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Enrichment of Phenolic Compounds from Palestinian Olive Mill Wastewater and *in-vitro* Evaluation of their Medicinal Activities

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Dedication

To my beloved parents Sami and Rudyna, to my teacher Miss Rania Anbtawi, to Miss Reem Yaghmour, to my brothers and sisters, to my supervisor Dr. Fuad Al-Rimawi and to my Co supervisor Prof. Saleh Abu-Lafi, to Dr.Mohammad Abu Al-Haj, and to all the staff members of the Chemistry Department at Al-Quds University.

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.

Name: Mahmoud Sami Hasan AlNatsheh

Signed 

Date: 16 / May /2017

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Abstract:

The production of olive oil generates huge quantities of by-product called olive mill wastewater (OMWW) about 200 thousand m^3 per year, which poses serious environmental problems. This effluent contains several polyphenols. In this work, liquid-liquid extraction of olive mill wastewater using ethyl acetate was studied for extraction of phenolic compound of OMWW from sureef (Hebron) during the harvested gene Results revealed that phenolic compounds are very abundant in olive mill wastewater. Total phenolic content and total flavonoid content of the OMWW extract were determined using spectrophotometric method. Antioxidant activity of the OMWW extracts were evaluated using four different testes (two measure free radical scavenging ability (DPHH and ABTs) and two measure reducing ability of OMWW (CUPRAC and FRAP).

Results showed that OMWW is very rich in phenolic and flavonoid compounds, which extent high antioxidants activities reflected by their ability as reducing agents (FRAP, and CUPRAC which were found to be as 2193 ± 15.5 mMFe+2/ liter of and 433 ± 5.5 mg Trolox /liter, respectively) and ability to scavenge free radicals DPPH, ABTs (1406±13.6 mg Trolox /liter, and 6.3± 0.2 mg Trolox /liter of OMWW respectively).

The OMWW extract which showed also positive activities as antibacterial (gram positive and gram negative), antifungal as well as activities against yeast.

HPLC analysis of the OMWW extract showed that it contain mainly hydroxyltyrosol (H.T) and tyrosol (T) but no oleurpein.

The OMWW extract was used as natural preservative and antioxidants for olive oil and cream preparation. This positive has an effect on the stability of olive oil reflected by its acid value, peroxide value, K_{232} and K_{270}. The acid value of the olive oil sample with 1 % of OMWW extract added to it was found to be 0.91 ± 0.05 compared to 1.1 ± 0.04 for oil sample without OMWW extract. Peroxide value was found to be 14.2 ± 0.9, 18.1 ± 1.0 for olive oil sample with 1% OMWW and without OMWW extract, respectively, showing the effect of OMWW on the stability of the olive oil which lowered the Peroxide value. K_{232} and K_{270} of olive oil which reflects primary and secondary oxidation products of oil was also found to be affected by the addition of OMWW extract where they found to be lower for oil samples with OMWW extract. K232 value was found to be 3.64 ± 0.09 and 3.46 ± 0.09 for olive oil sample without and with 1% OMWW extract, respectively, while K270

was found to be 0.49 ± 0.04 and 0.43 ± 0.04 for olive oil sample without and with 1% OMWW extract, respectively.

OMWW extract was also added to a cream as natural preservative and compared to a cream with chemical preservative (positive control), and with a cream without any preservative (negative control). Results showed that OMWW extract can work as natural preservative exactly as conventional chemical preservative compared to the cream without any preservative (chemical or natural) where this cream was not stable.

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List of Abbreviations

OMWW: Olive mill waste water

AA : Acid value

PV: Peroxide value

TPC: Total phenolic content

TFC: Total flavonoid content

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

CUPRAC: Cupric reducing antioxidant power

ABTs: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

FRAP: Ferric reducing antioxidant power

AA: Antioxidant

PUFA: Poly unsaturated fatty acid

Chapter one:

Introduction:

1.1 Olive Mill Wastewater (OMWW) in Palestine

In Palestine, olive oil production generates large amounts of Olive Mill Wastewater (OMWW) remnants. It is mainly composed of polyphenolic compounds. The OMWW is claimed to be one of the most polluting effluents produced by the agro-food industries, because of its high polluting phytotoxics (figure1.1). It also exhibits high toxicity towards plants, bacteria, and aquatic organisms, due to its composition of organic substances (14-15%) and phenolic compounds (up to 10 g/l). (R.Adham Msc theses Al-Najah University 2012). However, this view is changing as OMWW has the potential to become a low-cost starting material rich in bioactive compounds, particularly phenolics which can be extracted, enriched and ultimately applied as natural antioxidants in food, cosmetics, and pharmaceutical industries. In Palestine, most olive millers either use common treatment methods like evaporation to manage OMWW or dispose it in an areas surrounding to their facilities.(R.Adham Msc theses Al-Najah University 2012)

Figure 1.1: Photo showing problem of OMWW

1.2 Antioxidants in OMWW

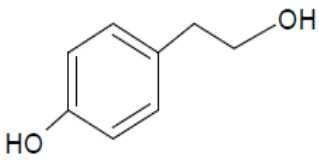
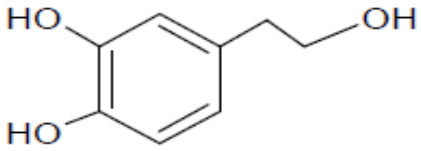
OMWW represents a great environmental problem, since this by-product is characterized by a high organic load; among the different organic substances found in OMWW, including sugars, tannins, phenolic compounds, organic acids and lipids. It is characterized by a dark-color caused by lignin polymerized with phenolic compounds, increased acidity (pH about 5), and high conductivity, among other factors .The phenolic content of OMWW is high up to 11.5 g/l with numerous interesting compounds. Many studies have been carried out on the recovery of polyphenols from OMW including adsorption on vegetable matrices. However, the aqueous extract of OMW (micro- or ultra-filtered OMW) can be used for the preparation of high value added products (Galanakis, Tornberg, & Gekas, 2010a). Besides its phenolic content, OMW contains many valuable nutrients such as sugar, proteins, and phosphorus; among others. OMW has also been referred to possess soluble dietary fibres and especially pectin material with excellent gelling ability (Galanakis et al., 2010a). The inorganic content of OMW is mainly composed of metals. Metals are important both from nutritional and toxicological viewpoints. Some metals, particularly iron, copper and zinc, are essential substances for the human body and their deficiency can have chronic and acute effects. However, even these elements can have

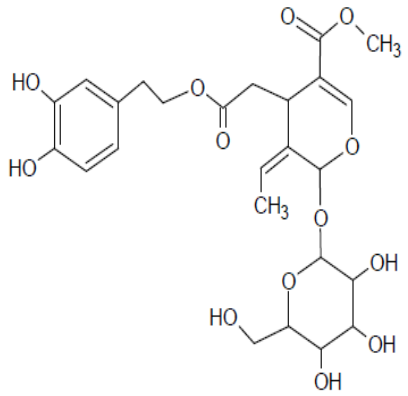
toxic effects depending on the chemical form, dose, route of absorption, and a host of other factors (Leung et al., 2010). OMW contains some important metal ions such as magnesium and calcium which are reported to minimize the risk of heart disease (Anne, 2011).OMWW composition varies broadly depending on many parameters such as olive variety, harvesting time, climatic conditions, and oil extraction process, among others. Consequently, characteristics of the raw material and extraction techniques have a significant effect on the composition of the final extracts, and so in the industrial process, the olive phenol extract composition must be standardized. An 'aqueous extract' of OMW suitable for foods, Olive mill wastewater generated from olive oil processing in the main olive oil producing.

The use of OMW aqueous extract in the preparation of functional beverages will certainly be governed by the quality and safety of the extracts. Some precautions should be considered during olive fruit harvesting, transportation and storage, and also during oil extraction.

The OMWW contains a lot of phenolic compounds, the main three phenolic compounds are Oleuropein, Tyrosol (T) and hydroxytyrosol (H.T) as shown in table (1.1).

Table 1.1 The main phenolic compounds in OMWW

Bio phenol	**Chemical structure**	**Properties**
Tyrosol		IUPAC name : 4-(2Hydroxyethyl)phenol Synonyms: 4-(2-Hydroxyethyl)phenol Molecular formula: $C_8H_{10}O_2$ Molecular weight :138.164
Hydroxytyrosol		IUPAC name : 4-(2Hydroxyethyl_-1,2-benzenediol Synonyms: 3-Hydroxyryrosol, 3,4-dihydroxyphenylethanol Molecular formula: $C_8H_{10}O_3$ Molecular weight: 154.16

Bio phenol	Chemical structure	Properties
Oleuropein		IUPAC name : (4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]- 5-ethylidene-6-[[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)- 2-tetrahydropyranyl]oxy]-4H-pyran-3-carboxylic acid, methyl este Molecular formula : C25H32O13 Molecular weight :540.514

1.2.1 Oleuropein:

Oleuropein is the active ingredient found in green olives, olive leaf and freash olive mill waste water. OMWW extracts are made from the fresh OMWW. The effectiveness of OMWW extract depends largely on the amount of oleuropein. Oleuropein and its metabolite hydroxytyrosol have powerful antioxidant activity in vivo and in vitro. (Nocolas Kalogerakis a, Maria Politia et al, 2013)

1.2.2 Hydroxytyrosol (H.T):

H.T is a phenylethanoid, a type of phenolic phytochemical with antioxidant properties in vitro. In nature, hydroxytyrosol is found in olive leaf, olive oil, and olive mill waste water in the form of its elenolic acid ester oleuropein and, especially after degradation, in its plain form.

Hydroxytyrosol itself in pure form is a colorless, odorless liquid. The olives, leaves and OMWW contain large amounts of hydroxytyrosol (compared to olive oil), most of which can be recovered to produce hydroxytyrosol extracts. However, it was found that black olives, such as common canned variety, containing iron(II) gluconate contained very little of the original hydroxytyrosol, as iron salts are catalysts for its oxidation. Hydroxytyrosol can also be produced endogenously as it is a product of dopamine oxidative metabolism known as DOPET (3,4-dihydroxyphenylethanol). Hydroxytyrosol is mentioned by the scientific committee of the European Food Safety Authority as one of several olive oil polyphenols that may contribute to protecting blood lipids against oxidation. (Nocolas Kalogerakis a, Maria Politia et al, 2013)

1.2.3 Tyrosol (T):

Tyrosol is an antioxidant that is naturally present in several foods such as wines and green tea. However, tyrosol is present most abundantly in olives. The antioxidant is present in the leaves, fruit, olive mill waste water, and, therefore, in the oil of the plant. It is classified as a phenolic antioxidant. Anything in the phenolic family has great antiseptic value.(Zafar, J et al 2012)

Although there are some very minor differences, tyrosol is considered almost identical to another antioxidant called hydroxytyrosol. In fact, the two antioxidants are often considered interchangeable.

The main job of any antioxidant is to protect cells and tissue from oxidative injury. Oxidative injuries to the brain can cause Parkinson's disease as well as Alzheimer's disease and other forms of dementia. Oxidative injuries can also cause cancer and heart.

Although tyrosol has not been proven to cure cancer, it can help prevent it. Also, studies have shown that those who regularly consume olive oil are at a lower risk of heart disease and mental impairment. In fact, it has been proven to greatly lower a person's risk of developing Alzheimer's disease.(White, E and Lockwood, B et al 2007)

Topically applied olive oil can also promote skin health and can keep skin from aging. However, particularly when treating acne, it should be remembered that tyrosol rich olive oil is not a "magic elixir". Also, modern medicine has proven that tyrosol does indeed have anti-aging properties similar to those of vitamin E. .(Nocolas Kalogerakis a, Maria Politia et al, 2013)

1.3 Antioxidant activity (AA)

OMWW are rich in natural secondary metabolites of phenolic compounds. The antioxidant activity of these phenolics have been attributed to different mechanisms, among which are prevention of chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction, binding of transition metal ion catalysts, reductive capacity and radical scavenging scheme1 shows mechanism of AA. There are many methods that have been proposed to evaluate antioxidant activity. Among are the DPPH assay, NO assay, reducing power assay, metal chelating, active oxygen species such as H_2O_2, O_2•− and OH• quenching assays.

The free radicals usually act by attacking the unsaturated fatty acid in the biological membranes which extend to membrane lipid peroxidation, decrease in membrane fluidity, and reduction of antioxidant defense enzymes, receptor activity and damage to membrane protein as shown in scheme (1). These destructive processes finally trigger the cell inactivation or death. The antioxidants generally scavenge the free radicals and detoxify the physiological system. The excessive production of free radicals, a decreased level of antioxidant defense enzymes and increased lipid peroxidation are responsible for producing oxidative stress and linked with various pathological conditions (Esterbuar et al 1990).

$LH + R^{\cdot} \rightarrow L^{\cdot} + RH$……..…….(1)

$L^{\cdot} + O2 \rightarrow LOO^{\cdot}$ …….…….(2)(Initiation)

$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$ ……..…….…….(3)(Propagation)

$LOOH \rightarrow LO^{\cdot} + LOO^{\cdot}$ + aldehydes ……..(4)

LH: is antioxidant

L:lipid:oil

Scheme 1: Mechanism of antioxidant activity

1.4 Methods of AA evaluation and principles:

There are different methods to evaluate the antioxidant capacity of food stuffs including olive oil and OMWW among which are: DPPH, FRAP, CUPRAC and ABTS.(YILDIRM, G et al 2009)

1.4.1 Free radical scavenging ability of OMWW:

1.4.1.1 ABTs assay (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)):

The ABTS cation radical (ABTS•+) which absorbs at 743 nm (giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS (2,2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding

the solution decolorization. ABTS can be oxidized by potassium persulphate (Pellegrini et al.2003) (Thaipong et al, 2006) see (Figure 1.2), giving rise to the ABTS cation radical (ABTS•+) whose absorbance diminution at 743 nm was monitored in the presence of Trolox, chosen as standard antioxidant (Pisoschi& Negulescu, 2012).

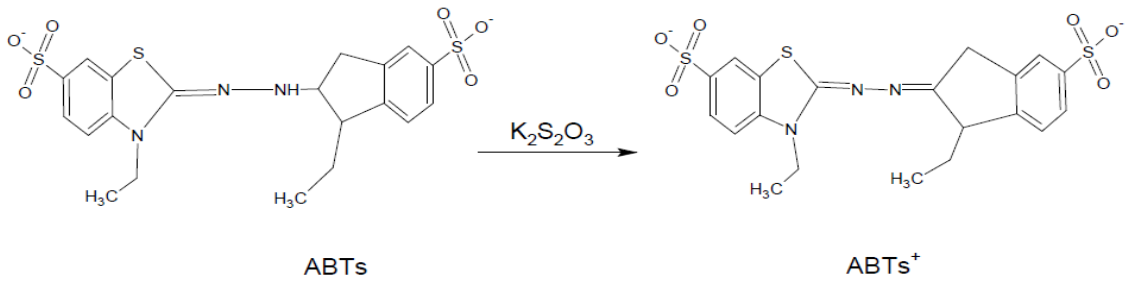


Figure 1.2: Oxidation of ABTS with $K_2S_2O_8$ and generation of $ABTS^+$(Miller et al, 1993)

1.4.1.2 2,2-Diphenyl1-1-picrylhydrazyl (DPPH):

This is known as a standard 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay.This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable DPPH radical (MacDonald-Wicks et al, 2006). Figure 1.3 shows the mechanism by which DPPH• accepts hydrogen from an antioxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals (Wicks et al, 2006). The antioxidant effect is proportional to the disappearance of DPPH• in test samples. Monitoring DPPH• with a UV spectrometer has become the most commonly used method because of its simplicity and accuracy. DPPH• shows a strong absorption maximum at 517 nm (purple).The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm. (Moon et al, 2009).

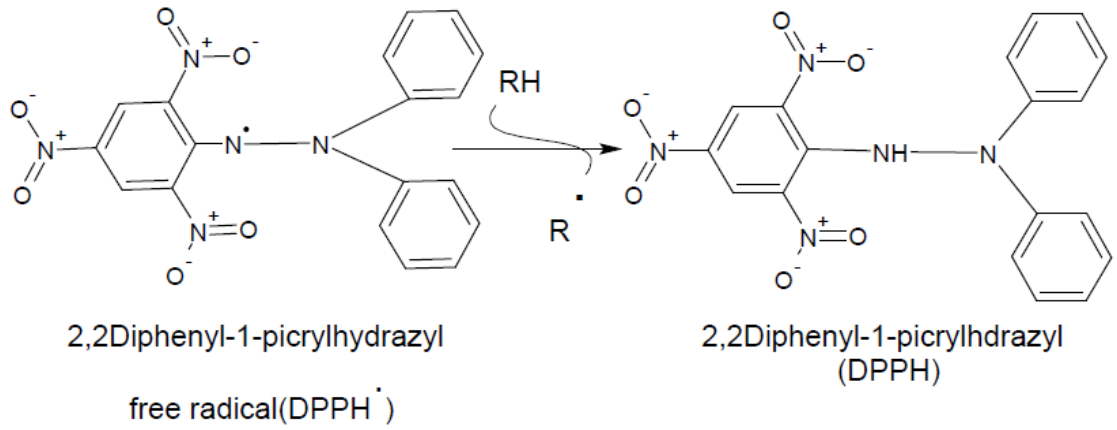


Figure 1.3: Mechanism of DPPH• free radical (Moon et al, 2009)

1.4.2 Reducing potential of the OMWW:

1.4.2.1 FRAP (Ferric Reducing Antioxidant Power):

Ferric Ion Reducing Antioxidant Power Assay (FRAP) is simple, fast, inexpensive, and robust method, and does not require specialized equipment. In the FRAP method the yellow Fe^{3+}TPTZ complex (2,4,6-tri (2-pyridyl)-1,3,5-triazine) is reduced to the blue Fe^{2+}TPTZ complex by electron-donating substances (such as phenolic compounds) under acidic conditions (Benzie et al, 1996), see Figure 1.4. Any electron donating substances with a half reaction of lower redox potential than Fe^{3+}/Fe^{2+} TPTZ will drive the reaction and the formationof the blue complex forward. The reaction detects compounds with redox potentials of <0.7 V (the redox potential of Fe^{3+}-TPTZ) (Prior et al, 2005)

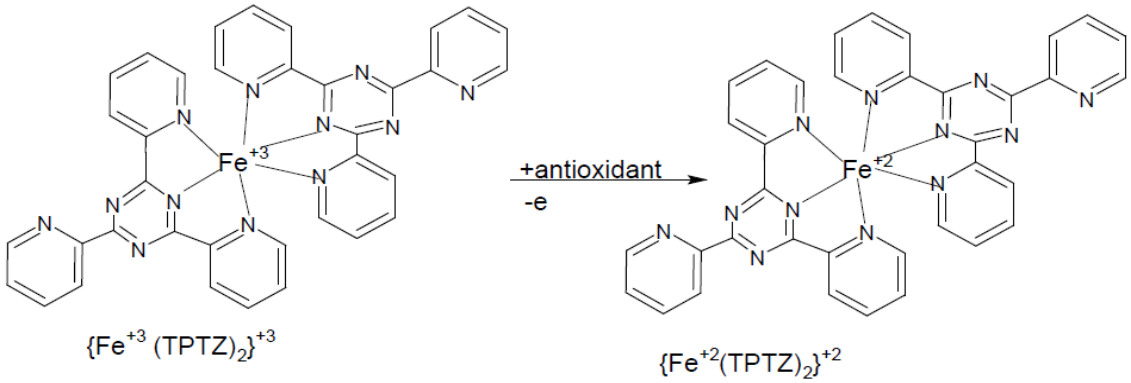


Figure 1.4: Reduction of yellow Fe^{3+} TPTZ complex (2,4,6-tri (2-pyridyl)-1,3,5-triazine) with antioxidants to the blue Fe^{2+} TPTZ complex by FRAP reagent (Prior et al, 2005)

1.4.2.2 CUPRAC assay:

The putative CUPRAC method was developed by (Apak et al, 2006). These assays are based on the reduction of Cu^{+2} to Cu^{+} by the combined action of all antioxidants or reducing in aqueous-ethanolic medium (pH 7.0) in the presence of neocuproine (2,9-dimethyl-1,10-phenanthroline), by polyphenols, yielding a Cu^{+} complexes with maximum absorption peak at 450 nm (Figure 1.6) (Lee et al, 2011). This method can be used for the determination of the antioxidant capacity of food constituent by the Cu^{+2}-neocuproine (Cu^{+2}-Nc) reagent as the chromogenic oxidizing agent. The reduction of Cu^{+2} in the presence of neocuproine by a reducing agent yields a Cu^{+} complex with maximum absorption peak at 450 nm (Tutemet al, 1991).

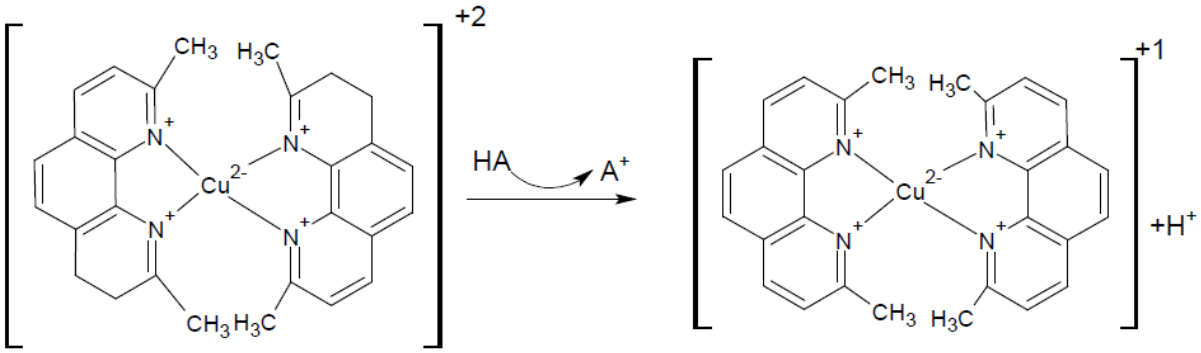


Figure1.5: CUPRAC reaction by an antioxidant molecule (HA: an antioxidant molecule, A+: an oxidized antioxidant molecule). Protons liberated in the reaction are neutralized by the ammonium acetate buffer (Tutemet al, 1991).

1.5 Total flavonoid content (TFC):

Flavonoids are phytochemicals that are naturally occurring chemicals produced by plants. They are biologically active and may affect health, however, unlike vitamins and minerals, they're not considered to be essential nutrients.(Uccella, N. et al 2000)

Dietary sources of flavonoids include fruits, vegetables, nuts, seeds, and legumes. While there is a fair amount of evidence that eating a diet rich in plant-based foods is beneficial for health, research hasn't explained how much of that benefit is due to the phytochemicals.

The benefit may be due to the nutrients, fiber, or because people who eat more plant-based foods may also be more likely to maintain their weight better and be more active. (Singleton V. L., and Rossi, J. A. et al 1965)

Despite a lack of evidence, some phytochemicals are available in supplemental form. These supplements are generally considered to be safe. The structure of flavonoid is shown figure (1.6).

The principle of aluminum chloride colorimetric method for TFC determined is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids. (Tutemet et al, 1991)

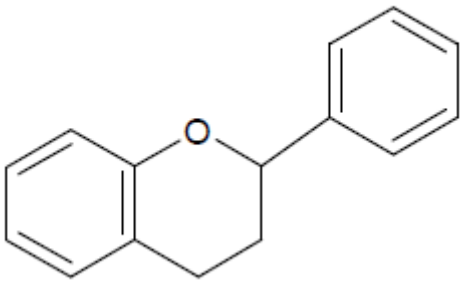


Figure 1.6: Structure of Flavonoid

1.6 Total phenolic content (TPC)

The Folin–Ciocalteu reagent which is a mixture of tungstates and molybdates works on the mechanism of oxidation–reduction reaction. The method strongly relies on the reduction of the mixture heteropolyphosphotungsates–molybdates by the phenolic compound which results in the formation of blue coloured chromogen. The phenolic compounds react with Folin–Ciocalteu reagent only under basic conditions adjusted by sodium carbonate solution. Under Basic conditions it has been observed that the phenolic compound undergoes dissociation to form a phenolate anion which reduces the Folin–Ciocalteu reagent i.e. the mixture of tungstates and molybdates rendering a blue coloured solution.

The colour intensity of the formed blue chromogen can be measured by the absorbance readings using a spectrophotometer.(Malheiro, R., Rodrigues, N., et al 2015)

1.7 Antibacterial activity

Antibiotics are important in fighting bacterial infections and have greatly benefited the health-related quality of human life since their discovery. However, over the past few decades, many commonly used antibiotics have become less effective against certain illnesses due to the emergence of drug-resistant bacteria. It is essential therefore to investigate newer drugs with lesser resistance that is derived from natural sources. (Benzie et al, 1996).

About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer. Natural products e.g. phenolic compounds of OMWW may give a new source of antimicrobial agents with possibly novel mechanisms of action. The in-vitro antibacterial activity of polyphenols phytochemicals present in OMWW represent a challenge to evaluate their effects as antimicrobial agents against human pathogens (Benzie et al, 1996).

1.8 Antifungal activity

Antifungal susceptibility testing remains an area of intense interest. Susceptibility testing can be used for drug discovery and epidemiology. With the demonstration that susceptibility of *Candida* spp. to antifungal agents (particularly fluconazole) generated correlations with clinical outcome for some forms of candidiasis that were qualitatively similar to that seen for antibacterial agents, and the steady introduction of new drugs of both preexisting and new classes, the interest in and need for clinically relevant susceptibility testing has increased. The need extends beyond testing *Candida* spp. With resistance demonstrated among such diverse fungi as *Cryptococcus Aspergillums* it is clear that the need for meaningful susceptibility test results is as great for the fungi as it is for the bacteria.(Manach, C., Scalbert, A., Mor and , C., Remesy C., et al 2004)

1.9 Effect of OMWW extract on olive oil quality

Free acidity is an important parameter that defines the quality of olive oil and is defined as a percentage as grams of free fatty acids (expressed as oleic acid, the main fatty acid present in olive oil) in 100 grams of oil. As defined by the European Commission regulation No. 2568/91 and subsequent amendments, the highest quality olive oil (Extra-Virgin olive oil) must feature a free acidity lower than 0.8%. Virgin olive oil is characterized by acidity between 0.8% and 2%, while lampante olive oil (a low quality oil that is not edible) features a free acidity higher than 2%. The increase of free acidity in olive oil is due to free fatty acids that are released from triglycerides.(Manach, C., Scalbert, A., Mor and , C., Remesy C., et al 2004)

Oxidation of vegetable oils during storage modifies their organoleptic properties, affecting the shelf life of this product. The oxidative process depends on illumination, fatty acid composition, availability of oxygen, temperature, and nature and concentration of the antioxidant and prooxidant minor components. However, oil stored in bulk is kept away from light and air, and bottled oil is exposed to light only at the retail outlet.

Therefore, the main factors affecting oil shelf life are the minor components, the fatty acid composition of the lipid matrix, and the storage temperature. In most seed oils, tocopherols are the main antioxidants, whereas in virgin olive oils, a fair correlation has been found between total phenols and oxidative stability, measured both at low temperature, and at high temperature (Benzie et al, 1996).

1.10 Peroxide value:

The peroxide value is defined as the amount of peroxide oxygen per 1 kilogram of fat or oil. Traditionally this was expressed in units of milliequivalents, or in millimoles per kilogram .The unit of milliequivalent has been commonly abbreviated as mequiv or even as meq.

The peroxide value is determined by measuring the amount of iodine which is formed by the reaction of peroxides (formed in fat or oil) with iodide ion.

$2\ I^- + H_2O + HOOH \rightarrow HOH + 2OH^- + I_2$

The base produced in this reaction is taken up by the excess of acetic acid present. The iodine liberated is titrated with sodium thiosulphate.

The acidic conditions (excess acetic acid) prevents formation of hypoiodite (analogous to hypochlorite), which would interfere with the reaction. (Jerman, T. et al 2014)

Detection of peroxide gives the initial evidence of rancidity in unsaturated fats and oils. Other methods are available, but peroxide value is the most widely used. It gives a measure of the extent to which an oil sample has undergone primary oxidation, extent of secondary oxidation may be determined from p-anisidine test

The double bonds found in fats and oils play a role in autoxidation. Oils with a high degree of unsaturation are most susceptible to autoxidation. The best test for autoxidation (oxidative rancidity) is determination of the peroxide value. Peroxides are intermediates in the autoxidation reaction.

Autoxidation is a free radical reaction shown figure (1.7) involving oxygen that leads to deterioration of fats and oils which form off-flavors and off-odors. Peroxide value, concentration of peroxide in an oil or fat, is useful for assessing the extent to which spoilage has advanced.

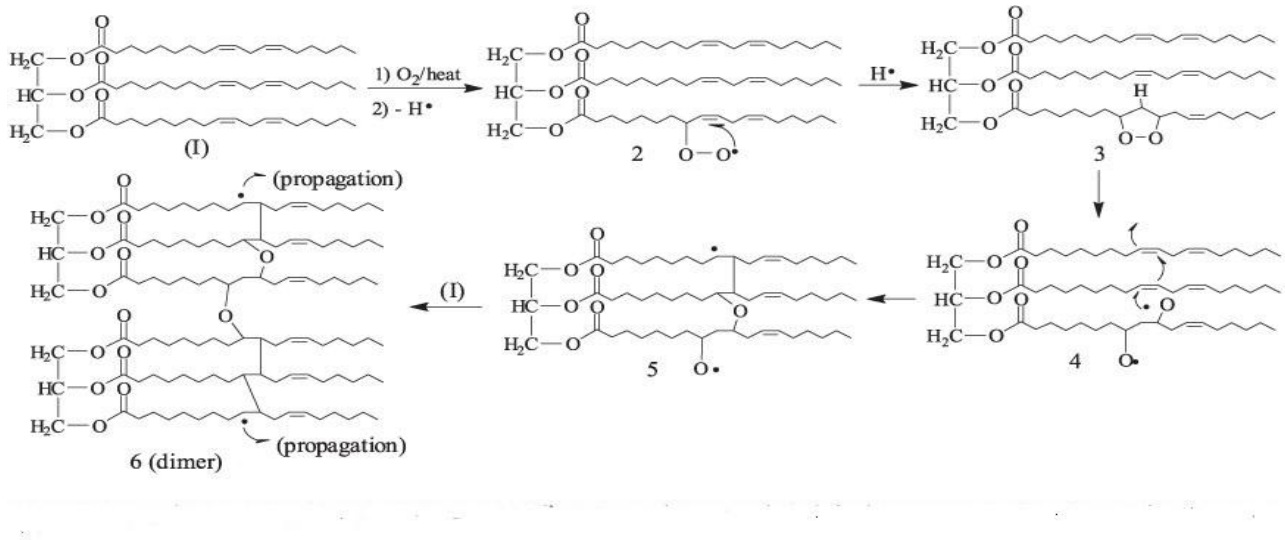


Figure 1.7: Mechanism of peroxide reaction (free radical reaction).

1.11 K_{232} and K_{270}:

The UV spectrum involves the electronic absorption of fatty acids; in particular, the 230-270 nm and shows high absorption when conjugated dienes and trienes formed in the autoxidation process from the hydroperoxides of unsaturated fatty acids and their fragmentation products are present. For this reason, the absorbances measured at 232 nm and 270 nm, namely K_{232} and K_{270}, provide an official method for olive oil quality control, which is capable of detecting product oxidation and adulteration by means of rectified oils, (Mignan et al, 2012; Angerosa et al, 2006) since they can give an indication of the level of oxidation to produce primary and secondary products incurred during production and/or storage (Afaneh et al. 2013).

Chapter Two:

Purpose of the present work

2.1 Main problem

Olive mills in West Bank generate about 200 thousand m^3/year of OMWW (R. Adham Msc theses Al-Najah University 2012), which is considered a big pollutant problem for soil and plants. Nonetheless, polyphenolic compounds which present in high concentrations in OMWW can be recovered and used as natural antioxidant and preservative.

2.2 Hypotheses

1. OMWW contains natural antioxidants (polyphenolic compounds).
2. The polyphenolic compounds extracted from OMWW can acts as natural antibacterial and antifungal.
3. They work also as natural preservatives and antioxidants for olive oil and cream.
4. Recovery of these polyphenolic compounds from OMWW reduces environmental problems of OMWW and therefore plays a role in the treatment of OMWW.

2.3 Objectives and aims

1. To extract polyphenolic compounds from OMWW.
2. To analyze polyphenolic compounds by HPLC-PDA instrument.
3. To evaluate antimicrobial activity of OMWW and to use it as natural antioxidant and preservative for olive oil and cream preparation.

2.4 Previous studies

There are number of reports in the literature that investigated the extraction and antioxidants from OMWW. A study from Greece for example has studied recovery of phenolic compounds, such as hydroxytyrosol and tyrosol, as well as total phenols from OMWW. Antioxidants were recovered by means of liquid-liquid solvent extraction. For this purpose, a laboratory-scale pilot unit was established and the effect of various organic solvents, namely ethyl acetate, diethyl ether and a mixture of chloroform/ isopropyl alcohol, on process efficiency was investigated. It was found that the performance of the three extraction systems decreased in the order: ethyl acetate > chloroform/isopropanol > diethyl ether, in terms of their antioxidant recovery yield. It was estimated that treatment of 13 liter of OMWW with ethyl acetate could provide 0.247 kg hydroxytyrosol, 0.062 kg tyrosol and 3.44 kg of TPh. Furthermore, the environmental footprint of the whole liquid-liquid extraction system was estimated by means of the life cycle assessment (LCA) methodology to provide the best available and sustainable extraction technique. From an environmental perspective, it was found that ethyl acetate and diethyl ether had similar environmental impacts (Nicolas Kalogerakis Maria Politi et al 2013).

Another study was conducted in 2014 about the extraction of polyphenols from OMWW from Morocco. (Hassan Ait Mouse, Mohamed Mbarki et al.2014). In this study, the OMWW was tested for its composition in phenolic compounds according to geographical areas of olive tree, i.e. the plain and the mountainous areas of Tadla-Azilal region (central Morocco). Biophenols extraction with ethyl acetate was efficient and the phenolic extract from the mountainous areas had the highest concentration of total phenols' content.

Additionally, the results of HPLC-ESI-MS analyses showed that phenolic alcohols, phenolic acids, flavonoids, secoiridoids and derivatives and lignans represent the most abundant phenolic compounds. Nüzhenide, naringenin and long chain polymeric substances were also detected. Mountainous areas also presented the most effective DPPH scavenging potential compared to plain areas; IC_{50} values were 11.7 ± 5.6 μg/ml and 30.7 ± 4.4 μg/ml, respectively. OMWW was confirmed as a rich source of natural phenolic antioxidant agents (Hassn Ait Mouse, Mohamed Mbarki et al.2014).

Another study was conducted in 2015 about the extraction of polyphenols from OMWW from Tunisia. In this work, liquid-liquid extraction was used using ethyl acetate. By a colorimetric assay, results revealed that phenolic compounds are very abundant in olive mill wastewater. The evaluation of the antioxidant activity of the extracts confirms its

potential to scavenge free radicals under fast kinetic behavior (1-2 min) compared with synthetic antioxidants. The enrichment of olive oil with phenolic fraction was performed showing that the antioxidant activity increases with the increase of phenolic fractions added, allowing the improvement of the antioxidant ability of olive oil. So in conclusion, this study demonstrate that low-cost natural phenolic extracts retain high antioxidant activity and fast kinetic behavior and can improve the antioxidant activity of olive oil instead of synthetic antioxidants (Asma Yangui, Mohamed Hedi Abessi, Manef Abderradda et al.2015).

To present, in Palestine there is no single investigation that investigated the use of OMWW as preservative in olive oil or cream. Moreover, the antioxidant activities, total phenolic and flavonoid content were investigated for the first time in Palestine.

Chapter Three:

Experimental work.

3.1 Methodology

3.1.1 OMWW collection:

Twenty liters of OMWW was collected from an olive mill in Surif, a village located in Hebron at the southern part of Palestine, Hebron.

3.1.2 Pretreatment of the sample:

The sample was filtered from solid particles using filter paper and then centrifuged at 3000 rpm for 15 min.

3.1.3 Treatment of sample:

n-hexane was added to the filtered sample (1:1, V/V) and left for one hour, then centrifuged at 4000rpm for 15 min to remove all fats and oils from the sample. The n-hexane layer was discarded.

3.1.4 Extraction of phenolic compound from treated sample:

The extraction procedure was performed using ethyl acetate (1:2, v/v ratio of OMWW/ ethyl acetate). The sample is then transferred to a beaker with large headspace and stirred at 120 rpm for 30min. The upper layer which contains the phenolic compounds was

separated. Finally ethyl acetate was evaporated using rotary evaporator at 40C^{o} until crude viscous extract was obtained.

3.2.1 Determination of total phenolic content:

Total phenolics were determined using Folin–Ciocalteu reagents (Singleton & Rossi, 1965). 40 µl of each extract was mixed with 1.8 ml of Folin–Ciocalteu reagent (pre-diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in the range of (100 – 500 ppm) were used for calibration. Results were expressed as g gallic acid equivalents (GAE)/ liter of OMWW sample. (Hamid, F., and Hamid, F. H. et al 2016)

3.2.2 Total flavonoids content (TFC):

The determination of flavonoids was performed according to the colorimetric assay of Kim, Jeong, and Lee, 2003. Distilled water (4 ml) was added to (1 ml) of OMWW extract. Then, 0.3 ml of 5% sodium nitrite solution was added, followed by 0.3 ml of 10% aluminum chloride solution. Test tubes were incubated at ambient temperature (25◦C) for 5 min, and then 2 ml of 1 M sodium hydroxide were added to the mixture. Immediately, the volume of reaction mixture was made to 10 ml with distilled water. The mixture was thoroughly vortexed and the absorbance of the pink color developed was determined at 510 nm. Aqueous solutions of known Catechin concentrations in the range of (50 – 100 ppm) were used for calibration and the results were expressed as mg catechin equivalents (CEQ)/ liter of OMWW sample. (Hamid, F., and Hamid, F. H. et al 2016)

3.2.3 Antioxidant activity by FRAP:

The antioxidant activity of the extracts were determined using a modified method of the assay of ferric reducing/antioxidant power (FRAP) of Benzie and Strain, 1999. Freshly prepared FRAP reagent (3.0 ml) was warmed at 37◦C and mixed with 40 µl of each extract and the reaction mixtures were later incubated at 37◦C. Absorbance at 593 nm was read with reference to a reagent blank containing distilled water which was also incubated at 37 ◦C for up to 1 hour instead of 4 min, which was the original time applied in FRAP assay.

Aqueous solutions of known Fe (II) concentrations in the range of (2 - 5 mM) (FeSO4.6H2O) were used for calibration.

3.2.4 Antioxidant activity by DPPH:

The percentage of antioxidant activity (AA %) of the extracts was assessed by DPPH free radical assay. The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams et al. The samples were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of DPPH radical solution (0.5 mM in ethanol). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light yellow) were read [Absorbance (Abs)] at 517 nm after 100 min of reaction using a UV/VIS spectrophotometer). The mixture of ethanol (3.3 mL) and sample (0.5 mL) serve as blank. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL). The scavenging activity percentage (AA %) was determined by the following equation:

3.2.5 ABTS:

Materials used were: ABTS (2,2-azino-di- (3-ethyl-benzothialozine-sulphonic acid)), potassium persulphate ($K_2S_2O_8$), Torolox,UV-Vis Spectrophotometer.

A modified procedure using ABTS (2,2-azino-di- (3-ethyl-benzothialozine-sulphonic acid)) as described by (Re et al, 1999) was used. The $ABTS^+$ stock solution (7 mM) was prepared through reaction of 7 mM ABTS and 2.45 mM of potassium persulphate ($K_2S_2O_8$) as the oxidant agent. The working solution of $ABTS^+$_ was obtained by diluting the stock solution in ethanol to give an absorption of 0.70 ± 0.02 at $\lambda = 734$ nm. Sample extract (50 µL) was added to 90 µL of $ABTS^+$ solution and absorbance readings at 734 nm were taken at 30^oC exactly 10 min after initial mixing.

The percentage inhibition of $ABTS^+$ of the test sample and known solutions of Trolox were calculated by the following formula: % inhibition = $(100\times (A_0\text{-}A)/A_0$ where A_0 was the beginning absorbance at 734 nm, obtained by measuring the same volume of solvent, and A was the final absorbance of the test sample at 734 nm. The radical-scavenging activity of the test samples was expressed as Trolox equivalent antioxidant capacity (TEAC) mg

Torolox/liter OMWW.

3.2.6 Cupric reducing antioxidant capacity (CUPRAC) assay:

The assay was conducted as described previously by Resat et al. To 0.5 ml of extract or standard of different concentrations solution, 1 ml of copper (II) chloride solution (0.01 M prepared from $CuCl_2.2H_2O$), 1 ml of ammonium acetate buffer at pH 7.0 and 1 ml of neocaproin solution (0.0075 M) were mixed. The final volume of the mixture was adjusted to 4.1 ml by adding 0.6 ml of distilled water and the total mixture was incubated for 1 hour at room temperature. Then the absorbance of the solution was measured at 450 nm using a spectrophotometer against blank.

3.2.7 HPLC-PDA analysis:

HPLC coupled to photodiode array (PDA) was used for analysis of H.T.T. and oleourpein in the crude extracts of OMWW. The HPLC is an excellent tool used to separate the nonvolatile polar compounds. Reversed phase columns (C18) was used along with water using acetic acid and acetonitrile as a mobile phase by using gradient mode for 70 minutes. The PDA detector was utilized to extract the maximum wavelength of each separated polyphenol. Standards were injected and their retention and spectra was compared to the peaks of interest. Calibration curve that covers linear interval was constructed to quantify all the polyphenols under investigation. The selected wavelength was at 279 nm.

3.2.8 Antibacterial Activity by well diffusion method

Antibacterial activities of the extracts were investigated by well diffusion method which depends on diffusion of the sample tested from a vertical cylinder through a solidified agar layer in a plate. (El Riach, M., Priego-Capot, F., Rallo, L., et al 2012)

In this method, the media was prepared by mixing 3.05 g agar in 100 mL of distilled water for each microorganism. Both gram positive (Staphylococcus aureus) and gram negative (Escherichia coli) bacteria were tested. The media boiled and then sterilized at 121 °C for 15 minutes. After sterilization, the media cooled, then at 45 °C the suspension of each microorganism added separately. The media is then distributed to plates (20 mL/plate), after the media solidified, two holes made using sterile cylinder (6±0.1 mm). 100 µL of

each extract was placed in each hole for each plate. The plates incubated at 37 ± 0.5 °C for 24 hours. After incubation period, the zone of inhibition was measured by a caliper.

3.2.9 Antifungal activity

Prepared an agar (MH) in tubes, then the tubes are melted by heating in water bath. After that the OMWW extract (100µl) is added to the medium at 50 ^{o}C. The mixture is poured in a petre dish and let to cool. There after a portion of an aspergilla's Niger colony was put in the center of the plate surface at 0.5mm. The plate was incubated at 37 ^{o}C for one week, and then the diameter of the colony was measured and compared to positive control (agar without OMWW extract). Then, inoculated with 0.5mm diameter of the colony portion of A Niger and let to be incubated with the sample for one week at 37 ^{o}C. (El Riach, M., Priego-Capot, F., Rallo, L., et al 2012)

3.3 Effect of addition OMWW extract on quality of olive oil:

OMWW extract was added to olive oil sample of different concentrations, and the effect on the oil quality was assessed by measuring different oil parameters (Acid value, Peroxide value, K_{232} and K_{270}).

Olive oil is considered to be relatively affected by storage conditions such as temperature and humidity, which could be monitored in terms of its acidity and rancidity. Quality of olive oil was evaluated by measuring (Acid value, Peroxide value, K_{232}, K_{270} and Total phenolic content).

3.3.1 Acid value:

Acid value was determined by using the AOAC method number 940.28 as follows, 7gm of oil sample was put into a dry and clean 250 ml Erlenmeyer flask, then 50 ml of 96% ethanol was neutralized with 0.1 N aqueous NaOH solution in presence of 2 ml phenolphthalein solution to produce faint permanent pink, then the neutralized ethanol was added to the oil in the flask, then the mixture was shaken vigorously and boiled on a hot plate for two minutes then titrated with 0.1 N aqueous NaOH solution until permanent faint pink color appeared and persisted one minute.(Lotfy, H R., Mukakalisa, C., et al 2015)

3.3.2 Peroxide value Test

Peroxide value of olive oil samples was determined by using the AOAC method number 965.33, about 5g of oil were weighed into 250 ml glass-stoppered conical flask, then 30 ml of glacial acetic acid-Chloroform solution (3:1 by volume) were added with swirling to dissolve oil completely, then 1 ml of saturated potassium iodide solution was added (Potassium iodide has a solubility of 144 g/ 100 mL of water at room temperature. Therefore, at room temperature more than 144 g of potassium iodide were dissolved in 100 mL of water, creating a saturated solution. Anything more than 144 grams will not dissolve), then the flask was quickly stoppard and let to stand with occasional shaking for 1 minute in the dark, thereafter, 30 ml of freshly boiled and cooled water were added and flask contents were titrated with 0.01 N sodium thiosulfate solution with vigorous shaking until yellow colour had almost disappeared, about 0.5 ml of starch solution was added and titration was continued with vigorous shaking to release all iodine from chloroform layer, until the blue color just disappeared. Blank determination is conducted in the same way without the sample (Blank is composed of all additions except oil sample). (Lotfy, H R., Mukakalisa, C., et al 2015)

3.3.3 K_{232} and K_{270}:

1% solution of olive oil samples was prepared in cyclohexane (0.25 g oil in 25 ml cyclohexane), and the absorbance of the solution were measured at lambda of 232nm and 270nm for K_{232} and K_{270} respectively. (Jerman, T. et al 2014)

3.4 Preparation cream

In a 150-mL beaker, 8g of stearic acid, 1g of cetyl stearyl alcohol, 4g of isopropyl myristato and 2g glyceril monosterate were weighed and heated to 75 C. In another beaker, 8 g glycerine, 1 g triethanolamine, 6 g sorbitol, and 65 g of water were weighed and heated to 75 C. Then the content of the second beaker was added to the first beaker with constant agitation until cream is obtained. In one portion of the cream methyl and propyl paraben were used as preservative at 0.1% of each). In another cream preparation, OMWW extract was used as preservative at 1% concentration and another cream was prepared without any preservative as negative control.

Chapter four:

Result and Discussion

4.1 Extraction of polyphenolic compounds

Upon using the extraction method adopted using ethyl acetate as solvent, poly phenolic extracts was recovered from OMWW with a yield of about 1% (wt/wt). The polyphenols were analyzed using HPLC-PDA after filtration using proper dilution.

4.2 HPLC analysis:

The quantitation of the diluted samples were achieved based on linear calibration curves. Two solvents were utilized namely ethyl acetate and a mixture of ethyl acetate and methanol (2:1; V/V).

Figure 4.1 shows the chromatogram of hydroxytyrosol where its retention time is 9.2 minutes.

The spectra UV/Vis of hydroxytyrosol is shown in figure 4.1 B, and the calibration curve was shown in figure 4.1 C

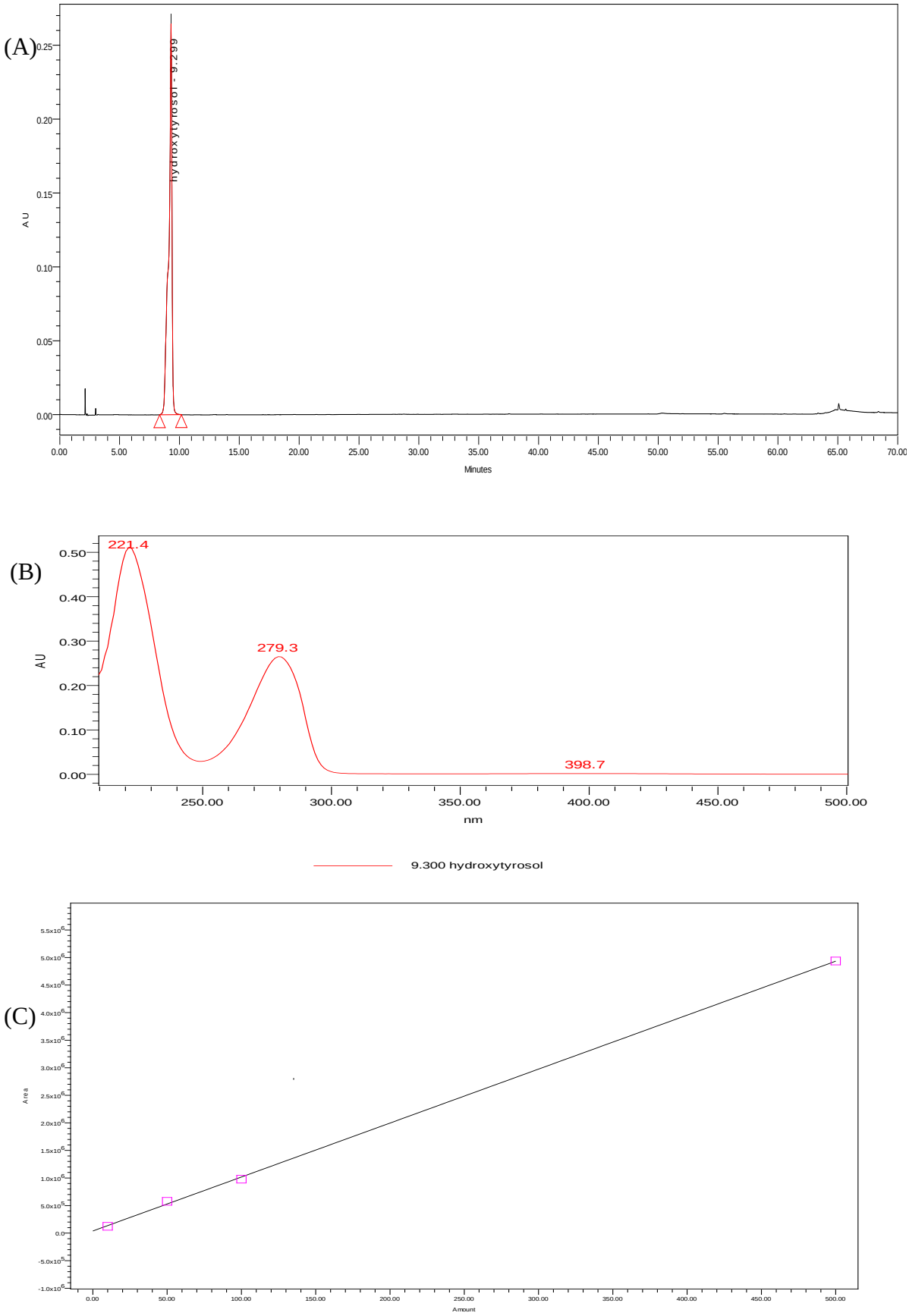


Figure 4.1: Chromatogram of hydroxytyrosol standard (100ppm concentration) (A), its spectrum (B) and its corresponding calibration curve (C)

Figure 4.2 A shows the chromatogram of tyrosol where the retention time is of 12.6 minutes .The UV/Vis spectra of is shown in figure 4.2 B, and its calibration curve is in figure 4.2 C.

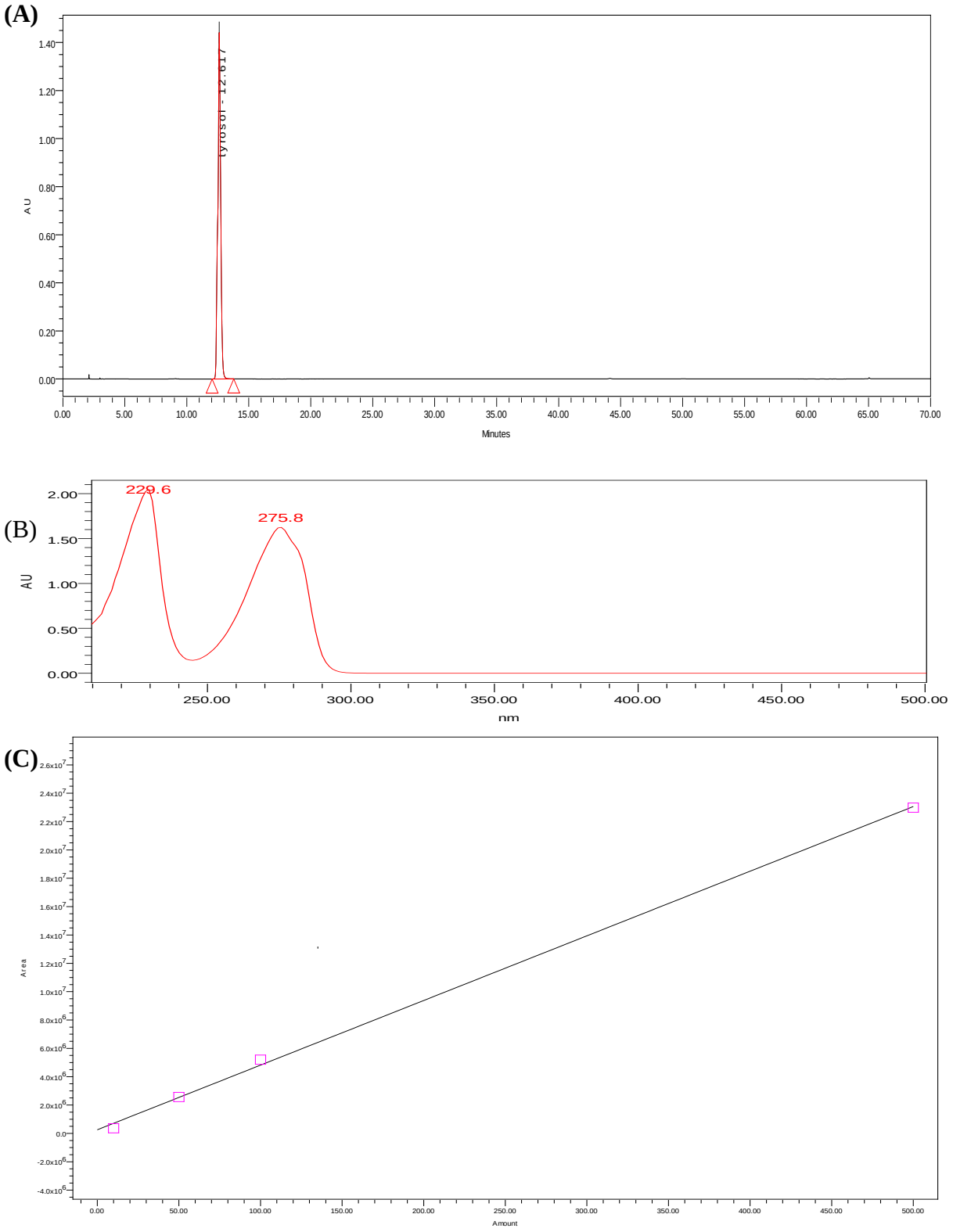


Figure 4.2: Chromatogram of Tyrosol standard (100ppm concentration) (A), spectrum. (B) and calibration curve for tyrosol (C).

Figure 4.3 shows the chromatogram of Oleurpein where the retention time was observed of 31.9 minutes. The spectrum of Oleurpein were shown in figure 4.3B, and the calibration curve was shown in figure 4.3C.

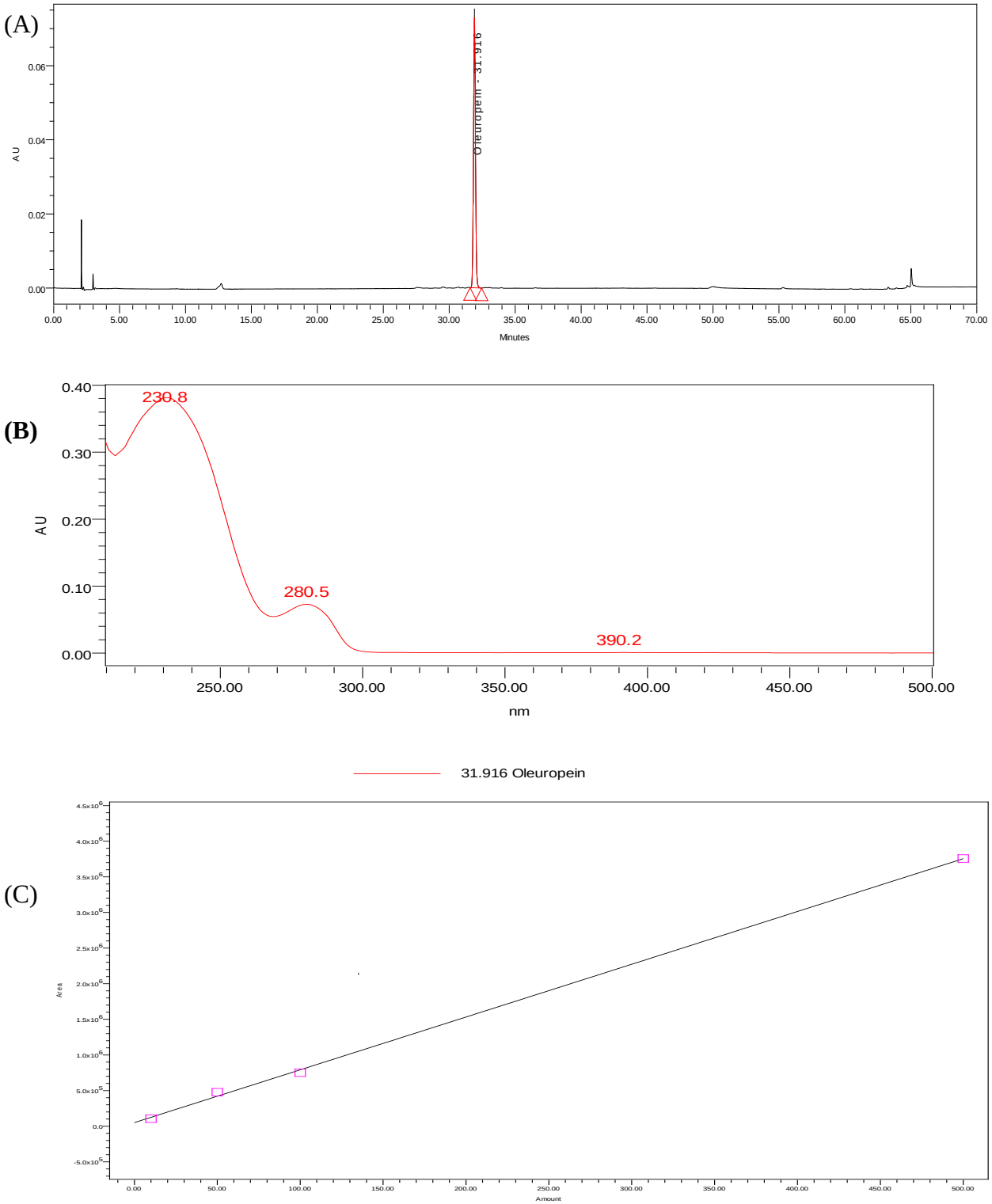


Figure 4.3: Chromatogram of oleuropin standard (100 ppm concentration) (A), its spectrum (B) and calibration curve for oleurpein(C).

Figure 4.4 shows a chromatogram for OMWW ethyl acetate extract showing that only hydroxytyrosol and tyrosol are detected at a concentration of 371 ± 2.3mg/L ,and 272.8 ± 2.1 mg/ L respectively .Oleurpein on other hand was not detected in the OMWW ethyl acetate extract.

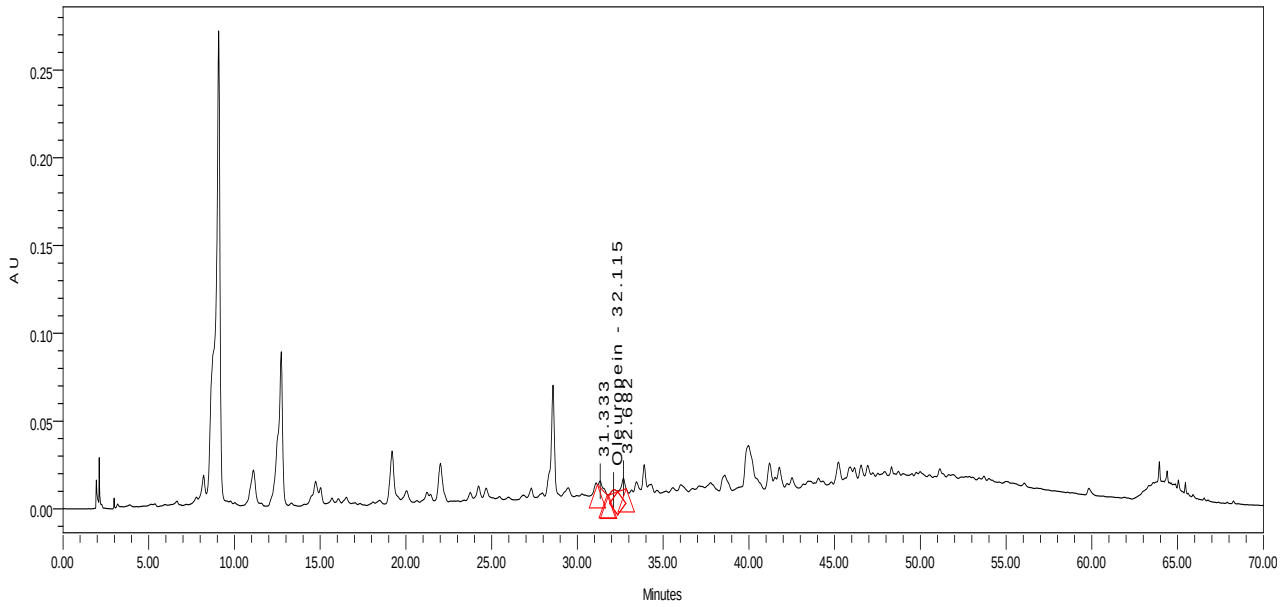


Figure 4.4: Chromatogram of ethylacetate extract analyzed by HPLC-PDA using the same conditions as in the standard samples

Methanol/ethylacetate (2:1 V/V) was used as extraction solvent of OMWW and the amount of hydroxytyrosol and tyrosol was determined based on the linear calibration curves. The amount of tyrosol and hydroxytyrosol in OMWW was found to be 180.51 ± 1.21 and 300.22 ± 2.31 mg/liter respectively. When comparing methanol/ethylacetate solvent mixture with ethylacetate as an extraction solvent for OMWW constituents, it turned out that ethylacetate alone is superior as reflected in figure 4.5.

The amount of hydroxytyrosol was found to be higher than that of tyrosol in the OMWW, while there was no oleurpein in OMWW extract as it was apparently hydrolyzed enzymatically to hydroxytyrosol and elenolic acid, as shown figure 4.6.

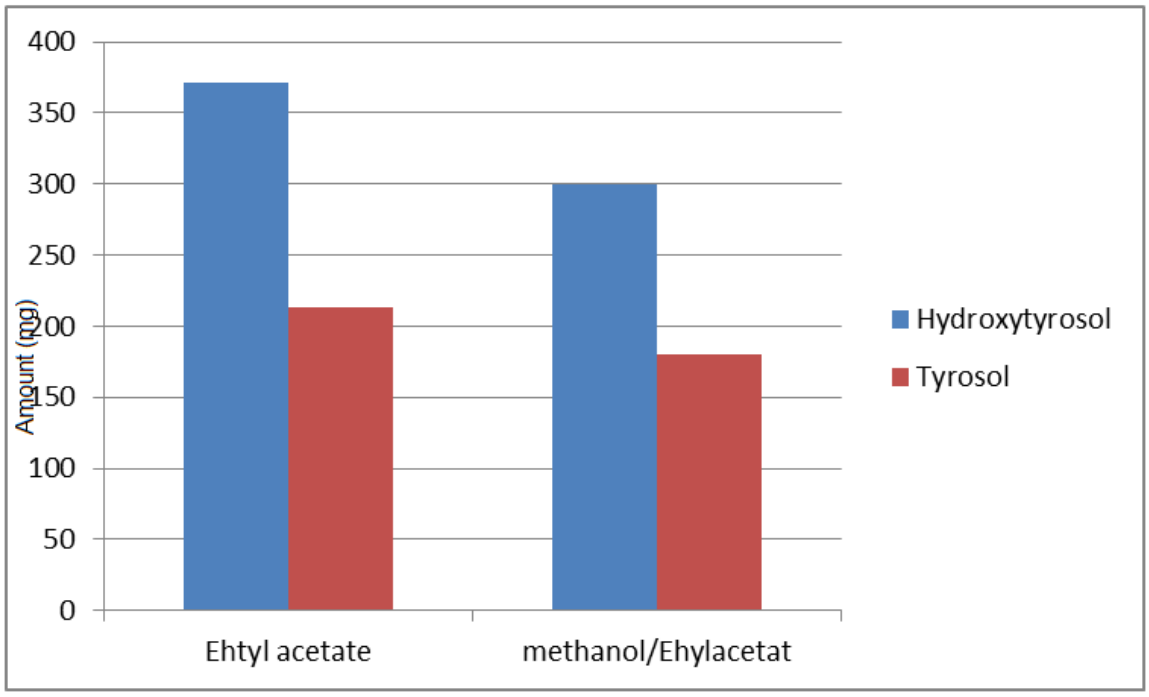


Figure 4.5: Amounts of hydroxytyrosol and tyrosol with different solvents (Ethylacetate and methanol/Ethyl acetate mixture 2:1; V/V)

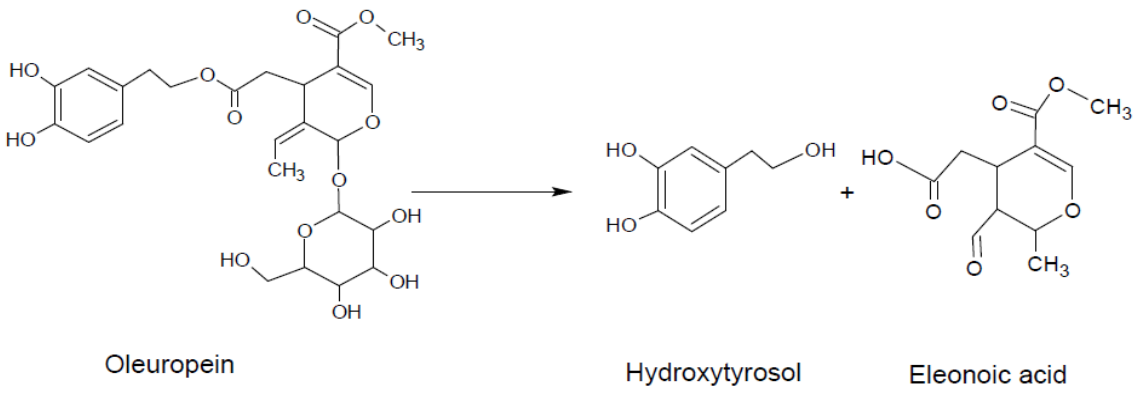


Figure 4.6: Enzymatic hydrolysis of Oleuropen

4.3 Determination of Total Phenolic content (TPC)

Total phenolic content of OMWW was determined by Folin-Ciocalteu method, and the results were expressed in (g GAE / liter of OMWW). Calibration curve was prepared by dissolving 0.5g of Gallic acid in 1L distilled water (500ppm). 10ml, 20ml, 35ml, 45ml was diluted with 50ml of distilled water in order to prepare different concentrations (Table 4.1). Then absorption was measured at 760nm (figure 4.7).

Table 4.1: absorbance of different concentration of gallic acid

Concentration of gallic acid(ppm)	Absorbance
100	0.132
200	0.426
350	0.830
450	1.070
500	1.199

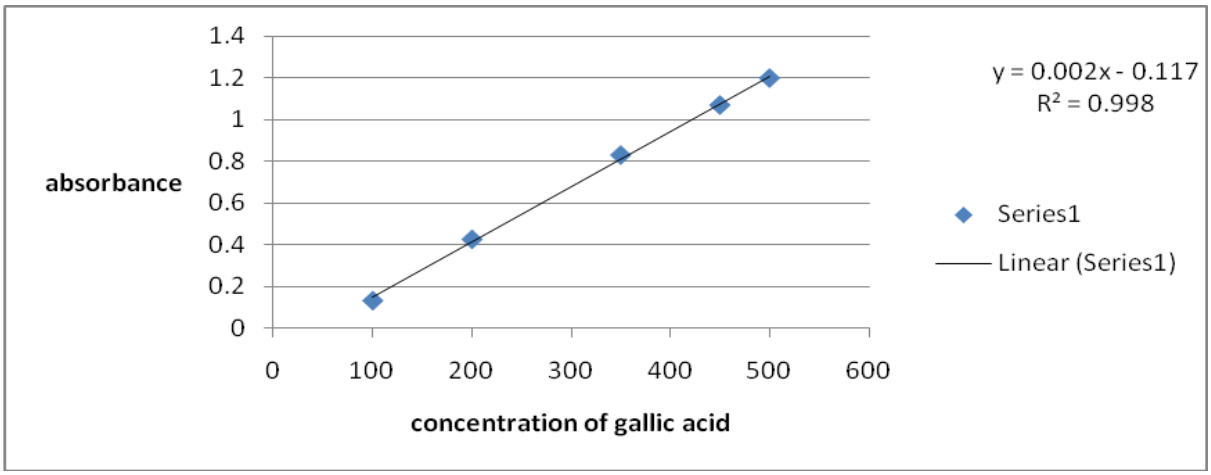


Figure 4.7: Calibration curve for total phenolic content

From the calibration curve above, the amount of total phenols were determined and expressed in grams of gallic acid per one liter of OMWW. Results revealed that TPC of OMWW analyzed in this study 2.5 ± 0.1 g gallic acid/liter OMWW. This amount is much higher than that in olive oil which is about 0.1-0.5 per liter) (W.Dweik Msc thesis , Al-Quds University 2016).

4.4 Total Flavonoid content (TFC)

TFC was determined by spectrophotometric method at 510 nm and results were expressed in mg of catechin per liter of OMWW using calibration curve at different concentration of catechin (table 4.2) and (figure 4.8).

Table 4.2: Absorbance for different concentrations of Catechin.

Concentration of catechin (ppm)	Absorbance
50	0.255
60	0.282
75	0.353
85	0.396
100	0.496

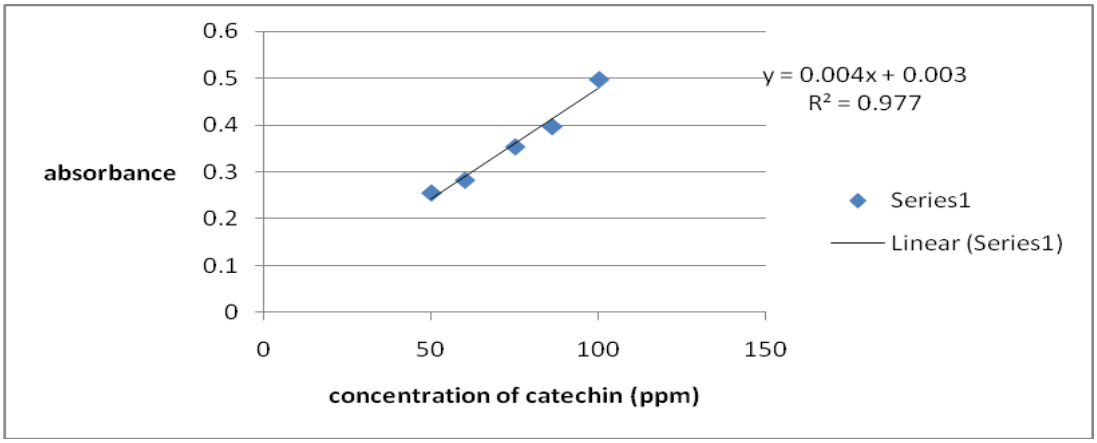


Figure 4.8: Calibration curve for total flavoniod content.

Results showed that TFC of is 499.3 ± 5.1 mg /L of OMWW. OMWW was found to be superior compared to olive oil which has TFC in the range of 19-129 mg /Kg oil (W.Dweik Msc., thesis, Al-Quds University, 2016).

4.5Antioxidant activity

4.5.1Ferric reducing antioxidant power (FRAP):

Results were expressed in (mM Fe^{+2} / g) . Calibration curve (figure 4.9) of different concentrations of Fe^{+2} was prepared and absorption was taken at 593nm (table 4.3).

Table 4.3: Absorbance of different concentrations of Fe^{+2}

Concentration of Fe^{+2} (mM)	Absorbance (593 nm)
2	0.279
2.5	0.299
3	0.400
3.5	0.511
4	0.627
4.5	0.745
5	0.848

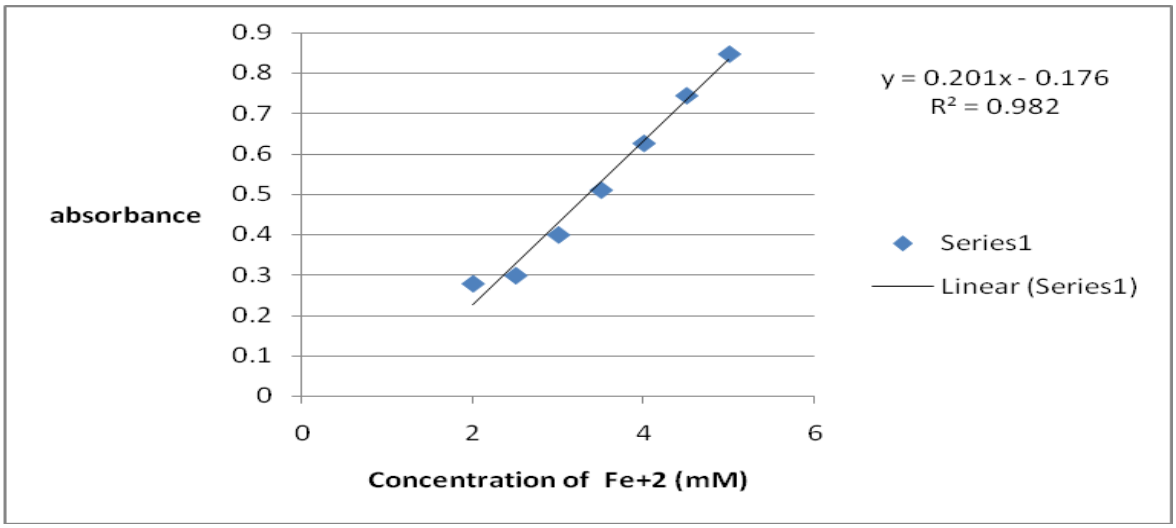


Figure 4.9: Calibration curve for FRAP antioxidant.

Result showed that FRAP of OMWW is 2193 ± 15.5 mM Fe^{+2}/ L OMWW. Recent study on olive oil gave 123.3mM Fe^{+2}/Kg oil which indicates the superiority of OMWW compared to olive oil. (W.Dweik Msc., thesis, Al-Quds University,2016).

4.5.2 Free radical scavenging activity using DPPH:

Results were expressed as (mgTrolox /liter of OMWW). Calibration curve (figure 4.10) of different concentrations of Trolox was prepared and absorption was taken at 515nm (table 4.4).

Table 4.4: Absorbance for different concentration of Trolex

Concentration in Trolex ppm	Absorbance
20	0.729
40	0.677
60	0.623
80	0.580
100	0.499
120	0.470
140	0.407
180	0.285

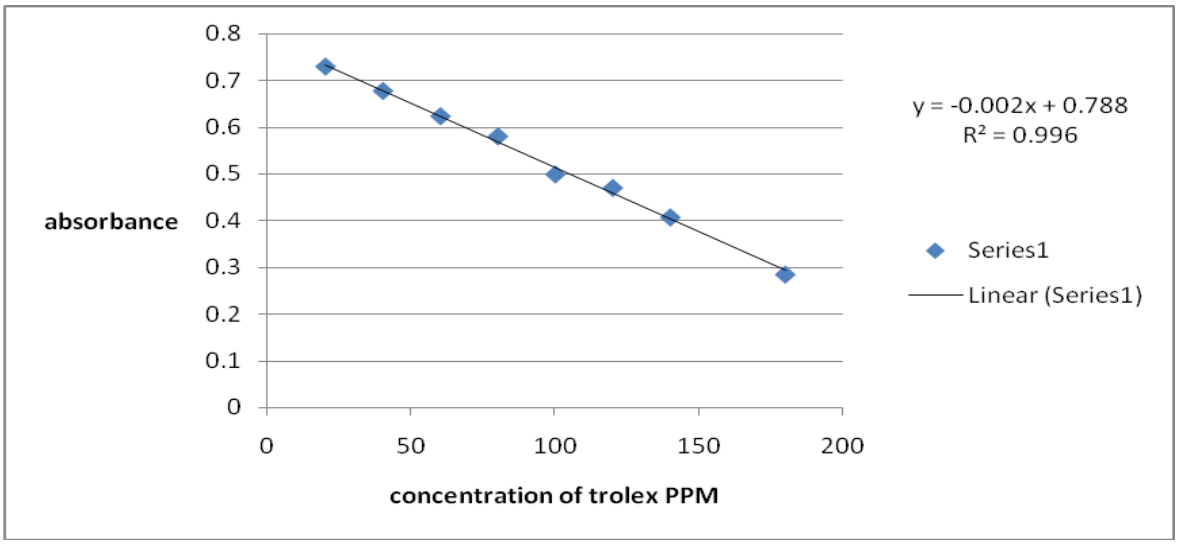


Figure 4.10: Calibration curve for DPPH

Result showed that DPPH of OMWW is1406 ± 13.6 mg Torolox/liter of OMWW.

Recent study on olive oil gave 677.7 mg Torolox/Liter of oil, which indicates the superiority of OMWW over olive oil (W.Dweik Msc., thesis, Al-Quds University, 2016).

4.5.3 Cupric reducing antioxidant power:

Results were expressed as mg Trolex/ l of OMWW). Calibration curve (Figure 4.11) of different concentrations of Trolex was prepared and absorption was taken at 450nm (table 4.5).

Table 4.5: Absorbance for different concentration of Trolex

Concentration of Trolex (ppm)	Absorbance
20	0.032
40	0.059
60	0.077
80	0.098
100	0.118
180	0.204
200	0.246

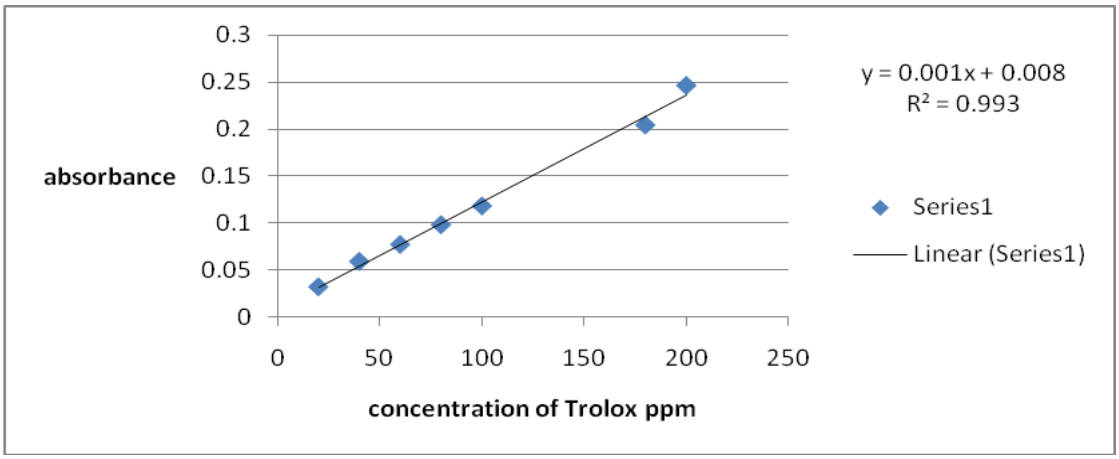


Figure 4.11: Calibration curve for cupric antioxidant powder

Result showed that CUPRAC of OMWW is 433 ± 5.5 mg Torolox/Liter of OMWW , recent study on olive oil gave 19.53 mg Torolox/g oil (W.Dweik Msc., theses, Al-Quds University,2016), which indicates the superior of OMWW over olive oil.

4.5.4 ABTs

The free radical scavenging capacity of OMWW extracts was also studied using the ABTS radical cation decolorization assay , which is based on the reduction of ABTS+• radicals by antioxidants of the OMWW extract tested, see Figure 4.12

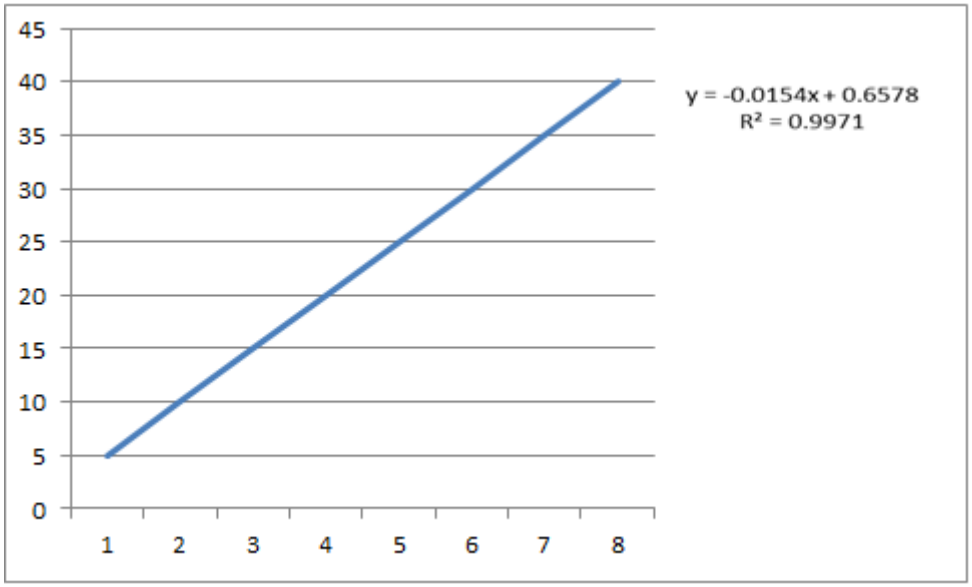


Figure 4.12: Calibration curve for ABTs

Result showed that ABTs of OMWW is 6.3±0.2 mgTrolex / liter of OMWW. Recent study on olive oil gave 699.2±0.94 mg Torolox/Kg oil (W.Dweik Msc., thesis, Al-Quds University, 2016), which indicates the superiority of olive oil over OMWW.

The following table summarizes the results of TPC, TFC, and A.A (FRAP, CUPRAC, ABTs and DPPH) of OMWW.

Table 4.6: The result of OMWW tests.

TPC g/L	TFC mg catechin/L	FRAP mM Fe^{+2}\ Liter of OMWW	CUPRAC mg Torolox/ liter of OMWW	ABTs mg Torolox/liter of OMWW	DPPH mg Torolox/ liter of OMWW
2.5±0.1 RSD=4.0%	499.3±5.1 RSD=1.0%	2193 ±15.5 RSD=0.7%	433±5.5 RSD=1.3%	6.3±0.2 RSD=3.1%	1406±13.6 RSD=1.0%

4.6 Antimicrobial Activity

4.6.1 Antibacterial activity:

OMWW extract was tested on both gram positive and gram negative bacteria and zone of inhibition was measured. Result showed that OMWW extract has an activity on gram positive bacteria (staph arouse) with a zone inhibition of 23 mm. Also it has an effect on gram negative bacteria (E-coli) with a zone of inhibition of 25mm, shown figure 4.13.The zone of inhibition of standard antibiotics is 10mm, 13 mm respectively. Result showed that OMWW extract has higher antibacterial activity against both gram positive and negative bacteria compared to well-known antibiotic (amoxicillin).

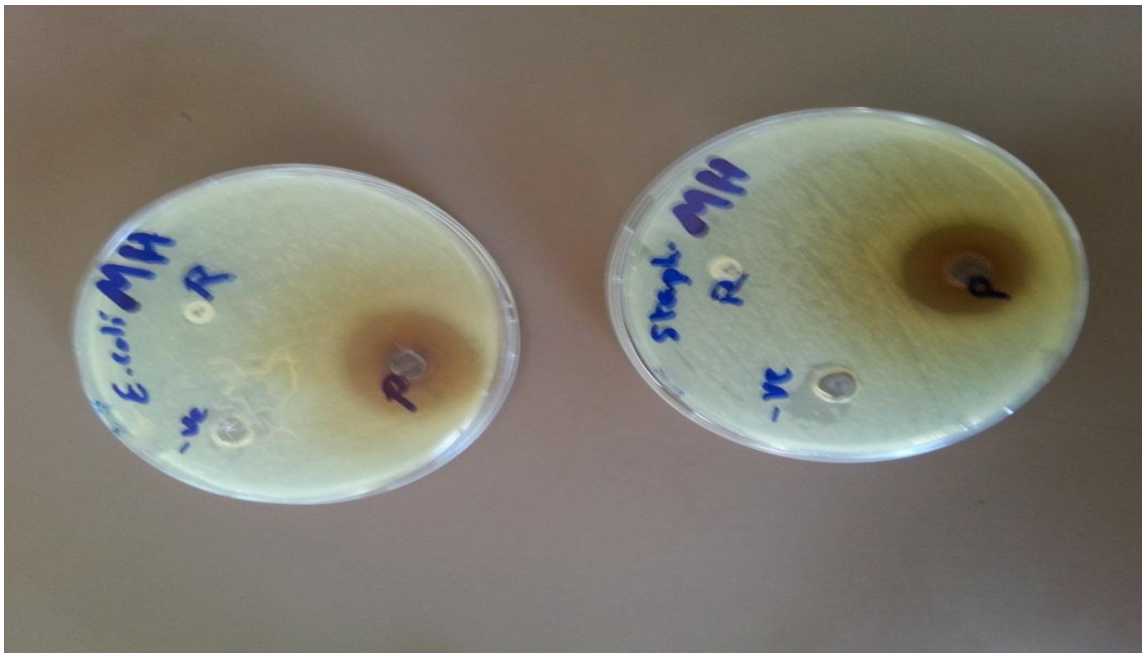


Figure 4.13: Inhibition zone of Staphylococcus aureus and E-coli bacteria.

4.6.2 Antifungal activity:

OMWW extract was also tested on a fungi and yeast. The OMWW extract was found to be effectives against Candidiasis (thrush), with zone inhibition of 23 mm as shown in (figure 4.14) .The OMWW extract found also effectives against Aspergillus (yeast), the zone inhibition is equal 20% of from total zone, shown (figure 4.15).

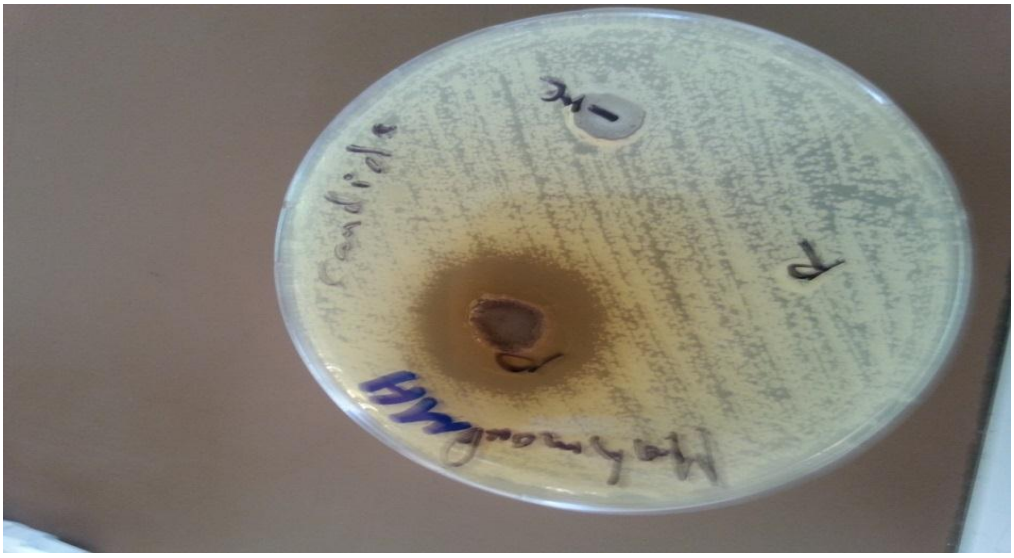


Figure 4.14: Zone inhibition of candida fungi.

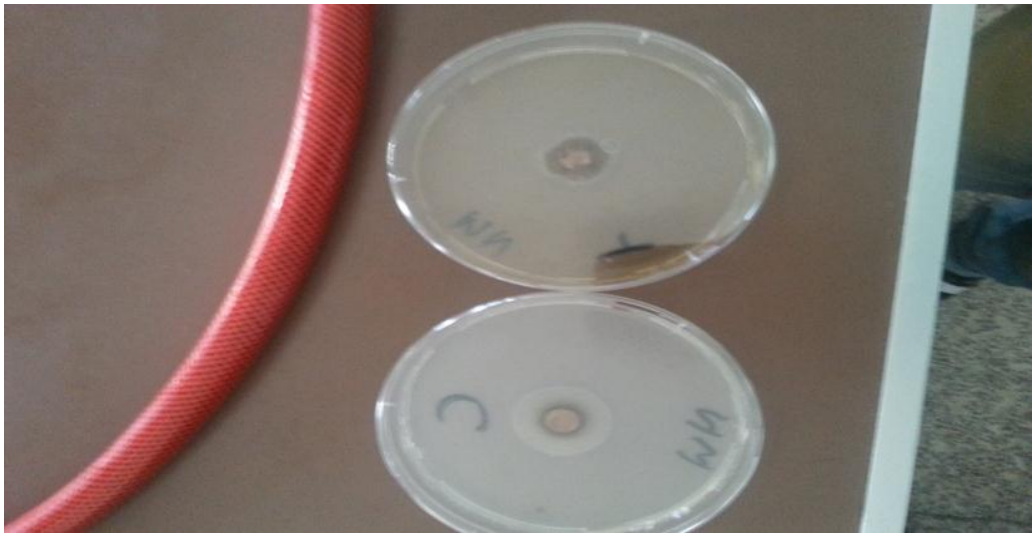


Figure 4.15: Zone inhibition of Aspergillus yeast.

4.8 OMWW extract as preservative:

Based on the results of this study which showed that OMWW extract is active against microbial and fungi, it can be used as preservative in food or creams.

4.8.1 OMWW extract as antioxidant (preservative of olive oil):

Olive Oil is considered to be relatively affected by humidity which could be monitored in terms of its acidity and rancidity Therefore, a material is fundamentally needed in order to maintain oil and preserve it from decay with time besides resisting the change in its acidity which is indicated in this study.

For that purpose, OMWW extract has been introduced and applied on olive oil which has been divided into three parts: First sample contain 1% OMWW extract, second sample contain 0.5% OMWW extract and third sample without extract. Then quality parameters of olive oil samples (Acid value, peroxide value, K_{232}, K_{270} and Total phenolic content) have been undertaken in the day of preparation (Time : zero) and after three months.

4.8.1.1 Acid value

As shown in Table 4.7, the Acid value of olive oil without OMWW analyzed at time zero was 0.9±0.1 which increased to 1.1 ± 0.1 after three months of storage at room temperature which indicates the increase in free acid with time, However percentage acidity of samples

with OMWW added at two concentration levels (0.5% and 1%) did not increase as in the samples without OMWW (Acid value are 0.93 ± 0.09 and 0.91 ± 0.1 for olive oil samples with 0.5% and 1% OMWW respectively.

{The oil acidity percentage when compared with the original sample is exposed to a change in the course of time.} However the value of such acidity in comparison with the samples to which OMWW extract has been added within three months has increased with time intervals; yet the increase has varied to be limited in the sample 1% and 0.5% of OMWW extract nevertheless the sample to which the normal aforesaid extract has not be added has displayed increase in the acidity values. In other words the application of the OMWW extract on olive oil would lessen the rate of acidity within the course of time and maintain oil to be restored in good condition.

4.8.2.2 Peroxide value:

The peroxide values was found to be affected by the addition of OMWW extract .While the PV of olive oil without OMWW extract increased from 14.1 ± 0.8 to 18.1 ± 1.1 after three months of storage, the Peroxide value of olive oil samples with 0.5% and 1% OMWW did not increase with the same rate as in the oil sample without OMWW extract (see table 4.7).

The peroxide value of olive oil with 1%, 0.5 % and 0% OMWW equal 14.2 ± 0.98, 15.2 ± 1.02 and 18.1±1.1 respectively. The peroxide value of olive oil when compared with the original sample is exposed to a change in the course of time. However the value of such rancidity in comparison with the samples to which OMWW extract has been added within three months has increased with time intervals; yet the increase has varied to be limited in the sample 1% and 0.5% of OMWW extract nevertheless the sample to which the normal aforesaid extract has not be added has displayed increase in the acidity values.

In other words the application of the OMWW extract on olive oil would lessen the rate of rancidity within the course of time and maintain oil to be kept in good condition.

4.8.2.3K_{232} and K_{270}:

K232 and K270 were also affected by the addition of OMWW to the olive oil. K232 and K270 values of olive oil increase after three months of storage. However the value of such rancidity in comparison with the samples to which OMWW extract has been added within three months has increased with time intervals see table 4.7; yet the increase has varied to be limited in the sample 1% and 0.5% of OMWW extract never the lese the sample to which the normal aforesaid extract has not been added has displayed increase in the acidity values.

In other words the application of the OMWW extract on olive oil would lessen the rate of rancidity within the course of time and maintain oil and kept in good condition.

4.8.2.4Total phenolic content of olive oil

As shown in table 4.7, TPC values has increased in olive oil samples that have been treated with OMWW extracts, because OMWW extract contain high amount of phenolic compounds. However, TPC samples without OMWW decreased after three months (see table 4.7) due to oxidation of antioxidants phenolic compounds originally present in olive oil.

The following table summarizes the results of AV, PV, K_{232}, K_{270} and TPC.

Table 4.7: Different tests for olive oil with different concentration of OMWW extract

Concentration of OMWW extract in olive oil		Acid value	Peroxide value (milliequivalents O_2 kg^{-1} oil)	K_{232}	K_{270}	TPC (mgGA/g oil)
0.0%	Time zero	0.9±0.1	14.1 ± 0.8	3.44±0.09	0.42±0.03	250.7±2.1
	Time after three months	1.1 ± 0.1	18.1±1.1	3.64±0.09	0.49 ± 0.04	241.2 ± 1.6
0.5%		0.93±0.09	15.2 ± 1.02	3.48±0.08	0.44± 0.03	260.3 ± 1.1
1.0%		0.91± 0.1	14.2 ± 0.98	3.46 ± 0.09.	0.43±0.04	267.2±1.6

4.8.2 Preservatives for simple cream:

Oily cream was prepared and divided it into three parts so that the first part contains chemical preservative (methyl parpen) at concentration 1% and the second contains OMWW extract at concentration of 1% and the third contains No preservative. Then the cream preparations were stored at room temperature for three months.

As shown in (figure 4.16), The cream without preservative was not stable as mold and fungi appeared in the cream. However the cream with preservative (either natural or chemical) was stable and no mold appeared in the cream. This results shown that OMWW can work as natural preservative for cream preparation.

(A)

(B)

(C)

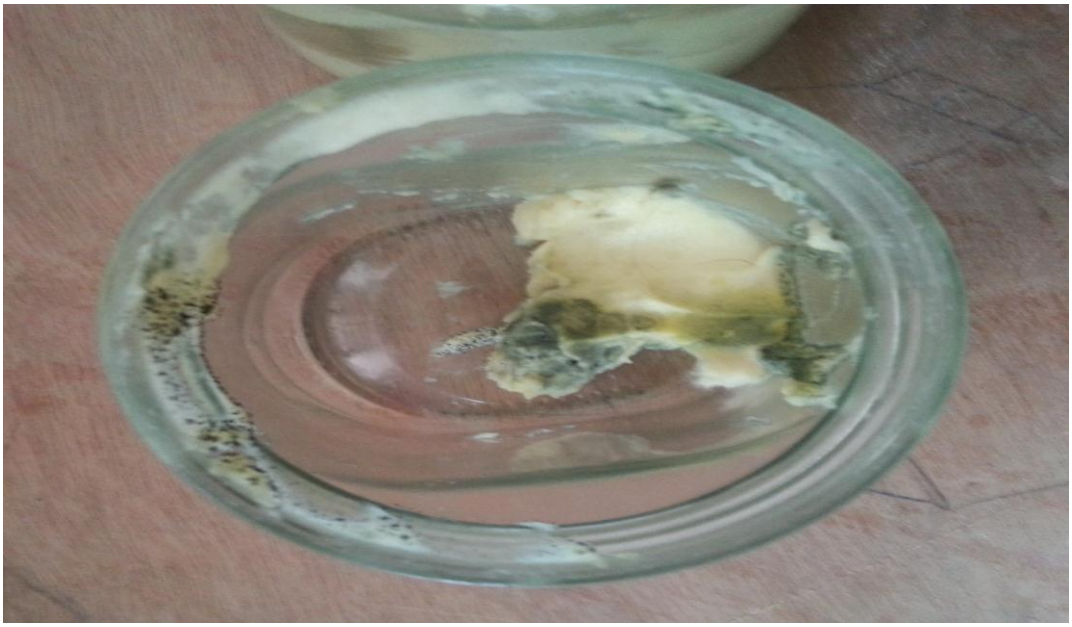


Figure 4.16: Cream with OMWW extract (A), with chemical preservative (methyl parapen)(B), and without any presevative (c).

Chapter five:

Conclusion

Ethyl acetate is found to be an excellent solvent for phenolic compound extraction from OMWW. OMWW phenolic compounds are higher than that of olive oil. OMWW mainly according to you expert contains hydroxytyrosol and tyrosol but not oleuropein as the HPLC-PDA results revealed. The phenolic fraction of OMWW give its high antioxidants activities as reflected by both the free radical scavenging ability of OMWW (DPPH, ABTs) and its reducing ability (FRAP, CUPRAC tests).

OMWW showed an antibacterial activity against both gram positive and gram negative bacteria, and it also proved to have a significant antifungal activity.

The current investigation revealed many benefits for OMWW including antioxidants for olive oil, and preservatives for cream preparation due to its phenolic content which as natural antioxidant. Further it can be useful as a natural source of antioxidants and a natural preservatives thus obviating the environmental problems and making use of it medically and nutritionally, useful research recommend to oil can be OMWW extract and also increase the rate of phenolic compounds which implies increasing antioxidants in it.

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استخلاص المركبات الفينولية من مخلفات عصر الزيتون الفلسطيني

وتقييم فاعليتها الطبية

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الملخص

إنتاج زيت الزيتون يولد كميات ضخمة من المياه العادمه الناتجه من عصر الزيتون والذي يسمى الزيبار، والذي يسبب مشاكل بيئية خطيرة. لكن هذه النفايات السائلة تحتوي على العديد من البوليفينولات المفيدة. في هذا العمل، تم دراسة استخلاص بعض المركبات الفينوليه من الزيبار باستخدام مذيب الاثيل اسيتيت. وكشفت النتائج أن مركبات الفينول وفيرة جدا في الزيبار كما اشار المحتوى الكلي الفينولي، والمحتوى الكلي للالفلافونويد .

وقد تم تقييم مضادات الاكسدة بواسطه اربع اختبارات مختلفه، حيث اظهرت النتائج وفرة مضادات الاكسدة في الزيبار. كما واظهر المستخلص أيضا فعالية ضد البكتيريا موجبة الجرام وسالبة الجرام، وفعالية ضد الفطريات، وكذلك فعالية ضد عفن الخميرة.

كما وأظهرت نتائج التحليل الكروماتوغرافي ان المستخلص يحتوي بشكل رئيسي على مركب الهيدروكسي تيروزل والتيروزل ولكنه لا يحتوي على مركب الاوليروبين.

تم استخدام مستخلص الزيبار كمادة حافظة لزيت الزيتون وخلطة كريم بشرة. فقد تم اضافة المستخلص الى عينة زيت زيتون بغرض حفظه واطالة عمره، وقد تم الاستدلال على ذلك من خلال فحوصات جودة الزيت المعروفة (القيمة الحامضية، قيمة البروكسيد، المحتوى الفينولي الكلي وقيمة K232 و K270. اظهرت النتائج ان القيمة الحامضية لزيت الزيتون تزداد بمعدل اقل في العينة المعالجة بمستخلص الزيبار مقارنة مع العينة بدون المستخلص، وهذا ينطبق ايضا على قيمة البروكسيد وقيم K232 و K270. اما بالنسبة للمحتوى الفينولي الكلي للزيت فقد وجد انه اعلى للعينات المعاملة بمستخلص الزيبار مقارنة مع العينة بدون المستخلص. اما بالنسبة للكريم الذي تم اضافة مستخلص الزيبار له فقد وجد ان المستخلص يحفظ الكريم من النمو البكتيري والعفن تماما مثل عينة الكريم التي تحتوي على المادة الحافظة الصناعية، اما بالنسبة لعينة الكريم التي لم يضاف اليها مادة حافظة فقد وجد انها غير مستقرة حيث تعرضت للنمو البكتيري والعفن.