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Phenolic compounds and antioxidant activity of olive leaves extract from Palestine and their biological activities

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Dedication

TO MY PARENTS,MOTHER, FATHER, SISTERS AND BROTHERS

TO MY SUPERVISOR

DR. FUAD AL-RIMAWI

Declaration

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

Signed……

Date……

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Abstract

Different olive leaf samples were collected from different areas of West Bank (north, middle, and south) at different maturation stages (June 2013, October 2013, and January 2014). The crude extracts of olive leaves were analyzed for their total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (AA) using standard assay methods (Folin-Ciocalteau method for TPC, ferric ion reducing antioxidant power (FRAP) for AA, and colorimetric assay method for TFC). The results revealed that maturation stage affected the composition of the olive leaves under investigation in terms of total phenolic, total flavonoid, and antioxidant activity. Additionally it was found that there is a significant difference in TPC, TFC, and AA of the olive leaves samples collected from north, middle, and south West Bank. Comparing both extraction media (water and acidified water) in terms of TPC, TFC, and AA, there was no clear trend because the differences were significant in all samples, but the higher values alternate between different extraction methods in different samples. Pearson correlation between TPC, TFC, and AA of samples collected from the three areas in West Bank showed that antioxidant activity is significantly correlated with total phenolic content in both extraction treatments, and weakly correlated with total flavonoids. But when all data was pooled, both TPC and TFC were highly and significantly correlated with AA. It was also found that no significant correlation existed between TPC and TFC in any sample under investigation.

الملخص

عينات مختلفة من أوراق الزيتون التي تم جمعها من مناطق مختلفة من الضفة الغربية (شمال، وسط، وجنوب الضفة الغربية) في مراحل النضج المختلفة (يونيو عام 2013، أكتوبر 2013 ويناير 2014). وتم في هذه الدراسة تحليل المحتوى الفينولي(TPC) ، إجمالي محتوى الفلافونويد(TFC) ، ونشاط مضادات الأكسدة (AA) لمستخلصات أوراق الزيتون باستخدام طرق الفحص القياسية (طريقة فولين Ciocalteau–لِTPC، أيون الحديديك المختزل لقوة مضادات الأكسدة (FRAP) لAA، وطريقة الفحص اللونية لِ TFC) وأظهرت النتائج أن وقت النضوج يؤثر على مكونات أوراق الزيتون من محتوى الفينولي الإجمالي، إجمالي الفلافونويد، ونشاط مضادات الأكسدة. بالإضافة إلى ذلك وجد أن هناك فرقا كبيرا فيTPC ، TFC، و AA لمستخلصات أوراق الزيتون في العينات التي تم جمعها من شمال ووسط وجنوب الضفة الغربية. وأيضاً أظهرت النتائج أن تأثير درجة حموضة وسط الاستخلاص (الماء العادي والماء الحامض)غير واضح حيث كانت القيم العظمى متبادلة بين كلا طريقتا الاستخلاص في العينات المختلفة. وكانت علاقة بيرسون بينTPC ، TFC، و AA للعينات التي تم جمعها من ثلاث مناطق في الضفة الغربية قد أظهرت أن النشاط المضادة للأكسدة يرتبط بشكل ملحوظ مع مجموع محتوى الفينول في كل العينات، وهناك علاقة ضعيفة لمضادات الأكسدة مع مركبات الفلافونويد. كما تبين عدم وجود ارتباط كبير بين TPC و TFC في جميع العينات التي تم فحصها.

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Fig. 11: Antioxidant activity (as µ mole FRAP /g of dry olive leaf) of olive leaves samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with acidified water (pH=3.0).

Fig.12. (2-7 numbers) are zone of inhibition of olive leaves samples against S. aureus and S. epidermidis bacteria.

List of Acronyms

AA: Antioxidant Activity.

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

ANOVA: Analysis of variance

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

DAD: Diode array detector

DNA: Deoxyribonucleic acid .

DPPH: Diphenyl-1- picrylhydrazyl

EC50: Half maximal effective concentration

EtOH: Ethanol

ESI/MS: Electrospray Ionisation Mass Spectrometry
FCR: Follin ciocalteu reagent

FRAP: Ferric Reducing Antioxidant Power.

GAE: Gallic Acid Equivalent.

HNE: Hydroxynonrnal

HORAC: Hydroxyl radical antioxidant capacity

HOSC: Hydroxyl radical scavenging capacity

HPLC: High-performance liquid chromatography

MDA: Malondialdehyde

OLE: Olive leave extract

ORAC: Oxygen radical absorbance capacity

PAL: Phenylalanine ammonia-lyase

pH: Power of hydrogen

PG: Propyl gallate

POD: Peroxidase.

PPO: Polyphenoloxidase.

RE: Rutin equivalents

RNA: Ribonucleic acid

RSD: Relative standard deviation.

SD: Standard deviation.

SFE : Supercritical fluid extraction

TFC: Total Flavonoid Content.

TPC: Total Phenolic Content.

TPTZ: 2,4,6-tri(2-pyridyl)-1,3,5-triazine.

Chapter One

Introduction

1.1 Introduction:

In the Mediterranean area, olive leaves are one of the by-products of farming of the olive grove; they can be found in high amounts in the olive oil industries (10% of the total weight of the olives) and they accumulate during the pruning of the olive trees [1].

The olive tree is among the oldest known cultivated trees in world. It is uncertain the exact origin of the olive tree, the genetic and archaeological studies indicated that the original centers of olive cultivation were Palestine, Lebanon, Syria, Cyprus and Crete. The olive tree was firstly wide spread on the Greek islands and the mainland Greece, Italy and then probably introduced into Spain by Greeks, Romans and Arabs [2].

Back cultivation of olive trees in Palestine to thousands of years, and this is what justifies the existence of long-lived olive trees in Palestine. The importance of this sector in Palestine, the enormity of the number of olive trees; since more than 11 million trees; and the enormity of the patch covered by this tree, with an estimated cultivated area of approximately 938 thousand acres, according to figures from the Ministry of Agriculture, and the Central Palestinian Bureau of Statistics for the year 2010 [3].

Olive leaves are considered as a cheap raw material which can be used as a useful source of high-added value products (phenolic compounds). Mediterranean diet is rich in olive drupes and olive oil is associated with the lower incidence of cardiovascular disease, cancer, inflammation and stroke [4,5]. Olive (Olea europaea L.) phenolics are known as powerful antioxidants, both in vitro and in vivo and it is known that olive oil represents a key healthy component of Mediterranean diet [6]. Not only olive oil, but olive leaf also has different beneficial effects on human health [7]. Compounds obtained from olive leave extracts (OLE) have been subject to numerous investigations. OLE from Mediterranean olive has been commercialized as a food supplement, which can be consumed in the form of tea, syrup and capsules. OLE is well-known for its hypotensive effects [8] and its antioxidant properties [9], hypoglycemic [10], cardioprotective [11], antimicrobial [12], radioprotective [13], antiatherogenic [14], antitumoral [15], anti-inflammatory [16], hypocholosterolemic, hepatoprotective [17] and anti-viral properties [18]. The main constituent of the olive leaf is the iridoid glycoside oleuropein, which is metabolized in the body to calcium elenolate, which is apparently responsible for many of the pharmacological actions of the olive leaf. Furthermore, the olive leaf contains triterpenes (oleanolic and maslinic acid), flavonoids (luteolin, apigenine, rutin), and chalcones (olivin, olivin-diglucoside). It has been traditionally used in hypertonia, arteriosclerosis, rheumatism, gout, diabetes mellitus, and fever [19].

Lipid oxidation has been one of the main subjects interests of the scientific community for centuries. Researchers are continuously seeking those natural antioxidants that will sufficiently protect fats and oils from oxidation. Commercial antioxidants are in high demand, and most of them are synthesized, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG). Although they are effective, inexpensive and stable under usual processing and storage conditions of oils. They are reported to be toxic and carcinogenic in animal models [20,21].

Thus, there is a growing request and interest for natural and safer antioxidants found in various kinds of land plants, such as leaves and fruit of olive trees, vegetables, fruits and herbs; in which tocopherol, vitamin C, carotenoid and polyphenols are good sources of antioxidant [22].

1.2 background

1.2.1 Polyphenols

Polyphenols are secondary metabolites of plants of which 8,000 polyphenolic compounds have been identified in various plant species. All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. Primarily they occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar (polysaccharide or monosaccharide) to an aromatic carbon also exist. Association with other compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common [23]. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans. Fig. 1 illustrates the different groups of polyphenols and their chemical structures.

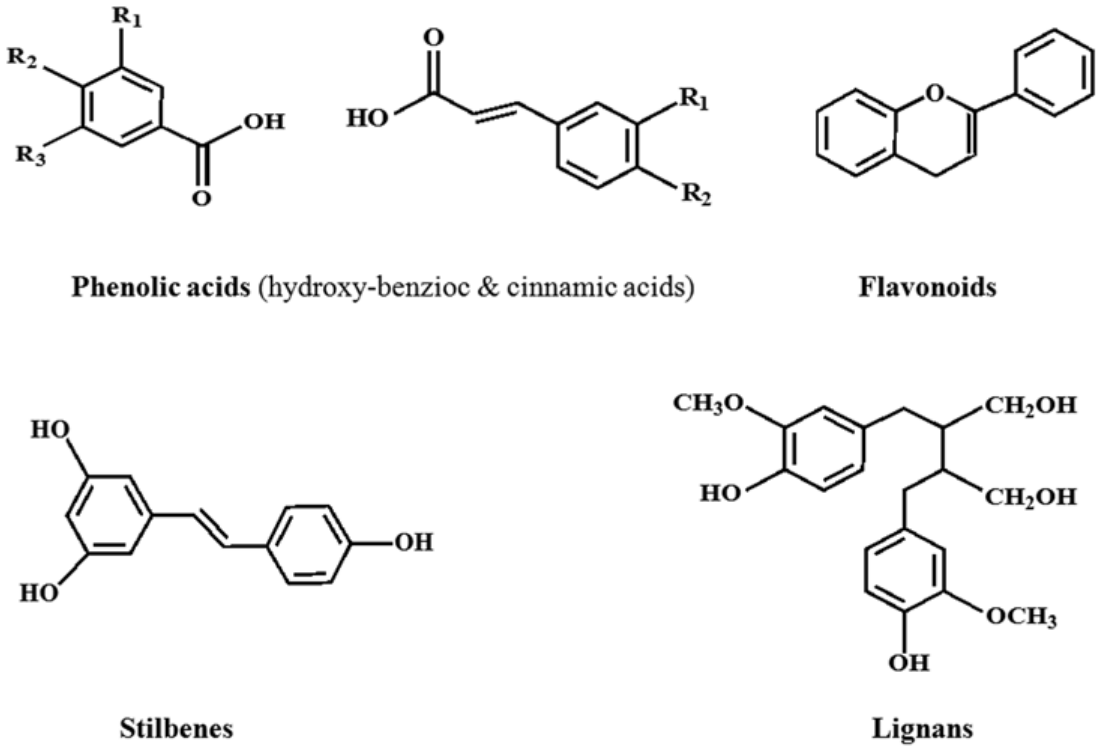

Fig.1. Chemical structures of the different classes of polyphenols, where R_1, R_2 and R_3 are H, OH or OCH_3.

1.2.1.1. Phenolic Acids

Phenolic acids are found abundantly in foods and divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid. The hydroxybenzoic acid content of edible plants is generally low, with the exception of certain red fruits, black radish and onions, which can have concentrations of several tens of milligrams per kilogram fresh weight [24]. The hydroxycinnamic acids are more common than hydroxybenzoic acids and consist chiefly of *p*-coumaric, caffeic, ferulic and sinapic acids.

1.2.1.2. Flavonoids

Flavonoids comprise the most studied group of polyphenols. This group has a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (Fig. 2). More than 4,000 varieties of flavonoids have been identified, many of which are responsible for the attractive colours of the flowers, fruits and leaves [25]. Based on the variation in the type of heterocycle involved, flavonoids may be divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones (Fig. 2). Individual

differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Quercetin, myricetin, catechins … etc, some most common flavonoids.

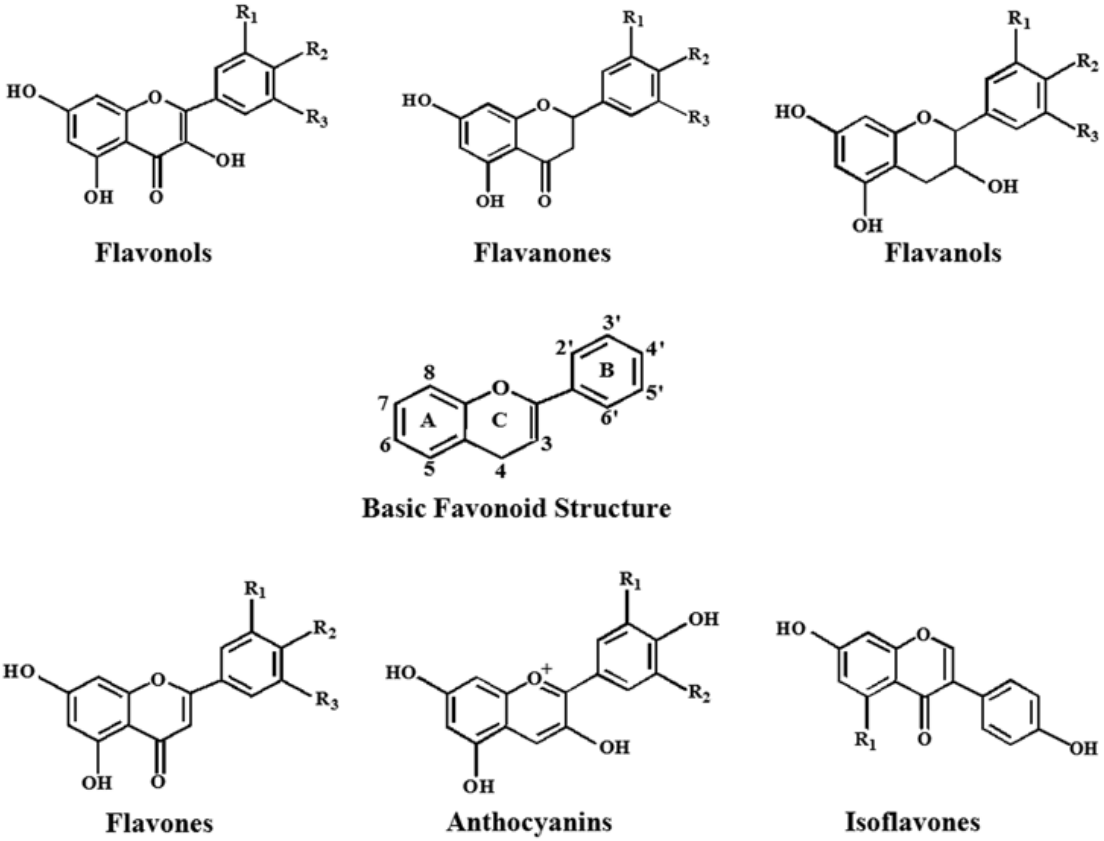

Fig. 2. Chemical structures of the different classes of flavonoids, where R_1, R_2 and R_3 are H, OH or OCH_3.

1.2.1.3. Stilbenes

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. Occurrence of stilbenes in the human diet is quite low. Most stilbenes in plants act as antifungal phytoalexins, compounds that are synthesized only in response to infection or injury. One of the best studied, naturally occurring polyphenol stilbene is resveratrol (3,4',5-trihydroxystilbene), found largely in grapes. A product of grapes, red wine also contains significant amount of resveratrol.

1.2.1.4. Lignans

Lignans are diphenolic compounds that contain a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues (Fig. 1). Several lignans, such as secoisolariciresinol, are considered to be phytoestrogens. The richest dietary source is linseed, which contains secoisolariciresinol (up to 3.7 g/kg dry weight) and low quantities of matairesinol [26]. [fig. 3]

Fig. 3. Structures of secoisolariciresinol and matairesinol.

1.2.1.5. Biological role of polyphenols in plants

Both natural phenols and the larger polyphenols play important roles in the ecology of most plants. Their effects in plant tissues can be divided into the following categories:

- Release and suppression of growth hormones such as auxin.
- UV screens to protect against ionizing radiation and to provide coloration (plant pigments).
- Deterrence of herbivores (sensory properties) and microbial infections (phytoalexins).
- Signaling molecules in ripening and other growth processes [27].

1.2.2. Chemical assays of olive leave extracts:

1.2.2.1. Antioxidant activity

An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by- products of cell metabolism [28,29]. Free radicals form when oxygen is metabolized or formed in the body and are chemical species that posses an unpaired electron in the outer (valance) shell of the molecule. This is the reason, why the free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA. These free radicals attack the nearest stable molecules, taking its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell [30]. Free radicals may be either oxygen derived (ROS, reactive oxygen species) or

nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are O_2^- [superoxide], HO[hydroxyl] ,HO_2 [hydroperoxyl], ROO[peroxyl], RO[alkoxyl] as free radical and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly NO [nitric oxide], ONOO [peroxy nitrate], NO_2 [nitrogen dioxide] and N_2O_3[dinitrogen trioxide] [31,32]. In a normal cell, there are appropriate oxidant: antioxidant balance. However, this balance can be shifted, when production species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Lipid peroxidation is oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading yield a wide range of cytotoxic products, most of which are aldehydes, like malondialdehyde (MDA), 4-hydroxynonrnal(HNE), Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's, Parkinson's, atheroscleorosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc [33].

The antioxidant activity from natural extracts can and must be evaluated with different tests. One of the most important methods used to measure antioxidant activity of plants extract is Ferric Ion Reducing Antioxidant Power Assay (FRAP)**,** It is simple, fast, inexpensive, and robust 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. Any electron donating substances with a half reaction of lower redox potential than Fe^{3+}/Fe^{2+} TPTZ will drive the reaction and the formation of the blue complex forward [34]. [fig. 4]

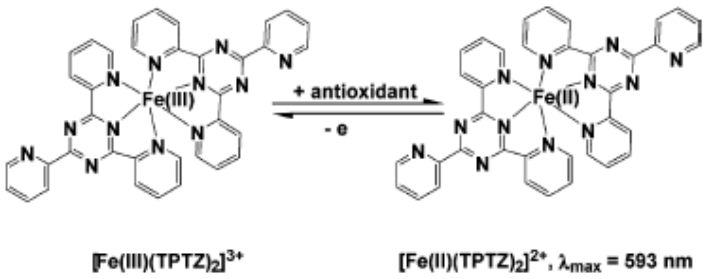

Fig. 4. Chemical structures of reaction of yellow Fe^{3+} TPTZ complex (2,4,6-tri(2 pyridyl)-1,3,5-triazine) with antioxidants is reduced to the blue Fe^{2+} TPTZ complex by electron-donating substances.

Other methods used to measure antioxidant activity of plants extract are 2,2-diphenyl-1- picrylhydrazyl DPPH and 2,2' – azinobis-(3-ethyl-benzothiazoline- 6-sulphonic acid) ABTS tests.

DPPH is a free radical that is stable at room temperature, which produces a violet solution in methanol. When the free radical reacts with an antioxidant, its free radical

property is lost due to chain breakage and its color changes to light yellow [35]. [fig. 5]

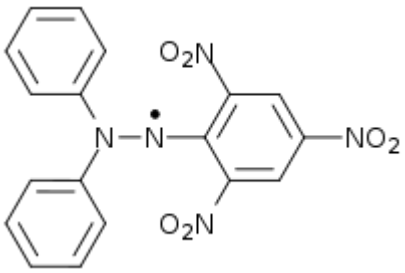

Fig. 5. Chemical structure of DPPH.

The ABTS assay, its based on the principle that when 2,2'-azinobis-(3- ethyl-benzothiazoline-6-sulphonic acid) {ABTS} is incubated with a peroxidase (such as metmyoglobin and H_2O_2, a relatively stable radical cation, $ABTS^{\cdot+}$, is formed.
The formation of $ABTS^{\cdot+}$ upon Interaction with ferryl myoglobin produces a relatively stable blue-green color, Measured at 600 nm. Antioxidants in the fluid sample suppress this color production to a degree that is proportional to their concentrations[36].

$$\text{metmyoglobin} + H_2O_2 \longrightarrow \text{ferryl myoglobin}^{\cdot} + H_2O$$

$$\text{ABTS} + \text{ferryl myoglobin}^{\cdot} \longrightarrow \text{metmyoglobin} + \text{ABTS}^{\cdot+}$$

Most of the chemical methods are based on the ability to scavenge different free radicals. Tests measuring the scavenging activity with different challengers, such as superoxide radical ($O_2^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$) have been developed [37]. The Oxygen Radical Absorbent Capacity (ORAC) assay measures the ability of the antioxidant species present in the samples to inhibit the oxidation of disodium fluorescein (FL), catalyzed by peroxyl radicals. Many reports have investigated the implementation of ORAC to natural extracts from food matrices, such as cherries, olives and oil seeds. In hydroxyl radical antioxidant capacity (HORAC) assay, a sample's capacity to prevent hydroxyl radical production is evaluated using FL as the probe. This assay also measures the scavenging capacity of antioxidants present in the samples, against hydroxyl radicals. There are few studies that apply the HORAC and hydroxyl radical scavenging capacity (HOSC) assays to evaluate the antioxidant activity from natural matrices [38].

1.2.2.2.Total Phenolic Assay by Folin-Ciocalteu Reagent.

Folin-Ciocalteu Reagent (FCR) was initially intended for the analysis of proteins taking advantage of the reagent's activity toward protein tyrosine (containing a phenol

group) residue [39]. Many years later, Singleton and co-workers extended this assay to the analysis of total phenolic in wine; since then the assay has found many applications [40]. The FCR-based assay gained popularity and is commonly known as the total phenols (or phenolic) assay.

The FCR is typically made by first boiling (for 10 h) the mixture of sodium tungstate ($Na_2WO_4.2H_2O$, 100 g), sodium molybdate ($Na_2MoO_4.2H_2O$, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL), and water (700 mL). After boiling, lithium sulfate ($Li_2SO_4.4H_2O$, 150 g) is added to the mixture to give an intense yellow solutions of the FC reagent. Contamination of reductants leads to a green color, and the addition of oxidants such as bromine can restore the desired yellow color. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates- molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI):

$$Mo(VI) + e \longrightarrow Mo(V)$$

Obviously, the FC reagent is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds [e.g., vitamin C, Cu(I), etc.]. Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH ~10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. This supports the notion that the reaction occurs through electron transfer mechanism. The blue compounds formed between phenolate and FCR are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds.

Despite the undefined chemical nature of FCR, the total phenols assay by FCR is convenient, simple, and reproducible [41].

1.2.2.3. Total flavonoid content Aluminium Chloride Colorimetric Method

The principle this method based on Formation of 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 with aluminium chloride. Aluminium chloride also forms acid labile complexes with the ortho - dihydroxyl groups in the A- or B-ring of flavonoids. For building the calibration curve , catechin is used as a standard materials. Various concentrations of standard catechin solution were used to make a standard calibration curve [42].

1.2.3 . Instrumental analysis :

In order to analyze the phenolic content in natural extracts, high performance liquid chromatography (HPLC) technique is widely applied, for both the separation and quantification of these compounds. The separation of different classes of phenolic compounds is achieved through the introduction of a reverse phase column, which enhances the process. Usually, diode array detector (DAD) is used for food phenolic compounds detection. HPLC coupled with mass spectrometry (MS) has commonly been used for structural characterization of phenols. Electrospray ionization mass spectrometry (ESI/MS) has been employed for the structural confirmation of phenols in peaches, nectarines, olives, grape seeds, cocoa, olive oil, etc [43].

Chapter two

Literature review

&

Objectives

Scientific literature does not contain any reports that deal with antioxidant activity and phenolics content or flavonoids content of olive either fruits or leaves from Palestinian territories. Therefore, a detailed study of the Palestinian olive leaves constitutes a valuable addition to the available literature. Abundant literature dealing with antioxidant activity came out of the middle east region especially Iran, turkey, Tunisia and Greek.

2.1. previous studies related to measurement of TPC, TFC, AA and antibacterial activities of olive leaves extracts:

Ryan et al., (2002) reported that there are differences in levels and type of phenolic compounds in Olea europaea L. leaves, fruits and seeds of Italy. Phenolic compounds are a diverse range of secondary metabolites derived from the shikimate pathway and phenylpropanoid metabolism. *Olea europaea* L. contains a number of unusual phenolics including various oleosides. The amounts and types of phenolics vary markedly between leaf, fruit, stone, and seed. The metabolic relationships between the various parts and phenolic content are poorly understood. Interest in this area is related to the importance of the phenolic profile to the aesthetics and quality of olive products, and to the use of olive leaves in phytomedicines. [44].

Briante et al., (2002) reported that there are also significant changes in the phenolic composition of fruits and leaves during the maturation period in two Italian cultivars of *Olea europaea.* During the ripening of two Italian cultivars of Olea europaea L. (AscolanaTenera and Frantoio), they are identified a β-glucosidase activity that contributes to the oleuropein degradation during maturation as well as an esterase activity, whose trend during the ripening is hypothesised to be linked to the fatty acid biosynthesis involved later in the maturation of fruits. This activity, in fact, is involved in the estereolysis of C_3 and C_4 esters that supply acetyl-CoA as the basic unit for fatty acid biosynthesis. The data obtained during the ripening indicate that polyphenol content and composition, in particular the oleuropein concentration, and their correlation with the recovered enzymatic activities, will be useful for a biochemical characterisation of different O. europaea L. varieties as important parameters in testing the quality of the obtainable oils [45].

Japon et al., (2006) reported the effect of temperature on the extraction of phenolics compound, who found a relationship between temperature and extraction yields. At 40 ^{o}C and for extraction times of 24 hours the amount of olive phenolics compounds extracted were 95% , while at 30 ^{o}C the amount of olive phenolics compounds extracted were 75% for 24 hours [46].

Isabel C.F.R. Ferreira et al., (2007) studied the antioxidant activity and phenolic contents of Olea europaea L. leaves from the northeast of Portugal sprayed with different copper formulations as pesticides. Results showed that leaves sprayed with this pesticides have low contents of phenolics and hence lower antioxidant properties. Olive leaves sprayed with copper oxychloride possessed the highest copper levels and

the lowest content in phenols, which influenced its antioxidant activity (higher EC50 values for reducing power, scavenging effect on DPPH radicals and inhibition of erythrocyte hemolysis). Leaves without copper residues proved to be a good natural source of antioxidants [47].

Hajimahmoodi et al., (2008) determined antioxidant activity, phenolic contents and reducing power in six Iranian olive cultivars. The highest antioxidant activity (28.699 mmol FeII/100 g dry plant), total phenolic contents (2997 mg gallic acid/100 g dry plant) and reducing power (8.331 g Vitamin E/100 g dry plant) were detected in Mishen and the lowest in Conservalina. A linear positive relationship existed between the antioxidant activity, total phenolic compounds ($r^2 = 0.976$) and reducing power of the tested olive pulp ($r^2 = 0.848$) [48].

Mylonaki et al., (2008) reported that the optimum working condition for conventional extraction of olive leaves phenolics compound was: 60% ethanol (v/v) with pH 2 for 5 hours, the temperature and solvent: solid ratio used in the study was 22±2 ^{o}C and 40:1 respectively [49].

Kostas Kiritsakis et al., (2010) determined the total amounts of phenols in the Olive Leaf Extracts from Greek Olive Cultivars. The results showed that 6094, 5579 and 6196 mg/kg (mg gallic acid/kg dried olive leaves) for the cultivars megaritiki, kalamon and koroneiki, respectively [50].

Essam A Abdel-Sattar et al., (2012) studied the chemical and biological properties of African Olive Leaf Extract which is a type of olive grown wild in south western regions of Saudi Arabia. They determined total phenolics content were 36 mg/g calculated as gallic acid equivalents (GAE) while the total amount of flavonoids was 1.35 mg/g calculated as rutin equivalents (RE). The in vitro antioxidant activity of African OLE showed an IC50 of 60.2 µg/ml using diphenylpicrylhydrazyl (DPPH) assay [51].

Theodora-Ioanna Lafka et al., (2012) studied the Phenolic Extracts from Wild Olive Leaves from Greece and Their Potential as Edible Oils Antioxidants . As solvents, methanol, ethanol, ethanol:water 1:1, *n*-propanol, isopropanol and ethyl acetate were used. The results showed that the most effective solvent was ethanol with optimum phenol extraction conditions 180 min, solvent to sample ratio 5:1 v/w and pH 2. Ethanol extract exhibited the highest antiradical activity among solvent and supercritical fluid extraction (SFE) extracts, which in addition showed the highest antioxidant capacity compared to synthetic and natural food antioxidants such as BHT, ascorbyl palmitate and vitamin E [52].

Myriam Ben Salah et al., (2012) has investigated olive leaves of five varieties (Limouni, Gerboua, Meski, Chetoui and Sevillane) for the total phenol and flavonoid content, for the major compound and for the in vitro antioxidant properties. Results

showed that olive leaves extract exhibited a good antioxidant activity and a reduced power, each variety showed its own feature. The means of TP in olive leaves extracts in terms of mg GAE/g of dry material ranged from 73.05 ± 15.52 to 144.19 ± 10.27 ($p < 0.05$). The highest amount was observed in the order Limouni > Gerboua > Meski > Chetoui compared to Sevillane content which had the lowest amount. The TF content was also expressed as mg CE/g of dry material and ranged from 56.57 ± 6.0 to 125.64 ± 3.36 ($p<0.05$). Varieties of Gerboua exhibited an important amount of TF followed by Limouni and Lucques. Sevillane still had the lowest level of TF compared to the other varieties [53].

Janina Diogo, (2013) determined the total phenolic content of wild olive leaves from turkey by colorimetric method. The values ranged from 249 to 906 mg GAE/L extract, varying in response to different solvents used. In general, highest values were exhibited by methanol extracts (ranging of 622.4 ± 19.3 to 906.8 ± 9.4 mg GAE/L extract) followed by EtOH (ranging of 366.5 ± 4.9 to 879.8 ± 10.8 mg GAE/L extract) and acetone (ranging of 251.0 ± 18.7 to 627.6 ± 13.3 mg GAE/L extract) extracts. The water extracts obtained the lowest values, ranging between 250.0 ± 2.2 and 566.9 ± 5.5 mg GAE/L of extract. The antioxidant activities of olive stone extracts from zambujeiro (as ORAC, HORAC and HOSC) ranged from 3872.3 ± 282.7 to 25755.2 ± 179.9 μmol TE/L, from 1030.3 ± 42.3 to 3704.0 ± 100.4 μmol CAE/L, and 641.6 ± 57.7 to 4468.9 ± 394.5 μmol TE/L, respectively [54].

Also there are many studies talking about antimicrobial activity of olive leave extracts:

Morteza Azizollahi Aliabadi et al, (2011) studied the antimicrobial activity olive leave aqueous extracts that were collected in winter 2011 from Gilan province (Northern Iran) against pathogenic bacteria (Staphylococcus aureus PTCC 1431, Salmonella typhimurium PTCC 1639, and Escherichia coli PTCC 1399, Klebsiella pneumonia PTCC 1053, Bacillus cereus PTCC1274). The results showed that the olive leave extract was found to be most active against Salmonella typhimurium PTCC 1639 with inhibitory, 11.5 mm. These findings suggest an antimicrobial potential for olive leaves [55].

Nahal Bouderba Nora et al, (2012) studied Antibacterial Activity of aqueous extract of olive leaves from Algeria , they used the diffusion method on solid medium and the direct contact method. The results showed that the aqueous extract reacted positively on all bacterial strains tested Escherichia coli ATCC25922, Escherichia coli 2, Staphylococcus aureus ATCC6538, Staphylococcus aureus ATCC25923, Klebsiella pneumoniae, Enterobacter cloacae ATCC13047, Pseudomonas aeruginosa ATCC10145 and Bacillus stearothermophilus ATCC 11778. Aqueous extract of Olea europaea leaves demonstrated the best inhibition against Escherichia coli 2 with MIC of 150

2.2. Objectives:

- To date no systematic study has been performed on Palestanian olive leaves. In this study we evaluated the antioxidant activity of a variety olive leaves extracts from three regions in West Bank in Palestine (north, middle, south) in different times of year (June 2013, October 2013, January 2014). Our investigation involved the use of Folin-ciocalteu, FRAP and the $AlCl_3$ colorimetric assays in order to determine the total phenolic content, total flavonoid content and antioxidant activity in the collected olive leave samples.

- We investigate the relationship between AA and TPC as well as relationship between AA and TFC in the samples tested.
- We investigate the effect of pH of medium extract on TPC, AA and TFC.
- We investigate the effect of extraction time on TPC, AA and TFC.
- We study the antibacterial activity of olive leaves extract (OLE).

Chapter three

Experimental

3.1. Chemicals, Reagents and Plant materials

3.1.1. Chemicals

The chemicals used for analyzing the antioxidant compounds (TPC, AA, and TFC) of olive leave are: 2,4,6-tripyridyl- S-triazine (TPTZ), ferric chloride hexahydrate, catechin, gallic acid, sodium hydroxide, hydrolic acid, acetic acid, sodium nitrite, aluminum chloride, iron (II) sulfate hexahydrate, sodium bicarbonate are purchased from Sigma-Aldrich.

3.1.2. Reagents

Folin–Ciocalteu reagent. FRAP reagent was prepared according to Benzie and Strain (1999) by the addition of 2.5 ml of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20mM FeCl3.6H2O and 25 ml of 0.3 M acetate buffer at pH3.6 [57].

Acetate buffer (0.3 M) at pH 3.6 was prepared according to British Pharmacopeia by dissolving 16.8g of acetic acid and 0.8g of sodium hydroxide in 1000 ml of water.

TPTZ (10 mM, M.wt = 312.34 g/mol) was prepared by dissolving 0.321g TPTZ in 100ml HCl. 40 mM HCl was prepared by diluting 3.77ml of stock HCl solution (10.6M) to 1000ml with water.

5% $NaNO_2$ was prepared by dissolving 5g of $NaNO_2$ in 100ml of water.

10% $AlCl_3$ was prepared by dissolving 10g of $AlCl_3$ in 100ml of water.

3.1.3. Plant materials

Olive leaves samples (Nabali cultivar) were obtained from three different regions of West Bank; south (Hebron), middle (Abu- Dies), north (Qalqelya). Olive leaves samples were collected at different maturity stages in June 2013, October 2013 and January 2014.

3.2. **Instrumentation**:

- Specord 40 UV VIS spectrum, versatile single-beam spectrophotometer for the measurement of 190-1100 nm conforms to Ph.Eur. quality, made by analytikjena company.
- Jenway pH meter (3310) with a combination glass electrode and a tolerance of ±0.01 pH units.

3.3. Procedure

3.3.1. Extraction

The olive leaves samples were dried at 30 °C, grinded with a blender and extracted with water or with acidified water (pH=3.0). Briefly, 5 g of the dried powdered olive leaves were macerated with 50 ml water for 2 hrs at 40 ^{o}C. The extracts were then filtered . Then the crude extracts were stored in Refrigerator at 4 ^{o}C until analysis.

3.3.2. Total phenolics content (Folin–Ciocalteu assay)

Total phenolics were determined using Folin–Ciocalteu reagents (Singleton & Rossi, 1965). Olive leave extract (40) µl 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 standing for 60 min at room temperature, and 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 mg gallic acid equivalents (GAE)/ g sample [58].

3.3.3. Total flavonoids

The determination of flavonoids was performed according to the colorimetric assay of Kim et al., 2003. Distilled water (4 ml) was added to (1 ml) of olive leave 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)/ g sample [59].

3.3.4. Measurement of Antioxidant Activity by FRAP assay

The antioxidant activity of olive leave extracts was 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) were warmed at 37◦C and mixed with 40 µl of olive leaf 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) ($FeSO_4.6H_2O$) were used for calibration [58].

3.3.5. Effect of Extraction Time on the studied parameters (TPC, TFC and AA)

Olive leaves powder were macerated in distilled water at 40 °C at various times including 1, 2, 4, 6, 8 and 24 h. Afterwards , OLE`s were analyzed for their TPC, TFC and AA.

3.3.6. Effect of pH on phenolic content Extraction

To optimize the extraction of phenolic compound by aqueous solvents, olive leaves powder were extracted with distilled water at 40 °C and various pH values to determine the optimum pH for extraction of phenolic and flavonoid compounds and AA.

3.4. Statistical analysis:

Statistical analysis were performed using SAS statistical program to test the difference between the measured parameters (TPC, TFC, and AA) of the olive leaf samples from north, middle, and south West Bank, as well as those collected with different maturation stages by treating main factors (maturation stage and location) separately using one-way analysis of variance (ANOVA). Additionally Pearson correlation was used to test the correlation between measured parameters (AA and TPC, AA and TFC, and between TPC and TFC).

3. 5. Antibacterial activities of the OLE by cylinder plate technique

Antibacterial activities of the olive leaf extracts was studied by cylinder plate technique which depends on diffusion of the sample tested from a vertical cylinder through a solidified agar layer in a plate. Pooled olive leaf extract sample from north, middle, and south West Bank (extracted with water or acidified water) were used for this study.

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 Staphylococcus epidermidis) and gram negative (Escherichia coli and Pseudomonas aeruginosa) 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 olive leaf 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.

Chapter four

Results
&
Discussion

Three olive leaves samples were collected from three different regions in the West Bank of Palestine (north, middle, and south) at three different harvesting times in the year (June 2013, October 2013, and January 2014) and extracted by two different media (water, and acidified water, pH = 3.0). The crude extracts of samples were then analyzed for their total phenolic content, total flavonoid content and antioxidant activity.

4.1. Total phenolic content (TPC)

Results showed that the harvesting date of the olive leaves affects significantly the total phenolic content (TPC), where TPC was found to decrease significantly with shifting from June 2013 through October 2013 to January 2014 for olive leaf samples in the three geographical areas of West Bank under study as seen in table (1), figures 6 and 7. Table 1 shows statistical analyses of the results showing statistical difference between the TPC of the leave samples of the three harvesting dates represented by different capital letters (A, B, C). The reduction of TPC in January 2014 compared to June 2013 sampling was 45-50% in water extracted samples, while in samples extracted with pH 3 solution, samples collected in January 2014 from north and middle west bank contained only half the contents of total phenolic compounds compared to samples collected from the same areas in June 2013, but this reduction was sharper in samples collected from the south West Bank (61% reduction). The highest TPC was found to be in olive leaf samples from north West Bank obtained in June 2013 that extracted by acidified water (50.10 mg GAE/g dry olive leaves), and the lowest value was for olive leave samples obtained from south West Bank collected in January, and extracted by acidified water (18.63 mg GAE/g dry olive leaves) as seen in Table 1.

The presence and amounts of phenolic compounds in olive leaves is subject to the influence of many factors, such as cultivar, environment and maturity stage during sampling. The decrease in total phenolic content is attributed to the oxidation of polyphenols by polyphenoloxidase during fruit maturity [60].

During the normal processes of growth and development, plants are subjected to different types of stress, such as drought, heat, ultraviolet light, air pollution, and pathogen attack [61-63]. Most plants suffer from both physiological and biochemical damage by exposure to temperatures higher or lower than optimal for growth [64,65]. The results of these injuries, which are reflected in most metabolic processes [66-67], may be a reduced growth capacity of the crops and therefore lower commercial yield [68]. It has been demonstrated that thermal stress induces the production of phenolic compounds, such as flavonoids and phenylpropanoids [69-72].

The metabolism of soluble phenolics is regulated by the activity of various enzymes. The first step necessary for the synthesis of the phenylpropanoid skeleton in higher plants is the deamination of the L-phenylalanine, giving rise to trans-cinnamic acid

and ammonium [73,74]. This reaction is catalysed by the enzyme PAL(Phenylalanine ammonia-lyase), which is commonly considered the principal enzyme in the biosynthesis of phenolic compounds [73,75]. PAL activity is affected by a great number of factors, both biotic and abiotic, including light, temperature, growth regulators, inhibitors of RNA and protein synthesis, drought and mineral nutrition [63,76,77,78]. It has been demonstrated that heat and cold stress induced the production of soluble phenolics and thereby increased PAL activity [69-72].

Rosa M. Rivero et al., has investigated the effect of temperatures (15°C, 25°C and 35°C) on phenolic metabolism in tomato plants. Their results showed that stronger shoot growth occurred at 15 and 25°C, the latter temperature giving the highest growth, which was twice that found at 35°C. The highest PAL activity in tomato plants was recorded at 35°C . Therefore, the PAL activity increased probability to heat stress. In addition, the metabolism of phenolic compounds also includes the action of oxidative enzymes such as POD and PPO, which catalyse the oxidation of phenols to quinones [79,80]. Some studies have reported that these enzyme activities increase in response to different types of stress, both biotic and abiotic [76,77]. In contrast to finding of these authors, POD and PPO activities were lowest in tomato plants at 35°C.

Haleno luo, 2011, evaluated the impact of cultivars and leave collection time from New Zealand on phenolic composition of olive leaves. Two cultivars "Frantoio" and "Barnea" were investigated , and leaves were collected from their respective tree in October and November 2010. The leaves collected in October had significantly higher total phenolic (25.17 and 17.17 mg caffeic acid / g dry matter) than those collected in November (17.20 and 15.75 mg caffeic acid / g of dry matter) respectively of cultivars [81].

This results support our findings. The higher values of phenolic content were in June with temperatures ranging from 25°C to 30°C and lowest values were in January with temperatures ranged from 5 °C to 12 °C (table 1).

Table 1: Total phenolic content (TPC) (as mg Gallic acid/g of dry olive leaf) of olive leaf samples obtained in June 2013, October 2013, and January 2014 , extracted with distilled water (pH ~ 7) and acidified water (pH = 3). Results are expressed as average ± SD. RSD is relative standard deviation of three samples of olive leaf).

	North West Bank		**Middle West Bank**		**South West Bank**	
	Water (pH = 7)	**Acidified water (pH=3)**	**Water (pH=7)**	**Acidified water (pH=3)**	**Water (pH=7)**	**Acidified water (pH=3)**
June 2013	**47.52 ± 0.9 (RSD: 1.9%) * A, a**	**50.10 ± 1.8 (RSD: 3.6%) A, a**	**46.13 ± 0.8 (RSD: 1.7%) * A, b**	**49.34 ± 0.8 (RSD: 1.6%) A, b**	**44.13 ± 1.0 (RSD: 2.3%) * A, c**	**48.30 ± 1.5 (RSD:3.1%) A, c**
October 2013	**31.42 ± 0.5 (RSD: 1.6%) * B, a**	**34.41 ± 0.9 (RSD: 2.6%) B, a**	**27.14 ± 1.2 (RSD: 4.4%) * B, b**	**26.33 ± 1.3 (RSD: 4.9%) B, b**	**23.87 ± 0.8 (RSD: 3.3%) * B, c**	**22.63 ±1.1 (RSD:4.9%) B, c**
January 2014	**26.14 ± 1.2 (RSD: 4.6%) * C, a**	**24.92 ± 1.0 (RSD: 4.0%) C, , a**	**25.42 ± 0.9 (RSD: 3.5%) * C, b**	**23.93 ± 1.7 (RSD: 7.1%) C, b**	**21.56 ± 1.4 (RSD:6.5%) * C, c**	**18.63 ± 1.0 (RSD:5.4%) C, c**

Different capital letters indicate significant differences within columns, different small letters indicate significant differences within lines of the same extraction treatments (water or pH 3). * indicate significant difference between different extraction treatments at each sampling date of the same sampling area.

Regarding the effect of the geographical region from where the olive leaf collected (north, middle, and south West Bank), TPC was found to be greater in samples collected from north followed by middle followed by south West bank, in both extraction methods (water and acidified water) in all sampling times (June, October and January). [table 1]. Statistically it was found that significant differences exist between the three geographical regions of West Bank (north, south, and middle).

Comparing both extraction methods in terms of total phenolic compounds recovery, there was no clear trend because the differences were significant in all samples (represented by * in table 1), but the higher values alternate between different

extraction methods in different samples. The differences for values of TPC with variation of PH of extraction solvent can be attributed to change of types of phenolic compounds at difference maturation stage and origin of the samples , this means changing polarity of phenolic compound and this effect on association/dissociation of phenolic compound with changes of PH, Hence pH affects TPC of olive leaves depending on cycling of biosynthesis and metabolism of phenolic compounds in plants.

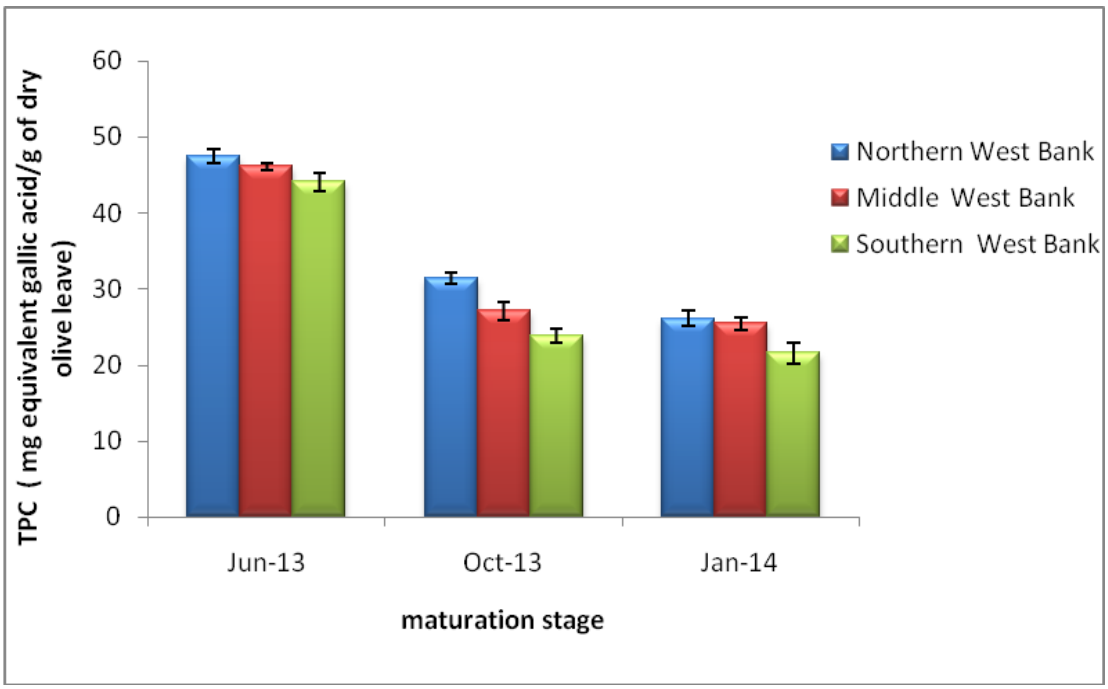

Fig. 6: Total phenolic content (TPC) (as mg Gallic acid/g of dry olive leaves) of olive leave samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with water (pH ~ 7). Error bars was added as standard deviation of three samples.

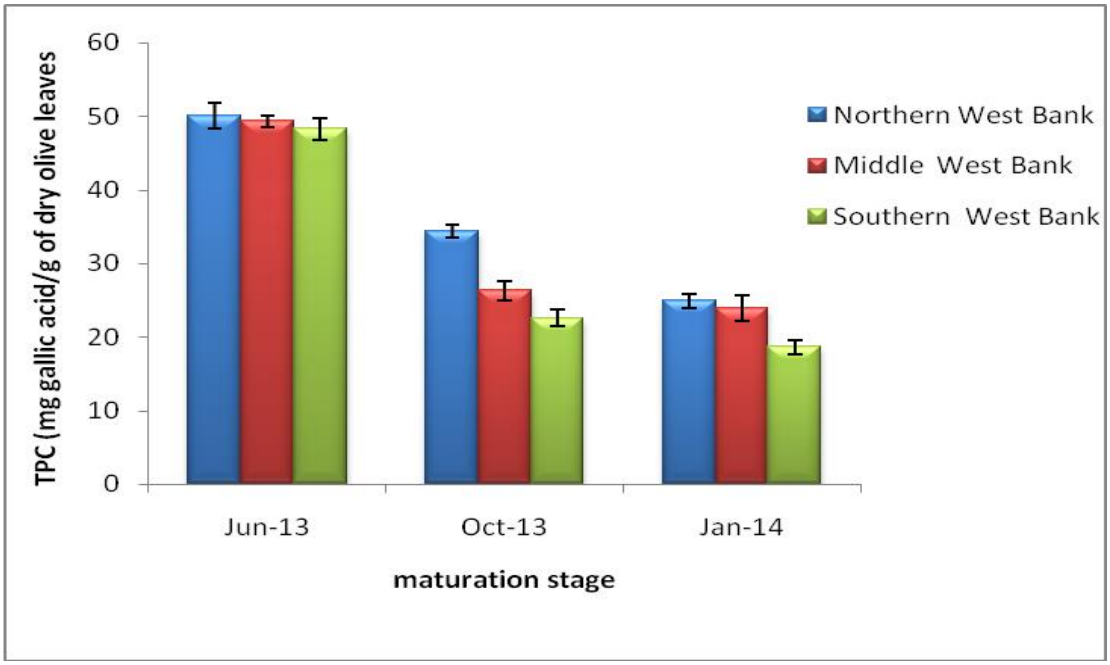

Fig. 7: Total phenolic content (TPC) (as mg Gallic acid/g of dry olive leaves) of olive leave samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with acidified water (pH=3.0). Error bars was added as standard deviation of three samples.

4.2. Total flavonoid content (TFC)

The total flavonoids contents were higher when samples were collected in June followed by samples collected in October, followed by samples collected in January in all collection areas and in both extraction methods in agreement with the results obtained for TPC. (see table 2 where this significant difference is represented by different capital letters (A, B, C)). The reduction of TFC in January 2014 compared to June 2013 sampling was 7-33% in water extracted samples, while in samples extracted with pH 3 solution, this reduction was from 15-38%, but this reduction was sharper in samples collected from the south and north West Bank (38% reduction) compared to middle West Bank (15%). The highest TFC contents were obtained from samples collected from southern West Bank in June when extracted with water (32.6 mg catechin/g of dry olive leaf), followed by northern West Bank when extracted at pH 3 (32.0 mg catechin/g of dry olive leaf), while the lowest value obtained when samples were collected in January (19.3 as mg Catechin/g of dry olive leaf) from middle West Bank and extracted with water.

During different maturation stages, the total flavonoid content varied during consecutive maturation stages and ranged between 19.4-26.5, 19.3-21.6, and 21.6-32.6 mg catechin equivalents/g dry of olive leaves (extracted with water) collected from north, middle, and south West Bank respectively.

It was interesting to compare the TFC of the samples obtained from the three regions of West Bank (north, middle, and south West Bank). It was found that there is no clear trend in the TFC in the three geographical regions, for example in June and January, the highest TFC was obtained in south West Bank when extracted with water, while in October, the highest TFC was obtained in north west bank when extracted with acidified water.

Comparing both extraction methods in terms of total flavonoid compounds recovery, there was no clear trend because the differences were in some samples significant while in other samples the differences are not significant, but the higher values alternate between different extraction methods in different samples. [table 2, fig. 8 and 9]

Table 2: Total flavonoids content (TFC) (as mg catechin/g of dry olive leaf) of olive leaf samples obtained in June 2013, October 2013, and January 2014 , extracted with distilled water (pH ~ 7) and acidified water (pH = 3).

	Northern West Bank		**Middle West Bank**		**Southern West Bank**	
	Water (pH = 7)	**Acidified water (pH=3)**	**Water (pH = 7)**	**Acidified water (pH= 3)**	**Water (pH = 7)**	**Acidified water (pH= 3)**
June 2013	**26.5± 0. 36 (RSD:1.36%) * A, b**	**32.0± 0.21 (RSD:0.66%) A, a**	**21.6± 0.26 (RSD:1.20%) * A, c**	**22.7± 0.47 (RSD:2.10%) A, b**	**32.6± 0.06 (RSD:0.18%) * A, a**	**31.4± 0.45 (RSD: 1.43%) A, a**
tober 2013	**22.4 ± 0.40 (RSD:0.47%) *B, b**	**25.5± 0.40 (RSD:0.39%) B, a**	**20.8± 0.46 (RSD:1.17%) B, c**	**21.3± 0.96 (RSD 2.10%) B, c**	**25.0± 0.99 (RSD:2.23%) B, a**	**24.6± 0.11 (RSD:0.25%) B, b**
January 2014	**19.4± 0.35 (RSD:1.63%) C, b**	**19.8± 0.06 (RSD:0.30%) C, a**	**19.3± 0.21 (RSD:1.03%) C, b**	**19.4±0.10 (RSD 0.52%) C, b**	**21.6± 0.21 (RSD:0.97%) C, a**	**19.6± 0.25 (RSD: 1.28%) C, b**

Different capital letters indicate significant differences within columns, different small letters indicate significant differences within lines of the same extraction treatments (water or pH 3). * indicate significant difference between different extraction treatments at each sampling date of the same sampling area (n.s indicate no significant difference).

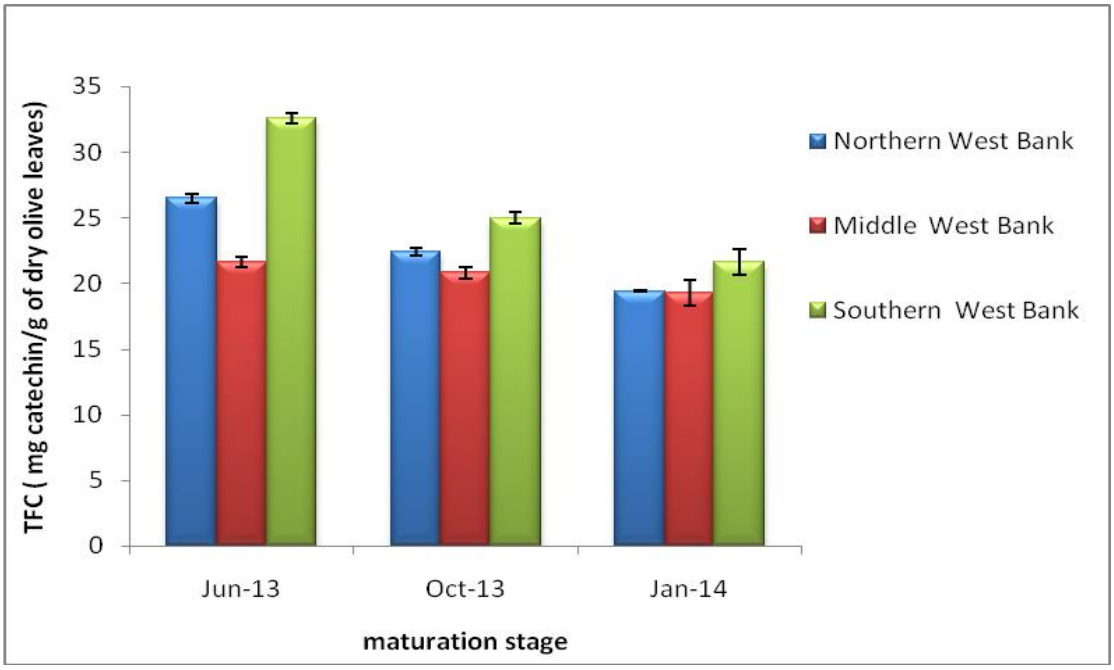

Fig. 8: Total flavonoid content (TFC) (as mg catechin/g of dry olive leave) of olive leaves samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with water (~pH=7.0). Error bars was added as standard deviation of three samples.

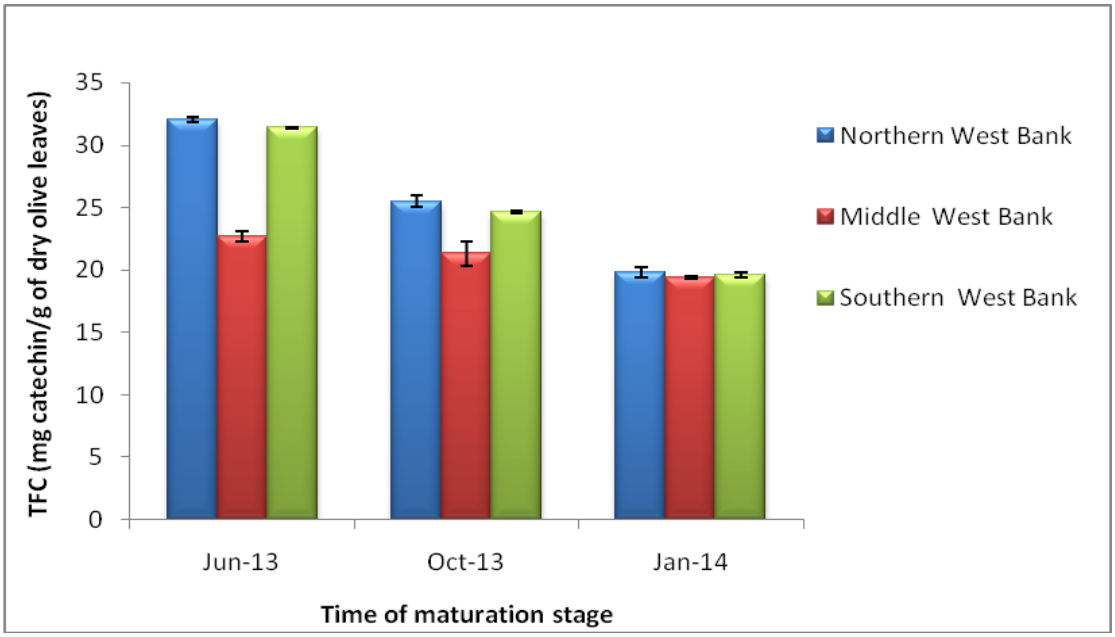

Fig. 9: Total flavonoid content (TFC) (as mg catechin /g of dry olive leave) of olive leaves samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with acidified water (pH=3.0). Error bars was added as standard deviation of three samples.

4.3. Antioxidant activity (AA)

Similar to TPC and TFC, antioxidant activity decreased with shifting date of sampling from June through October to January, this was shown in all sampling areas and both extraction methods see table 3, Figures 10 and 11. Show how the AA activity of samples collected in January diminished by about 55-70% compared to those collected in June. The highest AA values were obtained in samples collected from Southern West Bank in June in both extraction methods (1106.43 and 1187.23 µ mol FRAP/g of dry olive leaf, for samples extracted with water and acidified water, respectively) followed by samples collected from Northern West Bank in the same sampling date, while the lowest value obtained when samples were collected in January (317.37 µ mol FRAP/g of dry olive leave) from middle West Bank when extracted with acidified water. During different maturation stages, antioxidant activity (FRAP assay) measured during different maturation stages was found to be in the range of 422.20 - 936.67, 337.53-747.70, and 318.53 – 1106.3 µmol FRAP equivalents/g of dry of olive leaves (extracted with water) collected from north, middle, and south West Bank, respectively.

Results showed that the extraction method did not favor any of the methods utilized in these assays. Furthermore, as for TFC, there is no clear trend in the AA of the three geographical regions.

Table 3: Antioxidant activity (AA) (as µ mole FRAP /g of dry olive leaf) of olive leaves samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with distilled water (pH ~ 7) and acidified water (pH = 3).

	Northern West Bank		**Middle West Bank**		**Southern West Bank**	
	Water (pH = 7)	**Acidified water (pH= 3)**	**Water (pH = 7)**	**Acidified water (pH= 3)**	**Water (pH = 7)**	**Acidified ater (pH=3)**
June 2013	**936.67 ± 5.8 (RSD:0.62%) A, b**	**935.33±13.2 (RSD:1.4%) A, b**	**747.70± 8.45 (RSD:1.1%) *A, c**	**643.87± 6.7 (RSD:1.0%) A, c**	**1106.43± 9.3 (RSD: 0.84%) *A, a**	**1187.23± 13.0 (RSD: 1.1%) A, a**
October 2013	**855.53± 13.6 (RSD:1.6%) * B, b**	**871.50± 5.1 (RSD:0.58%) B, a**	**656.50± 6.6 (RSD: 1.0%) *B, c**	**616.00± 4.0 (RSD: 0. 65) B, c**	**867.23± 11.9 (RSD: 1.4%) * B, a**	**850.63± 12.9 (RSD: 1.5%) B, b**
Jan 2014	**422.20± 11.0 (RSD: 2.6%) * C, a**	**412.50± 22.9 (RSD: 5.6%) C, a**	**337.53± 5.7 (RSD:1.7%) *C, b**	**317.37± 15.2 (RSD:4.8%) C, c**	**318.53±8.6 (RSD:2.7%) *C, c**	**355.33± 7.0 (RSD: 2.0%) C, b**

Different capital letters indicate significant differences within columns, different small letters indicate significant differences within lines of the same extraction treatments (water or pH 3). * indicate significant difference between different extraction treatments at each sampling date of the same sampling area (n.s indicate no significant difference).

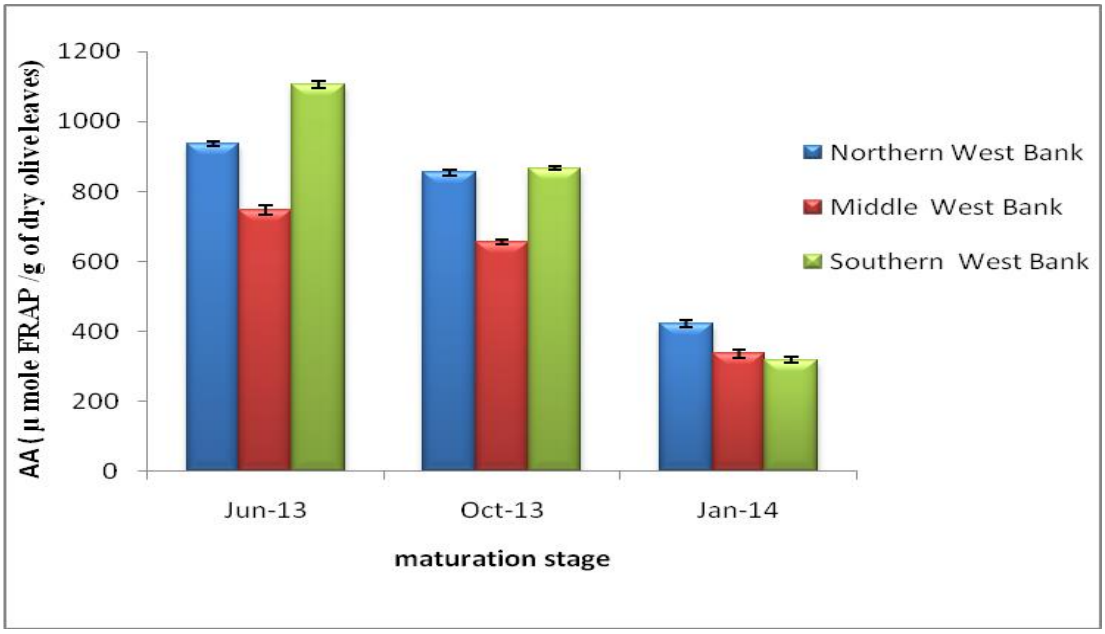

Fig. 10 : Antioxidant activity (AA) (as µ mole FRAP /g of dry olive leaves) of olive leaves samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with water (~PH=7.0). Error bars was added as standard deviation of three samples.

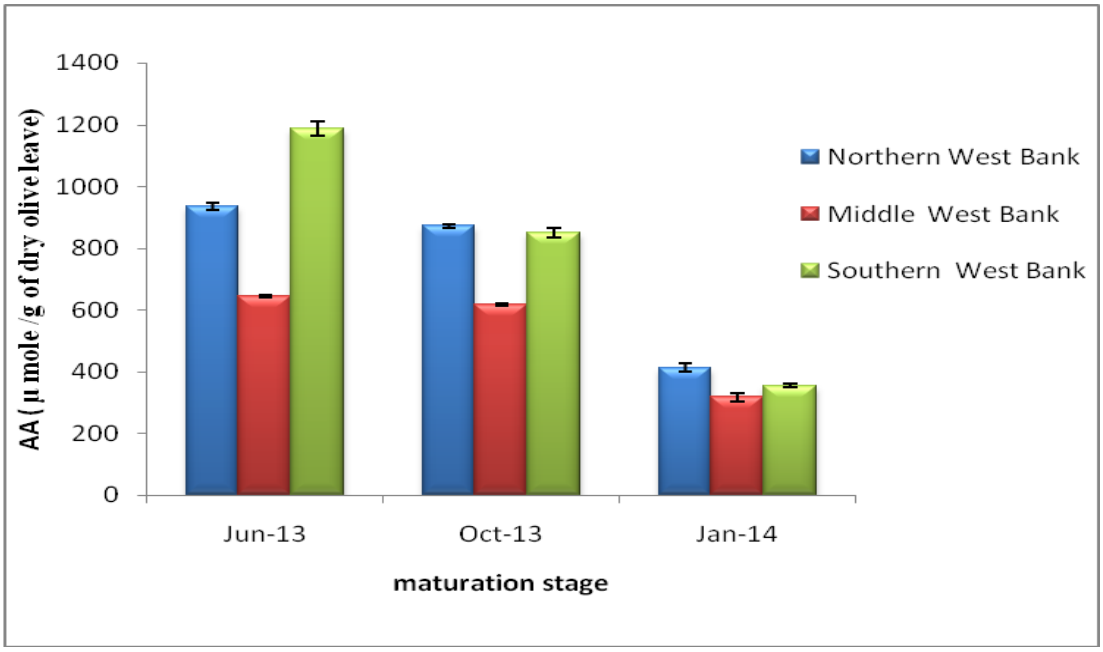

Fig. 11 : Antioxidant activity (as µ mole FRAP /g of dry olive leaves) of olive leaves samples obtained from north, middle, and south West Bank in three different maturation stages (June 2013, October 2013, and January 2014), extracted with acidified water (PH=3.0). Error bars was added as standard deviation of three samples.

4.5. Comparison of TPC, TFC, and AA of olive leaves of this study with literature values

It is interesting to compare our values total phenolics content, total flavonoids antioxidant activities of Palestinian olive leaves investigated in this study with those obtained by other investigations in different countries, see table 4. The total phenolics content varied between 50.10 and 24.01 mg gallic acid per g of dry leaves. The highest value is obtained by our study (palatine) while the lowest value was obtained by a study carried out in Greek. Similar trends were observed for TFC and AA. It appears that a multitude of factors affected the a for mentioned results. Since the studies were carried out in climatically different countries. Various factors such as olive variety, growing condition, maturity, season, geographic origin, fertilizers, soil type, amount of sunlight received, and experimental conditions (storage, extraction) appear to be responsible for the observed differences. [49, 51, 52, 82,83]

Table 4 : TPC, TFC, and AA of olive leaves of this study and other countries.

Country	TPC (as mg Gallic acid/g of olive leaves)	TFC (mg catechin /g of dry olive leaves)	AA (µ mol FRAP/g dry olive leaves)
Palestine (this study)	**50.10**	**32.6**	**1106.43**
Iran	**29.97**	**---**	**286.99**
Greek	**6.196**	**---**	**---**
South Saudi Arabia	**36.0**	**1.35**	**---**
North Tunisia	**24.01**	**21.47**	**---**
Turkey	**45.86**	**---**	**---**

4.6. Pearson correlation

Pearson correlation between TPC, TFC, and AA of samples collected from the three areas in West Bank showed that antioxidant activity is significantly correlated with total phenolic content in both extraction treatments, and weakly correlated with total flavonoids, see tables 5-8. But when all data was pooled, both TPC and TFC were highly and significantly correlated with AA, see table 8. It was also found no significant correlation between TPC and TFC in any sample under investigation. Similar results were obtained by Leila Abaza et. al. (2011) where they got a good positive correlation between antioxidant activity and total phenolics content or total flavonoids content. Similar results were obtained also by Manish S. Bhoyar et al., (2010) where a positive correlation was obtained between total phenolics content and

FRAP antioxidant activity assay of Caper (Capparis spinosa) leaves extract with a correlation coefficient of 0.649 [84, 85]. It is expected that TPC and TFC is correlated with AA since AA is mainly due to the presence of polyphenolic and/or flavonoid which have mong hydroxyl groups which has the ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations [68, 87].

Table 5: Pearson correlation between TPC, TFC and AA in samples collected from north West bank extracted by water (above diagonal) and at pH 3 (lower diagonal).

	TP	**TF**	**AA**
TP	**---**	**-0.20947**	**0.78967***
TF	**-0.00399**	**---**	**0.43442**
AA	**0.85113****	**0.52152**	**---**

Significance indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, n = 9.

Table 6: Pearson correlation between TPC, TFC and AA in samples collected from middle West bank extracted by water (above diagonal) and at pH 3 (lower diagonal).

	TP	**TF**	**AA**
TP	**---**	**-0.37870**	**0.72574***
TF	**-0.19651**	**---**	**0.36148**
AA	**0.63385***	**0.63183**	**---**

Significance indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, n = 9.

Table 7: Pearson correlation between TPC, TFC and AA in samples collected from south West bank extracted by water (above diagonal) and at pH 3 (lower diagonal).

	TP	**TF**	**AA**
TP	**---**	**0.06913**	**0.79377***
TF	**0.10274**	**---**	**0.66072**
AA	**0.87344****	**0.57385**	**---**

Significance indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, n = 9.

Table 8: Pearson correlation between TPC, TFC and AA in samples collected from all geographical areas and both extraction methods (pooled).

	TP	TF	AA
TP	**---**	**0.00776**	**0.68174*** **
TF	**0.16072**	**---**	**0.42914****
AA	**0.66332**	**0.57385**	**---**

Significance indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, n = 54.

4.7. Effect of extraction time on TPC, TFC, and AA

Consistent with Janina Diogo observant, we found that the phenolic compounds are more abundant after one hour of extraction, and their concentrations decrease along the time. Thus, the dried olive leaves collected from the southern part of the West Bank, were extracted with distilled water at 40 ^{o}C and for different time intervals (1, 2, 4, 6, 8 and 24 hrs). Our results are supported by the previously reported studies [55]. [table 9]

Table (9): TPC, TFC, and AA of olive leaves at different extraction time.

ime of extraction	**TPC (as mg Gallic acid/g of sample)**	**TFC (mg catechin /g of dry olive leaf)**	**AA (µ mol FRAP/g dry olive leaf)**
1 hour	**34.57 a**	**25.0 a**	**867.22 a**
2 hour	**34.63 a**	**25.1 a**	**872.23 a**
4 hour	**34.370 a**	**24.9 a**	**861.23 a**
8 hour	**34.182 b**	**25.0 a**	**866.05 a**
24 hour	**34.097 b**	**24.9 a**	**867.09 a**

4.8. Antibacterial activities of OLE

In this study, the antibacterial activities of pooled olive leaf extract samples from north, middle, and south West Bank (extracted with water or acidified water) were tested using two types of bacteria: gram positive and negative bacteria. Results have showed that the olive leaf extract from West Bank has showed antimicrobial activities against the two gram positive bacteria studied in this study, see Table 10 and11.

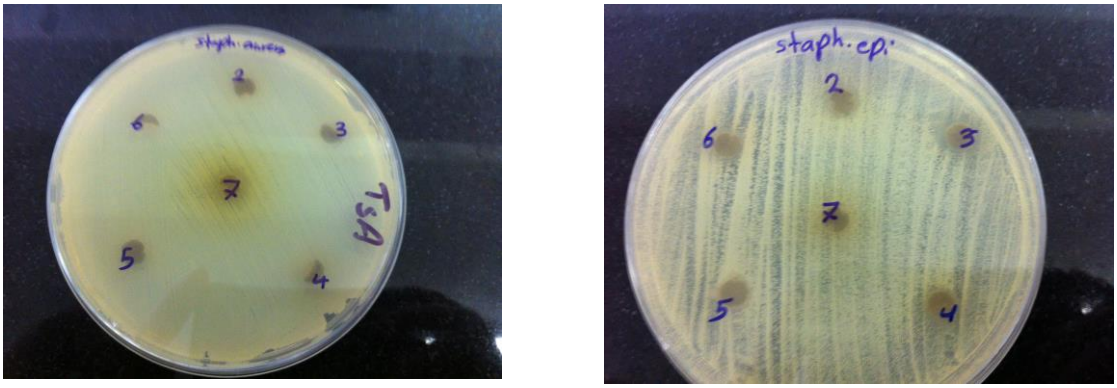

Fig.12. (2-7 numbers) are zone of inhibition of olive leaves samples against S. aureus and S. epidermidis bacteria.

This activity was compared with control positive antibiotic (neomycin) which gave zone of inhibition of about 16 mm. Comparing the zone of inhibition of olive leaves extract in this study with Neomycin zone of inhibition which is a well known antibiotic shows that olive leave extract has about 80-90% of Neomycin antibacterial activity.

However this extract did not show activities against gram negative bacteria.

Table 10: Zone of inhibition (S. aureus) resulting from olive leaf extracted with water (pH 7) and acidified water (pH 3).

Zone of inhibition (mm)		
Sample	**Olive leaf extracts (pH 3)**	**Olive leaf extracts (pH 7)**
north	**12.2**	**13.1**
middle	**12.3**	**13.2**
south	**12.1**	**13.0**

Table 11: Zone of inhibition (S. epiderrmidis) resulting from olive leaf extracted with water (pH 7) and acidified water (pH 3).

Zone of inhibition (mm)		
Sample	**Olive leaf extracts (pH 3)**	**Olive leaf extracts (pH 7)**
north	**14.0**	**14.3**
middle	**14.7**	**14.9**
south	**14.4**	**14.3**

Chapter five

Conclusions

Conclusions

- The Palestinian olive leaves are richer in TPC and TFC than those from other countries particularly Tunisia, Saudi Arabia, Greek, Iran and Turkey.

- The best method of extraction involves the use of water as the extracting solvent and heating to 40 ^{o}C for one hr.

- There is a positive correlation between AA and TPC.
- The concentrations of TPC, TFC and AA are highest in samples collected in June and lower in January.

- On the basis of these findings, it is concluded that olive leaves from Palestine is a rich source of phenolics, flavonoid compounds and constitutes a natural source of potent antioxidants that may prevent many diseases and could potentially be used in food, pharmaceutical, cosmetics formulations as additives, preservatives, antioxidant, …etc. However, it is very interesting to accomplish this study by other interventions to determine optimum method for extraction polyphenolic from olive leaves and also to know more about the different compounds (phenolic acids, flavonoids) responsible for the antioxidant activity, and also to investigate the mechanism of their action in vitro and in vivo.

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