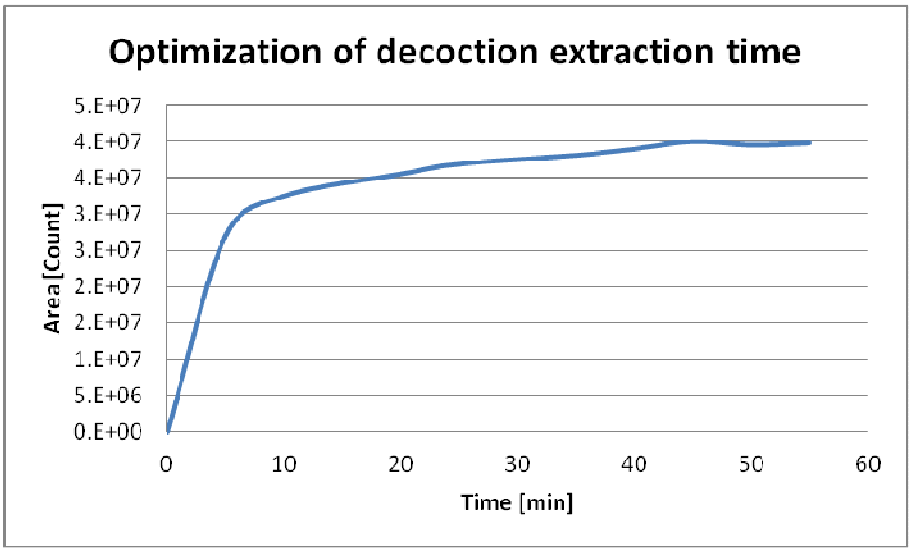


Figure 4.12: Optimization of decoction extraction time.

4.5 Olive leaves extract stability

The stability of olive leaves main phenolic compounds (Verbascoside ,Rutin , Luteolin 7-glucoside and Oleuropein) has been investigated at different temperatures ,pHs and solvents . Three samples prepared for the study, the first two samples contain 6g of olive leaves macerated with 100 ml of ultra pure water at pH 1 and 7 for 4 hours in a water bath at 60 C^0, and the third sample weight 1 gm extracted using soxhlet apparatus (Soxtest) using Ethanol as a solvent for one hour.

4.5.1 Analyzing methods used in the study:

As OLE contains many polyphenoyl compounds, validated methods of analysis should be used to determine quantitatively the amount of these compounds. Method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Several publications outline guidelines to validate pharmaceutical methods such as the United States Pharmacopeia (USP) [U.S. Pharmacopeia' 1994], International Conference on Harmonisation (ICP) [International Conference on Harmonisation, 1995] and the Food and Drug Administration (FDA) [Food and Drug Administration, 1994]. In general, methods must include studies on selectivity, linearity, accuracy, precision, dynamic range of response, limit of detection (LOD) and limit of quantification (LOQ).In this work we checked the linearity ,LOD and LOQ, and having good separation and resolution .

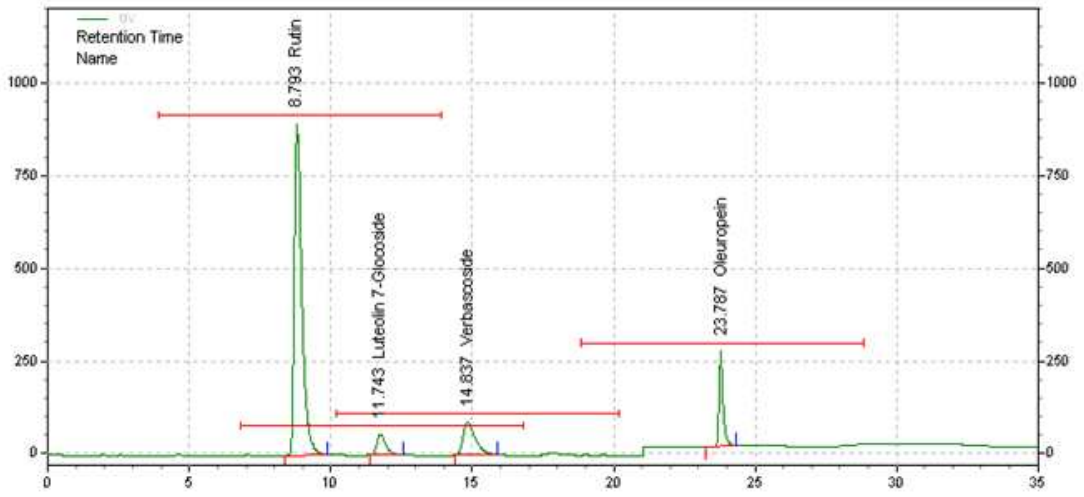


Figure 4.13: HPLC Chromatogram of reference standard for Rutien,Verbascoside ,Luteolin 7-Glocoside.

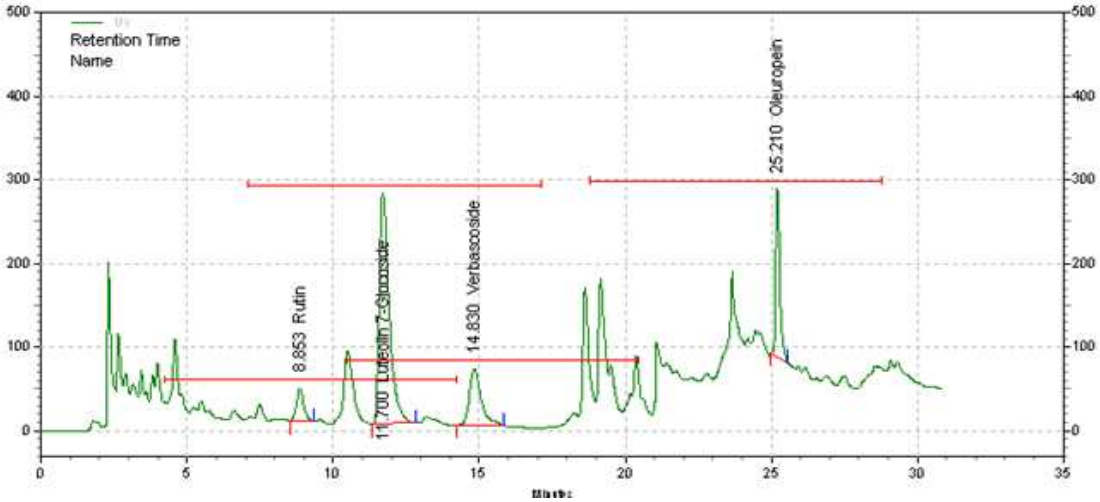


Figure 4.14: HPLC OLE Chromatogram showing Rutien, Verbascoside, and Luteolin7Glocside

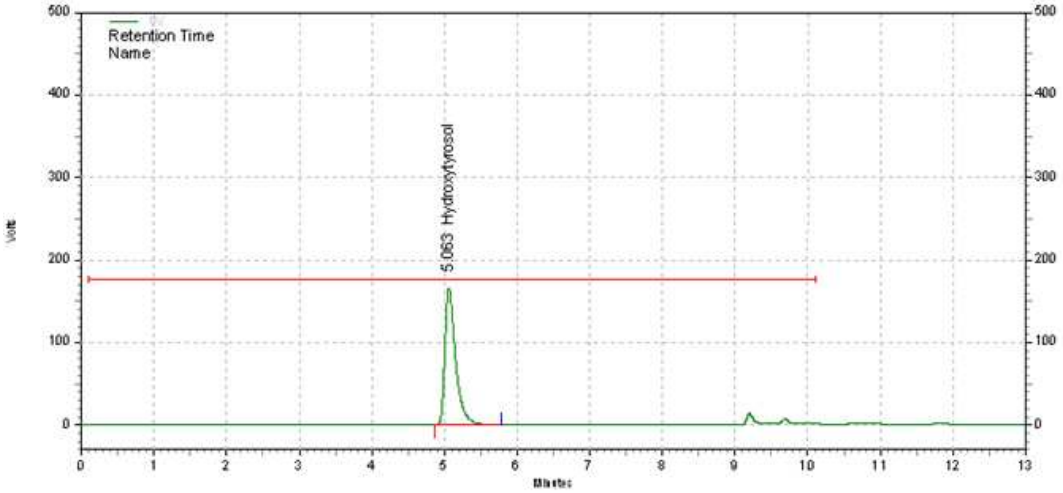


Figure 4.15: Hydroxytyrosol reference standard chromatogram

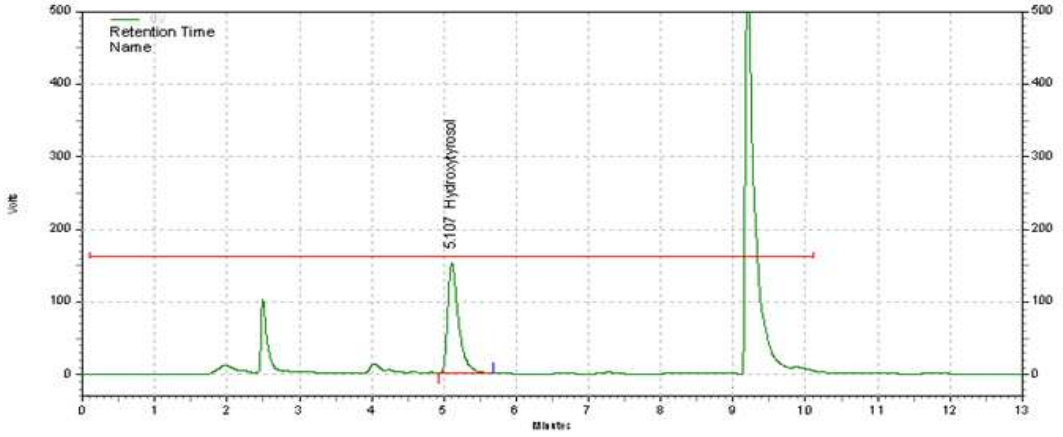


Figure 4.16: HPLC OLE sample chromatogram showing hydroxytyrosole peak

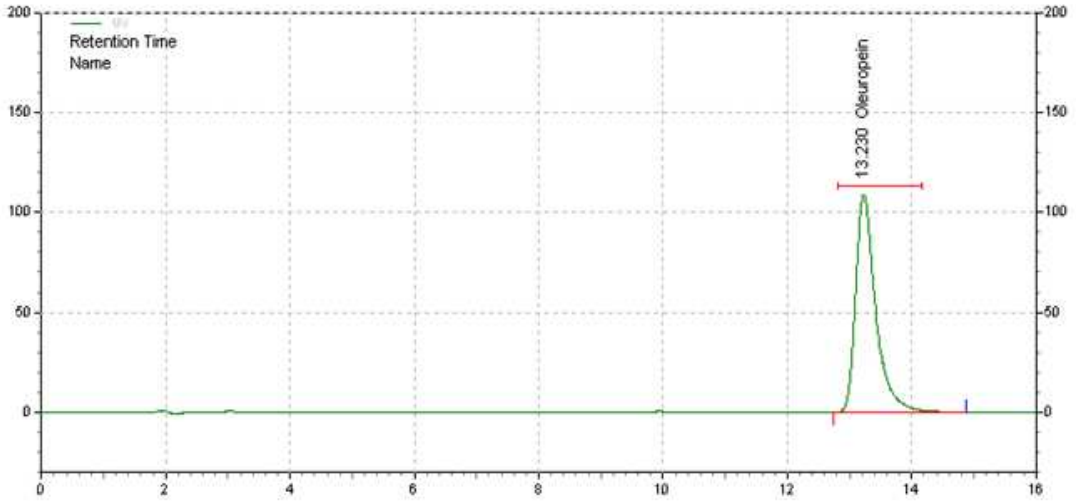


Figure 4.17: Chromatogram of Reference standard of oleuropein for the stability analysis

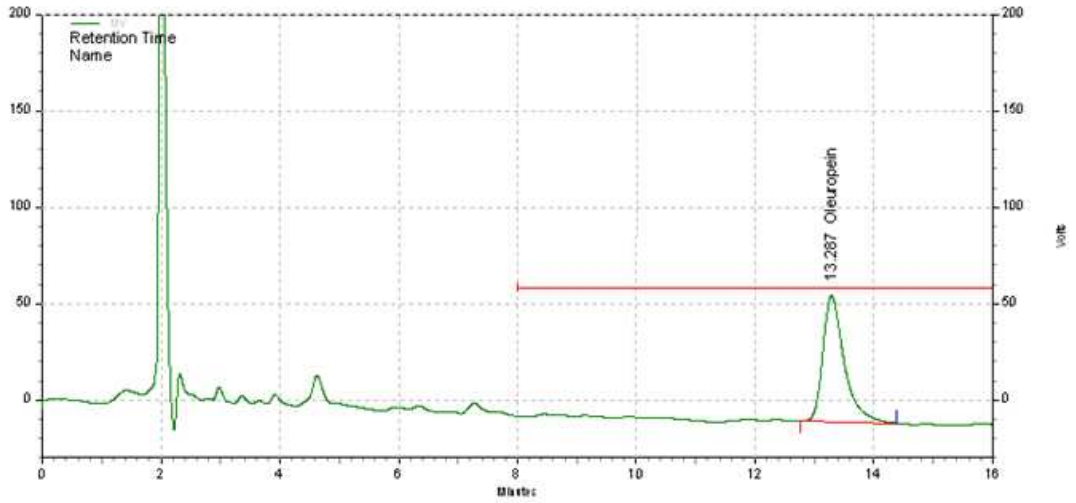


Figure 4.18: HPLC OLE Chromatogram showing oleuropein peak for the stability analysis

4.6 Stability of Rutien,Verbascoside ,Luteolin 7-Glocoside,Hydroxytyrosol and Oleuropein

The stability of Rutien, Verbascoside, Luteolin 7-Glocoside,Hydroxytyrosol and Oleuropein have been investigated according to the pH effect (pH 1,pH7) ,Temperature (4 ,

25, 35 C^0) effect and water and ethanol solvents effect the sample have been taken at each day of analyses and preserved at -80 C^0 .

4.6.1 Stability at pH 1 and 7

It is evident from figures 4.19 to 4.23 that at low PH's (PH 1, 3) the stability of OLE components is higher at high pH.

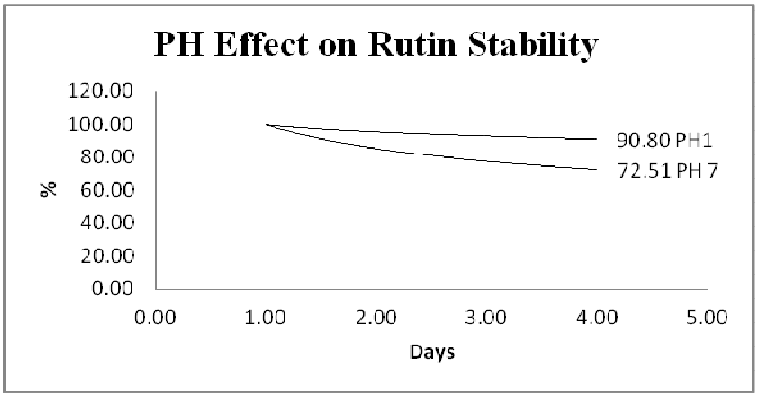


Figure 4.19: PH effect on rutin Stability

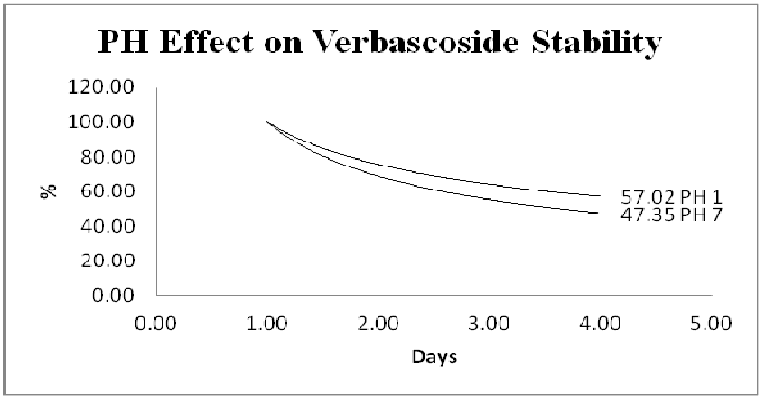


Figure 4.20: PH effect on verbascoside stability

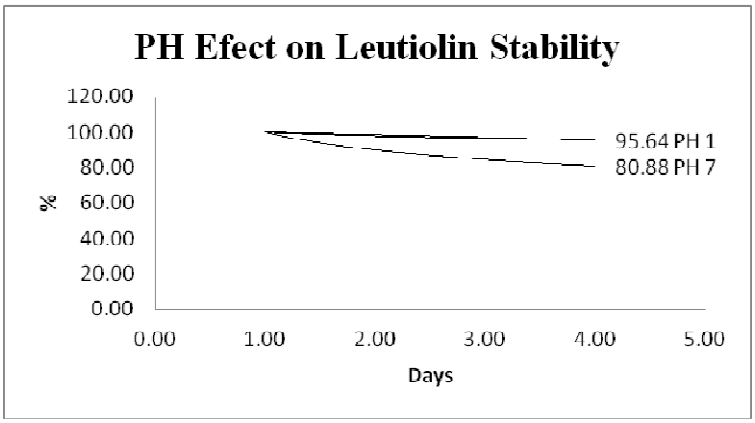


Figure 4.21: PH effect on leutiolin stability

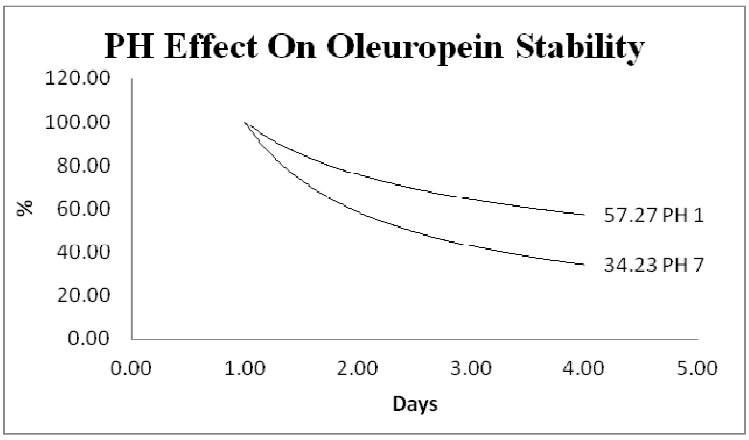


Figure 4.22: PH effect on oleuropein stability

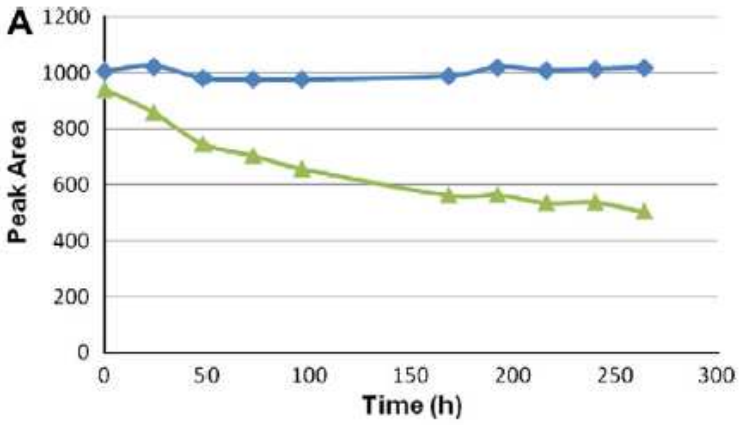


Figure 4.23: Hydroxytyrosol stability study at room temperature at PH 3 and PH 7 [Rambla-Alegre,M. et.al. (2011)].

4.6.2 Stability at different temperatures

From figure 4.24 to figure 4.28 we can see that as the temperature decreases the stability of OLE biophenols increase.

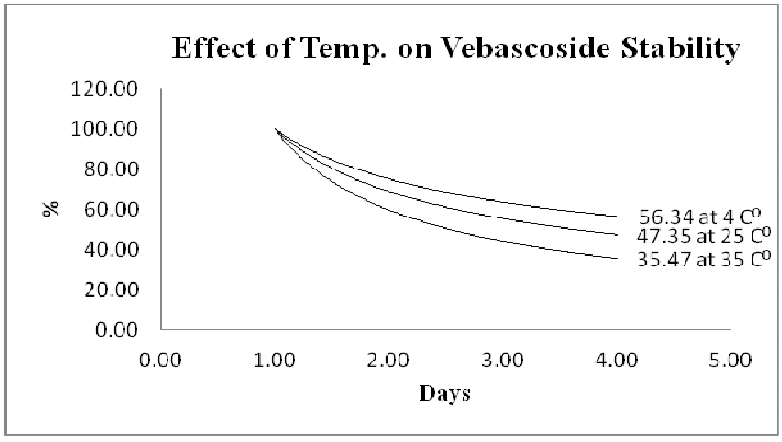


Figure 4.24: Effect of Temperature on verbascoside stability.

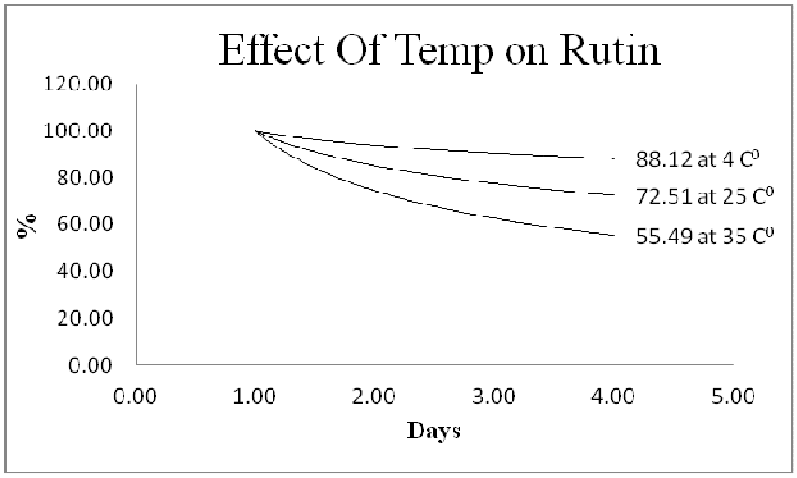


Figure 4.25: Effect of Temperature on rutin stability.

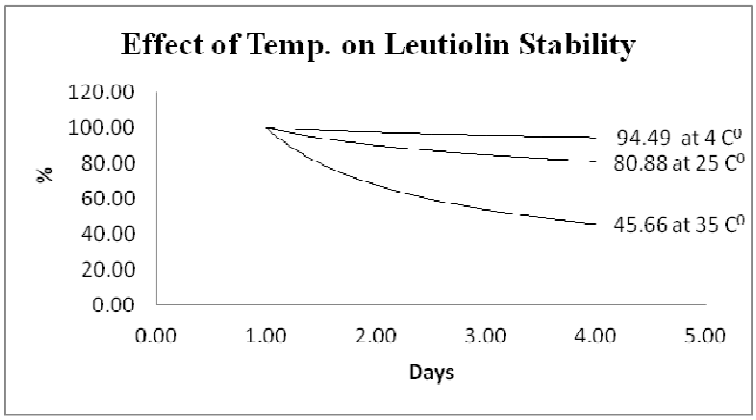


Figure 4.26: Effect of temperature on leutioline stability.

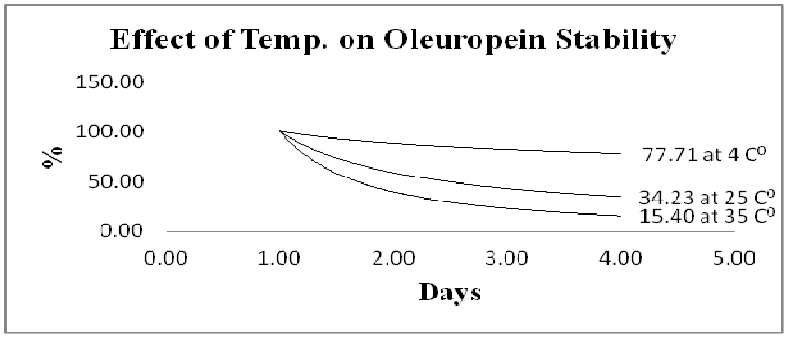


Figure 4.27: Effect of Temperature on Oleuropein stability.

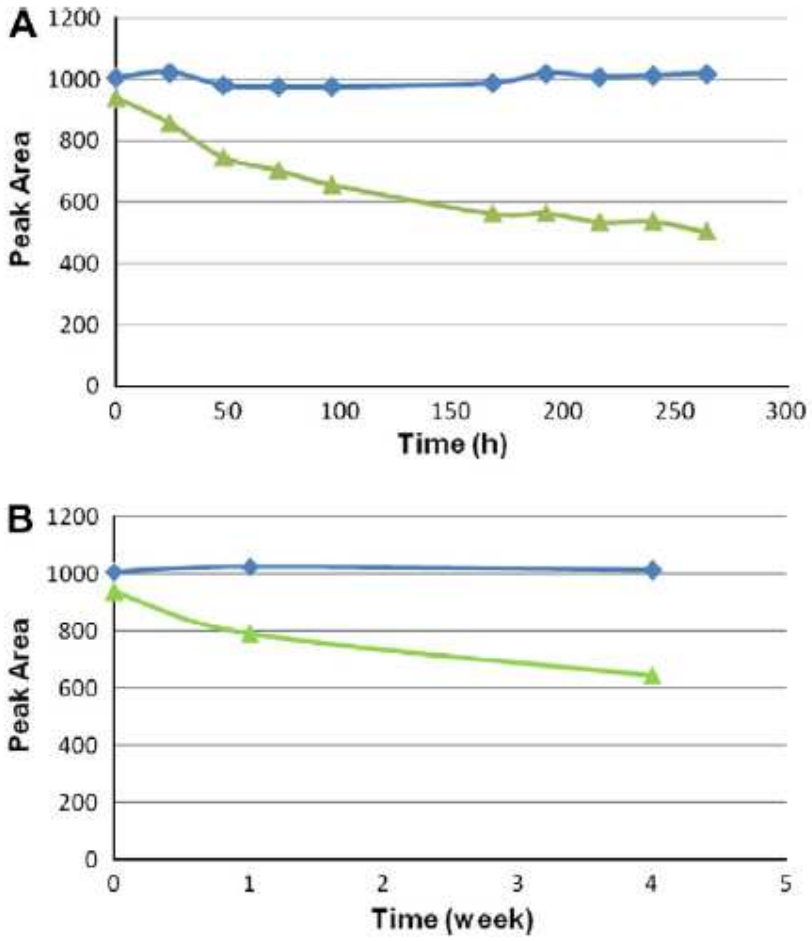


Figure 4.28: Effect of Temperature on Hydroxytyrosol stability .A room temp. and B at 4 C^0 [Rambla-Alegre,M. et.al. (2011)].

4.6.3 Stability using Ethanol as solvent

By making a comparison with water pH1at 4 C^0 and ethanol at 4 C^0 .From Figures 4.29 to figures 4.31 we can notes that Water is better than Ethanol.

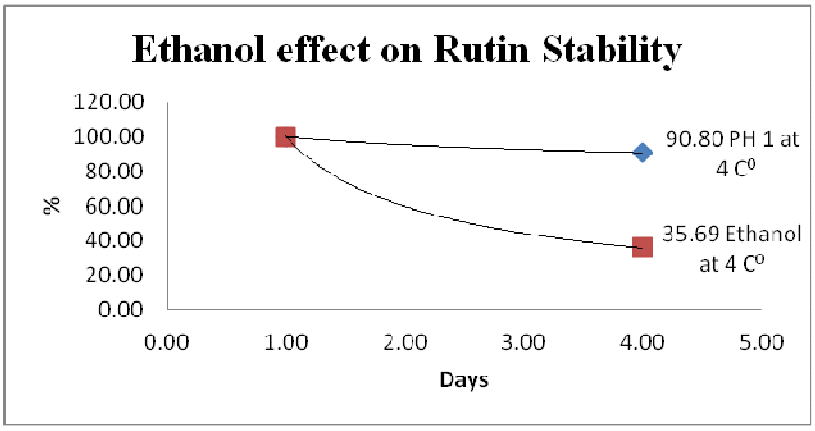


Figure 4.29: Ethanol effect on rutine stability

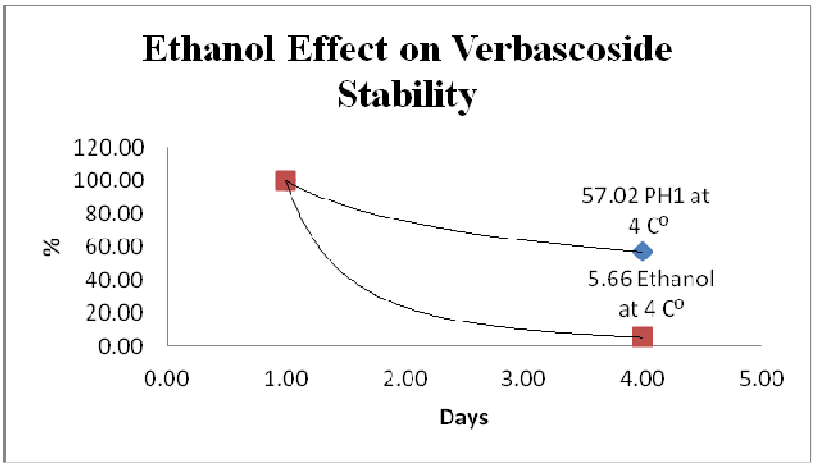


Figure 4.30: Ethanol effect on Verbascoside stability

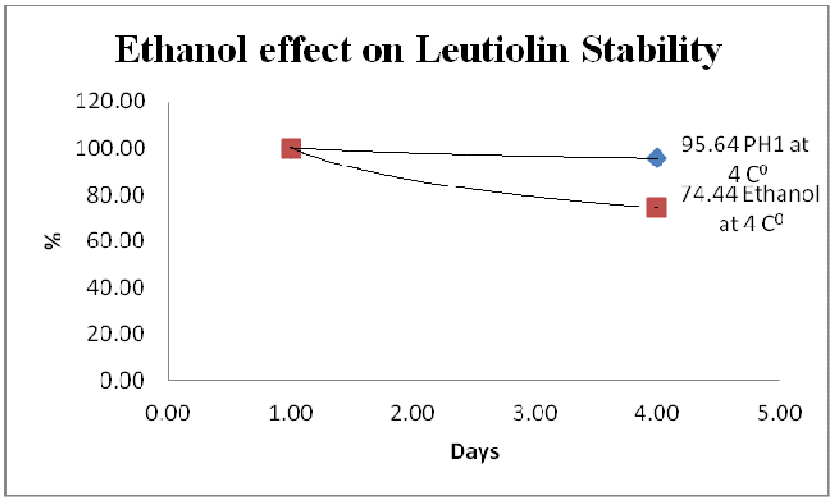


Figure 4.31: Ethanol effect on Leutioline stability

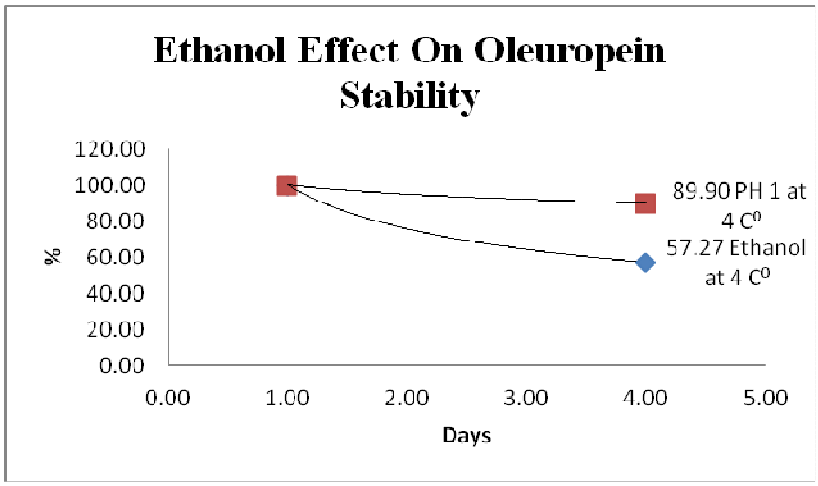


Figure 4.32: Ethanol effect on Oleuropein stability

4.6.4 Stability of Oleuropein ,Rutin ,Verbascoside, Hydroxytyrosol and leutiolin 7-Glocoside after 15 Days

After fifteen days the stability of Oleuropein ,Rutin ,Verbascoside, and leutiolin 7-Glocoside goes to zero but from figure 4.28 and 4.33 we can notice that even after 15 and 30 days the degradation hydroxytyrosol is not completely by the conversion of Oleuropein to Hydroxytyrosol [Santos M.M ,et.al,2012].

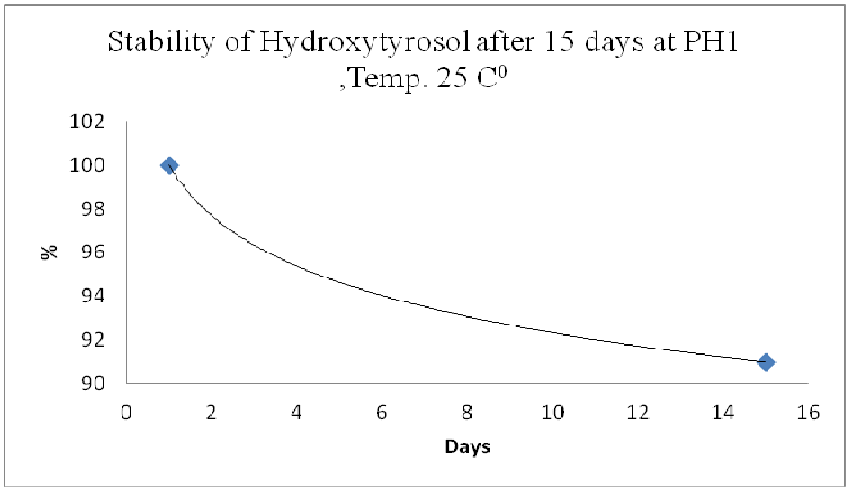


Figure 4.33: Stability of Hydroxytyrosol after 15 days at PH1 and Temperature 25 $C^{0.}$

4.7 Amount of Oleuropein ,Rutin ,Verbascoside, Hydroxytyrosol and leutiolin 7-Glocoside in Ramallah district

Olive leaves have been collected at March 2013 from Ramallah district, grind , preserved at 4 C^0 and analyzed at September 2013.Three samples prepared, the first two samples contain 6 gm of olive leaves macerated with 100 ml of ultra pure water at PH 1 and 7 for 4 hours in a water bath at 60 C^0, the third sample weight 1 gm extracted using soxhlet apparatus (Soxtest) using Ethanol as a solvent for one hour. The sample of Hydroxyltyrosol has been stored under -80 c^0 and analyzed after 18 days .

Component	Water pH1	Water pH 7	Ethanol
	mg/g	mg/g	mg/g
Oleuropein	47.15	2.64	32.26
Verbascoside	1.44	0.24	0.485
Hydroxytyrosol	0.524	0.00713	.098
Leutiolin 7-Glocoside	0.44	0.088	0.059
Rutin	0.1424	0.084148	0.0274

Table 4.4: Amount of Oleuropein ,Rutin ,Verbascoside, Hydroxytyrosol and leutiolin 7-Glocoside in Ramallah district.

4.8 Phase diagram of OLE /Olive oil / Tween 80.

The phase behavior of OLE investigated with olive oil and tween 80 .The first phase diagram contains 1.7% OLE.4% sugar and the second phase diagram contains 4% OLE and 14% sugar.

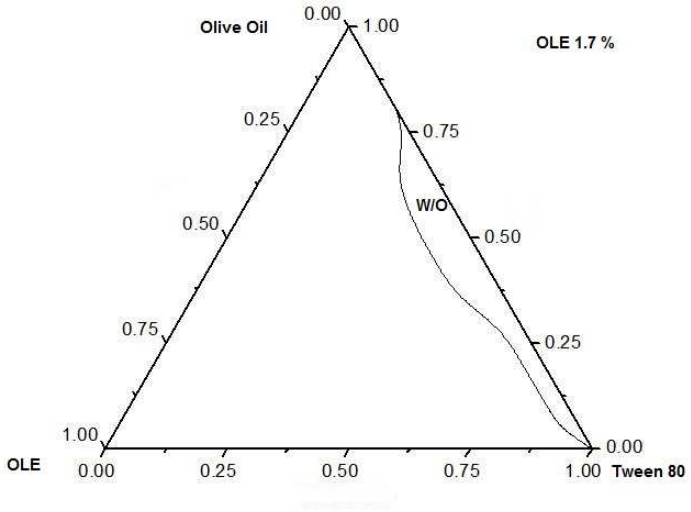


Figure 4.34: Phase diagram of OLE 1.7%/Olive Oil/Tween 80.

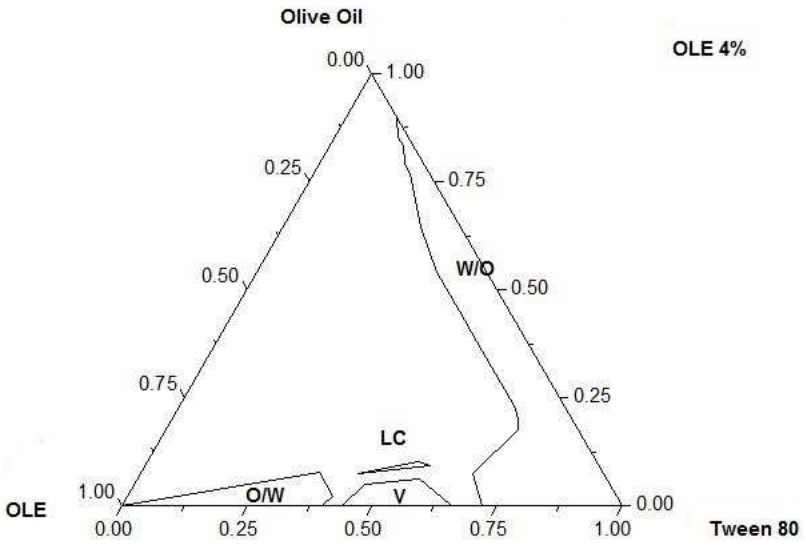


Figure 4.35: Phase diagram of OLE 4% /Olive oil/Tween 80

From the first diagram Figure 4.34 (1.7 % OLE) we can see that we have one region, but in the second diagram there are many regions W/O, O/W, LC.

Polychniatou V. et.al investigate the phase behavior for olive oil /Tween 40 and water using galic acid as co-surfactant and without it from the next figure we can see small zone of water in oil microemulsion.

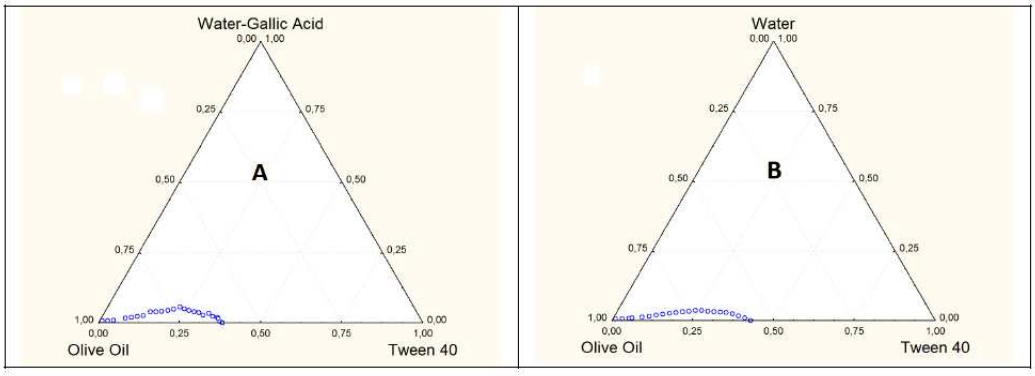


Figure 4.36: olive oil /Tween 40 and water.

Using OLE play as co surfactant and the polyphenol compounds improve the miscibility of water with the olive oil. So from figure (4.35) we can see that we get water in oil zone with low percentage of surfactant.

It was clear that increasing the amount of OLE increase the region of W/O. from figure (4.35) we can see the fermions of liquid crystal region in the range 84-85% of tween 80 surfactant. Also at low percentage of tween 80 surfactant we can get water in oil W/O at 4% of OLE but as the amount of OL decreases to 1.7% the percentage of surfactant need become high, that means that also OLE act as co-surfactant .

Chapter 5

5.1 Conclusions

1. Olive leaves extract rich in health benefits.
2. Decoction (boiling), soxhlet , Ultrasound Extraction (Sonication) and reflux distillation are the most effective extraction methods can be used for olive leaves extraction.
3. Polar solvents are the most suitable solvent for extracting the biophenol compounds. Methanol 100% is the best solvent for extraction Oleuropein but as it is toxic solvent Ethanol 100% is the suitable solvent. Water/Ethanol 75/25 % is the suitable solvent for extraction the total biophenol compounds.
4. Increasing the temperature up to 100 C^0 reduce the amount of biophenol compounds.
5. Using acidic pH solvents are the suitable pH for extracting the biophenol compounds.
6. Acidic media is the suitable media for elongate the stability of OLE.
7. Preserving OLE in a low temperature also elongate the stability life of OLE.
8. Increasing the amount of OLE increase the region of water/oil at low amount of surfactant.

5.2 Future work

1- Making full validation for the HPLC methods of biophenol compounds.

2- Analyzing the biophenol compounds during the four seasons from different districts in Palestine.

3- Investigating the phase behavior of the olive leaves extract with different surfactants, oils and different percentage of OLE.

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استخلاص و تحليل بعض مركبات الفينول المتعدد من اوراق الزيتون و دراسة سلوك الطور لمستخلص اوراق الزيتون/زيت الزيتون/تويين 80

اعداد : مصطفى احمد لبادة
اشراف : د. ابراهيم الكيالي

الملخص:

في هذا البحث تم جمع اوراق الزيتون من منطقة رام الله في شهر اذار وغسلها و تجفيفها في درجة حرارة الغرفة وتم بعد ذلك طحنها لاستخلاص مركبات البوليفينولز الموجودة فيها وذلك بعدة طرق مثل الغلي, النقع, الاستخلاص الساخن المتكرر (soxlet) ,الاستخلاص بواسطة طاقة الصوت (Sonication) ,التحريك المستمر والتقطير الراجع (reflux distillation), و تم استخدام جهاز الكروماتوجرافيا السائلة (HPLC) لتحليل هذه المركبات وتحديد كمياتها. وفي هذا البحث ايضا تمت دراسة تأثير النسب المختلفة لمستخلص اوراق الزيتون على سلوك الطور(behavior phase) لانظمة زيت الزيتون /تويين 80 (Tween 80).

توصل البحث الى ان طرق الاستخلاص مثل الغلي, الاستخلاص الساخن المتكرر, الاستخلاص بواسطة طاقة الصوت, التحريك المستمر, والتقطير الراجع تعتبر فعالة في استخلاص مركبات الفينول المتعدد في وقت قصير. أما بخصوص نوع المذيب فقد كان الايثانول هو الافضل لاستخلاص اكبر كمية من مركبات المتعدد الفينول وكذلك فان اضافة 75% من الماء الى الايثانول تزيد من نسبة فاعلية الاستخلاص الكلي لأوراق الزيتون .لقد اثبتت الدراسة كذلك بأن زيادة درجة الحرارة الى $100\ C^o$ خلال عملية الاستخلاص يؤثر سلبا على اسخلاص مركبات الفينول المتعدد. أما بخصوص استخدام وسط حامضي خلال عملية الاستخلاص فلقد وجد انه يزيد من كمية المواد المستخلصة وكذلك فانه يزيد من فترة ثباتية هذه المركبات وبخاصة عند حفظها في درجات حرارة منخفضة مثل $4\ C^o$. وأخيرا فقد وجدت الدراسة ان زيادة نسبة مستخلص اوراق الزيتون يؤدي الى زيادة منطقة الماء المذاب في الزيت باستخدام كمية قليلة من منشط السطوح (surfactant).
يوصي هذا البحث المزارعين بعدم القاء اوراق الزيتون في الحقل لما له تاثير سلبي على خصوبة التربة . تحليل مركبات البوليفينولزفي اوراق الزيتون خلال فصول السنة الاربعة لايجاد اعلى تركيز لهذه المركبات . دراسة تأثير النسب المختلفة لمستخلص اوراق الزيتون على سلوك الطور(phase behavior) باستخدام زيوت مختلفة و منشطات اسطح (Surfactants) مختلفة.