INT. J. BIOL. BIOTECH., 13 (2): 283-291, 2016.

BIOLOGICAL ACTIVITIES OF OLIVE LEAVES EXTRACT FROM NABALI BALADI VARIETY AGAINST LIPID AND PROTEIN OXIDATION

K. Al-Marazeeq[1], M.S.Y. Haddadin[2]*, B. Abdulla[2] and J.S. Haddadin[3]

[1]*Department of Nutrition and Food Technology, Al Huson University College, Al-Balqa' Applied University, Jordan.*
[2]*Department of Nutrition and Food Technology, The University of Jordan, Amman, Jordan.*
[3]*Department of Food and Nutrition Sciences, King Faisal University, Kingdom of Saudi Arabia*.
(* corresponding author: haddadinmaliks@yahoo.com)

ABSTRACT

Polyphenols, flavonoids, and tannins of Nabali Baladi olive leaves were extracted by using four different solvents (80 % methanol, 80 % ethanol, acetone, and distilled water). The total polyphenols were in the range of 158-392 mg gallic acid equivalent (GAE.g^{-1} extract, the total flavonoids were 11-71 mg rutin equivalent.g^{-1} extract, and the total tannins were 8-18 mg GAE.g^{-1} extract. The highest value of extraction was obtained with 80 % methanol and the lowest one was obtained with the distilled water. The antioxidant activity of the 80% ethanol olive leaf extract (OLE) was tested using DPPH (1,1-Diphenyl-2-Picryl-Hydrazyl) free radical scavenging activity, total antioxidant activity in the linoleic acid system, and protein oxidation by measuring the carbonyl content. The free radical scavenging activity of the 40-2800 µg OLE.mL^{-1} ranged between 9.62-93.15 %, with IC50 at 1082.35 µg.mL^{-1}. Inhibition of peroxides formation in the linoleic acid system reached 76.36 % using 2400 µg OLE.mL^{-1} after incubation for 108 hours. Inhibition of protein oxidation was 70.80-97.33 % using 50-2400 µg OLE.mL^{-1}. These results indicate the capacity of the Nabali Baladi OLE against lipid and protein oxidation, as well as the formation of free radicals which were dose dependent.

Keywords: Nabali Baladi olive – Leaves extract - Polyphenols – Lipid oxidation – Protein oxidation.

INTRODUCTION

The 'Nabali baladi' olive variety is the most common olive genotype grown in Jordan, due to its high oil content (20 to 35 %), an oleic acid content of around 67–71% , pickling, and adaptability to the rain-fed condition of Eastern Mediterranean area (IOC, 2012).

Recently, olive tree leaves have attracted increasing concern to the scientific and researchers (Erbay and Icier, 2010; Quirantes-Piné *et al.*, 2013). Olive tree leaves can be classified among the byproducts of the olive trees cultivation and can reside largely in olive mills (10 % of the total weight of olives) and through the pruning of olive trees (Tabera *et al.*, 2004; Xynos *et al.*, 2012). Whereas olive oil is quite popular for its flavor and essential health benefits, the olive leaves extracts (OLEs) are usually related to a wide number of the medicinal allegation (Abaza *et al.*, 2011). Studies concern olive leaf showed that their health general characteristics are assigned to a category of secondary metabolites that they contain, which are biophenols, which display a wealth of both structural variety and variousness of significant activities (Luque de Castro and Japón-Luján, 2006). OLEs hold in phenolic such compounds as flavones, flavonols, flavan-3-ols, substituted phenols and secoiridoids (Aouidi *et al.*, 2012; El and Karakaya, 2009; Farag *et al.*, 2003; Garcia *et al.*, 2000; Meirinhos *et al.*, 2005; Paiva-Martins *et al.*, 2007;Savournin *et al.*, 2001).

Epidemical researches have mentioned that oleuropein presents anti-ischemic, antioxidative, hypolipidemic, antiviral, antimicrobial, antiatherogenic, cardioprotective, antihypertensive and anti-inflammatory characteristics (Andreadou, *et al.*, 2006; Covas, 2007; Singh *et al.*, 2008; Visioli, and Galli, 1998). The reason for this activity is the presence of polyphenol's strong antioxidant power that they are capable of removing free radicals (Shi *et al.*, 2005). Bahloul *et al.*, (2014) have shown that the physical and chemical characteristic of olive leaves may get them interesting for industrial purposes. Ghanbari *et al.*, (2012) have reviewed the medicinal and preservation effect of olive leaves and their extracts.

Almost all parts of the olive tree can contain polyphenolic compounds, but its nature and content differs significantly among different parts. They have significant function in the whole sensory characteristics of foods. A great attention has been paid to natural resources, including olive leaf and olive leaf extracts to be employed in food industries as food additives and functional food materials instead of synthetic chemicals that may induce health problems, because natural ingredients have more uses to promote consumer acceptance, palatable, endurance and period of validity of foodstuffs.

Accordingly, the search for natural food additives, mainly of plant sources, has recently increased in a significant manner (Ghanbari *et al.*, 2012; Naveena *et al.*, 2008). The antioxidative role of olive leaves extracts against lipid and protein oxidation in raw and cooked pork during refrigerated and frozen storages has been evaluated (Botsoglou *et al.*, 2012; Botsoglou *et al.*, 2014a). Hayes *et al.*, 2010 have been studied the impact of adding olive leaf extract to raw and cooked pork patties on lipid oxidation during refrigeration.

The aim of this research was, therefore, to compare the efficiency of different extraction solvents to extract polyphenols, flavonoids, and tannins from olive leaves "Nabali Baladi variety". Furthermore, the goal of the present research was to investigate the antioxidant capacity of the extract against lipid and protein oxidation. The free radical scavenging activity of "Nabali Baladi variety" was also evaluated.

MATERIALS AND METHODS

Plant material

Olive leaf was randomly obtained from the olive trees (Nabali Baladi variety) from a local farm in the northern part of Jordan. 25 years old trees, rain-fed only and lived in an organic way in the last 10 years. The collection of leaves on a reasonable height around the entire circumference of each tree. The collected leaves were kept in special container. Then the leaves were washed to eliminate any dirties, then drying in the dark at room temperature and then grounded (Moulinex Miller, France) (20 mesh).

Extraction of olive leaves

The ground leaves were extracted in deionized water, 80 % acetone, 80 % ethanol, and 80 % methanol at a concentration of 20 % (w/v). The mixtures were mixed at room temperature for 2 hours using a rotary shaker (New Brunswick Scientific, USA) at 180 rpm and then at 37 °C for 15 minutes in an ultrasonic bath (Bandelin Electronic-RK-103 H, Germany). The ground leaves and the extraction solution mixtures were filtered through Whatman no:4 and then through a membrane filter (0.45 µm). Supernatant was concentrated under low pressure at 38°C for 3 h by applying a rotary evaporator (Büchi, RE 121, Switzerland) (Haddadin, 2010).

Total phenolic content

The total content of phenol compounds of Olive leaves extracts (OLE) was measured by using Folin-Ciocalteu method according to Singleton *et al.*, (1999). OLE solutions were made by solving 15 mg of each crude extract in 15 ml dimethyl sulfoxide (DMSO) (Fine-Chem, Mumbai, India). Then, 0.5 ml of each solution was mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich, USA) for 5 min at room temperature, then 2 ml of sodium carbonate solution (7.5 % in deionized water, w/v) were added. Placed the mixture in a dark place for two hours at 25±1 ˚C, and then measured the absorbance at 760 nm (Elico, SL-150, India). The concentrations between 0.01-0.05 $mg.ml^{-1}$ of gallic acid (Sigma-Aldrich, USA) were used for the standard curve. The total content of phenol compounds was expressed as mg gallic acid equivalent.g^{-1} OLE.

Total flavonoids content

The total amount of flavonoids in OLE was evaluated in accordance with the method of Zhishen *et al.*, (1999). To this end, 0.5 ml of each OLE solutions prepared in total phenolic assay was mixed with 0.3 ml of sodium nitrite 5 $g.l^{-1}$ (Labchem, USA), thereafter 0.3 ml of 1 $g.l^{-1}$ aluminum chloride solution (Labchem, USA) was added after 5 minutes. Six minutes later, two ml of 1M sodium hydroxide was poured to the mixture. Total volume was completed to 10 ml with distilled water and sonicated immediately after preparation. Determine the absorbance was achieved by measuring the samples at 510 nm against water blank using UV/Visible spectrophotometer (Elico, SL-150, India). Calibration curve was prepared by preparing rutin solution (Sigma-Aldrich, USA) (0-200 $\mu g.ml^{-1}$). Concentrations were expressed as mg rutin equivalent. g^{-1} of OLE.

Total tannins content

Total tannins were measured according to the method of Folin and Ciocalteu (Tamilselvi *et al.*, 2012). One hundred microliters of each OLE solutions prepared in total phenolic assay were mixed with 7.5, 0.5 and 1 ml of distilled water, Folin phenol reagent and sodium carbonate solution 35 % (m/v) respectively. Then the mixture completed to 10 ml with distilled water. Shaking well the mixture, and keep it at 25±1 ˚C for 30 minutes and then reading the absorbance spectrophotometrically at 725 nm (Elico, SL-150, India). As mentioned above a series of standard solutions of gallic acid were prepared and read against water as a blank. The results were expressed as mg gallic acid equivalent. g^{-1} of OLE.

DPPH free radical scavenging activity

The free radical scavenging activity of OLE on 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma-Aldrich, USA) was assessed as stated by Hsu *et al.,* (2006). One hundred microliters of various concentrations of 80 % ethanol OLE (40-2800 µg crude extract. ml^{-1} 80 % ethanol) was poured over 1.9 ml of 0.1 mM DPPH methanolic solution. The mixture was strongly shaken and left to stand for 30 minutes at 25±1 ˚C, and then reading the absorbance spectrophotometrically at 517 nm against a blank. For positive controls, butylated hydroxytoluene (BHT) (Sigma-Aldrich, USA) and ascorbic acid (Sigma-Aldrich, USA) were used (Duan *et al.,* 2007). The DPPH radical scavenging activity was calculated using the following formula (Chung, 2009):

DPPH radical scavenging activity (%) = $[(1- A_1/A_0) \times 100]$

Where: A_0 and A_1 absorbance of control and of sample, respectively.

Total antioxidant activity in linoleic acid system

A linoleic acid system was used to measure the total antioxidant activity of OLE (Mitsuda *et al.,* 1996). The emulsion of linoleic acid was made by mixing 0.28 g of linoleic acid (Labchem, USA), 0.28 g of Tween-20 emulsifier (Labchem, USA) and 50 ml of phosphate buffer (0.2 M, pH 7.0). The mixture was then run through the homogenizer (Ultra-Turax T25, IKA-Labortechnik, Germany). One hundred microliters of 100, 400, 800, 1600, 2000 and 2400 $\mu g.ml^{-1}$ of OLE in methanol was mixed with linoleic acid emulsion (2.5 ml, 0.2 M, at pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0). To enhance the peroxidation reaction the mixture was left to stand in the dark at 37 ˚C.

The peroxide level was measured based on thiocyanate method by successive addition of 5ml ethanol (75 % v/v), 0.1 ml ammonium thiocyanate (30 % w/v), 0.1 ml sample solution and 0.1 ml ferrous chloride (Labchem, USA) (20 mM in 3.5 % v/v HCl) (Vasuki, R. 2015). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Torolox) (Sigma-Aldrich, USA) was used as positive control. After mixing for 3 minutes, the peroxide values were determined by reading the absorbance at 500 nm, using UV/Vis spectrophotometer (Elico, SL-150, India). The percent of inhibition of peroxide value was determined by the following formula: I (%) = $[(1- A_1/A_0) \times 100]$

Where: I, A_0 and A_1: inhibition, absorbance of control and of sample, respectively.

Protein oxidation assay

The effect of OLE on the oxidation of protein was realized in accordance with the slightly revised method of Ardestani and Yazdanparast, (2007). Bovine serum albumin (BSA) (Sigma-Aldrich, USA) was oxidized by a Fenton-type reaction (Kızıl, *et al.,* 2011). The composition of the reaction mixture (1.2 ml) was the following: (50-2400 µg crude extract. ml^{-1}), potassium phosphate buffer (20 mM, pH 7.4), BSA (4 mg. ml^{-1}), ferric chloride (50 µM) (Labchem, USA), hydrogen peroxide (1 mM) and ascorbic acid (100 µM). The incubation of the mixture was performed for 30 min at 37 ˚C (Kızıl, *et al.,* 2011). The results were expressed as the percentage of inhibition; the control test was without the addition of OLE sample to the reaction mixture. The following formula was used to calculate percentage of inhibition

$I (\%) = A_1/A_0 \times 100$

Where: I, A0 and A1: inhibition, absorbance of control and of sample, respectively.

Statistical analysis

All tests were realized in triplicate. The results are expressed as a mean and standard deviation of three analysis values. Results were undergoing to statistical analysis applying SPSS program (SPSS, version 15 Chicago, IL, USA). The one-way analysis of variance (ANOVA) was employed. Differences between the means of treatments were assessed using Least Significant Differences (LSD) test at $p < 0.05$.

RESULTS AND DISCUSSION

The contents of total phenol, flavonoids, and tannins

Phenolic compounds are secondary metabolites present in plants with beneficial effects assigned to their antioxidant property (Brahmi *et al.,* 2012; Heim, *et al.,* 2002; Randhir, *et al.,* 2004).

Table 1 presents the concentration of total phenolic, flavonoids, and tannins in olive leaf extracts (OLE), obtained with different solvents. Based on the obtained results shown in Table 1, the solvent type significantly ($P \leq 0.05$) influenced the total contents of the polyphenols, flavonoids and tannins extracted from olive leaves. The highest contents ($P \leq 0.05$) of these compounds in the obtained extracts were in the descending order of methanol> ethanol> acetone> deionized water.

The results mentioned that the total quantity of polyphenols, flavonoids, and tannins in the olive leaves extracted by various types of solvents (methanol, ethanol, acetone and deionized water) occurred in the range of 158-392 mg GAE.g^{-1}extract, 11-71 mg rutin equivalent.g^{-1}extract, and 8-18 mg GAE.g^{-1}extract, respectively (Table 1). However, the contents of total phenolic and flavonoid compounds in Nabali Baladi variety were significantly higher than those reported by Abaza *et al.,* (2011) of Chétoui Tunisia olive leaf extracts, which were 16.52-24.93 mg.g^{-1} dry matter and 6.23-21.47 mg.g^{-1} dry matter, respectively. Lee *et al.,* 2009 found that the total amounts of flavonoid extracted by using hexane, chloroform, ethylacetate, butanol, and water fraction were 15, 26, 71, 75, and 19 mg.g^{-1} naringin equivalents, respectively. It can be observed that these results are inferior to those found in this study.

Solubility of phenolic compounds in the extraction solvent is the major factor that influenced the recovery of these compounds. Moreover, solvent polarity has been found to improve phenolic solubility (Naczk and Shahidi, 2006). Ziogas *et al.,* (2010) have been found that olive fruits genotype, period of maturity and the grown altitude are substantial parameters affecting phenolic extract content. In accordance to consumer acceptance, ethanol and water extracts are generally used in food applications because these solvents are not toxic and have not harmful side effects. As the water extracted less phenolic compounds compared to other solvent as shown in Table 1. Methanol is known to be toxic to human; therefore, its extract is not acceptable to be used in food although of its ability to extract high contents of phenolic compounds. Therefore, the choice of 80% ethanol to be used as an extractor in this study based on its high extractability and non-toxicity.

Determination of antioxidant activity of OLE

DPPH radical scavenging activity

Many researchers have been widely applied DPPH radical activity to determine the ability of free radicals scavenging of different types of natural product (Ljubuncic *et al.,* 2005). The concentration that can engender a reduction in the initial DPPH concentration by 50 % is designated as IC_{50} (Prior *et al.,* 2005).

Figure 1 shows the percent of DPPH radical scavenging capacity with BHT and ascorbic acid as references. The obtained results shown that at different concentration levels, OLE seems to have scavenging property. It can also be observed that a dose–dependent was found in the DPPH radical scavenging capacity; the activity increased with increasing concentration of OLE (Figure 1). The results in Figure 1, also indicated that ascorbic acid has greater free radical scavenging activity than that of OLE and BHT and that's goes for all the used concentrations. It was also noted that OLE at concentrations of 1400 µg.ml^{-1} and more start gaining high free radical scavenging activities. The IC_{50} value of OLE reached at concentration of 1082.35 µg.ml^{-1}, while IC_{50} values of BHT and ascorbic acid were 825.86 µg.ml^{-1} and 285 µg.ml^{-1}, respectively. These results were similar to those obtained by Abaza *et al.,* (2011) for Chétoui Tunisia olive leaf extracts. They found that the IC_{50} values of DPPH varied from 170 to 970 µg.ml^{-1}. It is important to note that with the increase in the value of IC50 accompanied by a decrease in antioxidant activity.

Although OLE was used as a crude extract comparing to the pure positive controls; BHT and ascorbic acid, the free radical scavenging activity of OLE at higher concentrations were comparable to that of BHT and ascorbic acid. These observations support the idea that the OLE possesses free radical scavenging activity. Brahmi *et al.,* (2012) have shown that phenolic compounds can directly participate in an antioxidant activity. They also mentioned that olive leaves could be considered as a major source of bioactive phenolic compounds in comparison to olive oil and fruits. This observation is in harmony with other report (Turkoglu *et al.,* 2007). In fruits a good relationship was found between free radical scavenging activity and the phenol amounts (Gao *et al.,* 2000; Kalt *et al.,* 2003; 2000; Wang and Lin, 2000; Wang *et al.,* 2008). Brewer, (2011) reported that phenolic antioxidants able to inhibit the generation of free radicals and / or to interrupt the propagation of autoxidation.

Plant extract containing phenolic compounds and flavonoids frequently has H-denoting activity, thereby rendering them extremely effective antioxidants. Generally, phenolic acids act as antioxidants by scavenging free radicals, while flavonoids are efficiently trapping both free radicals and chelate metals (Brewer, 2011). Moreover, the phenolic compounds scavenging activity seems to rely on the configuration of both numbers and location of free –OH groups. The phenolic compounds that contain more than one –OH groups are more effective than the ones with mono- hydroxyl group (Brewer, 2011). The results of this study confirm that at lower concentration, the OLE possesses less DPPH radical scavenging activity than that of ascorbic acid. Whereas, at higher concentration it presented a stronger scavenging activity. Consequently, the OLE might play the role of a natural antioxidant.

Total antioxidant activity in linoleic acid system

Figure 2 presents the antioxidant capacity of OLE in linoleic acid system using the ferric thiocyanate method (FTC). The formation of peroxides was detected in the course of linoleic acid peroxidation and thereof the oxidation of ferrous iron to ferric iron was occurred. The ferric ion constitutes complex compounds due to the interaction with

SCN^-. These formed compounds have optimum absorbance at 500 nm (Benzie and Strain, 1996). Thus, a high absorbance value indicates that there is a formation of high peroxide in the course of incubation of the emulsion.

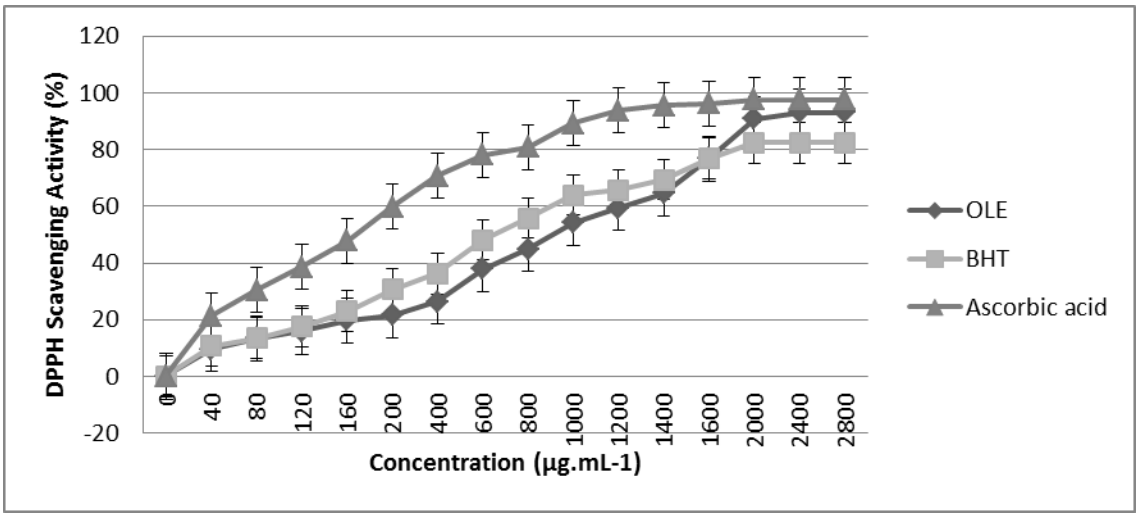

Fig. 1. Scavenging activity of olive leaf extract. Results are mean and standard deviation of triplicates. Significant differences at $P<0.05$.

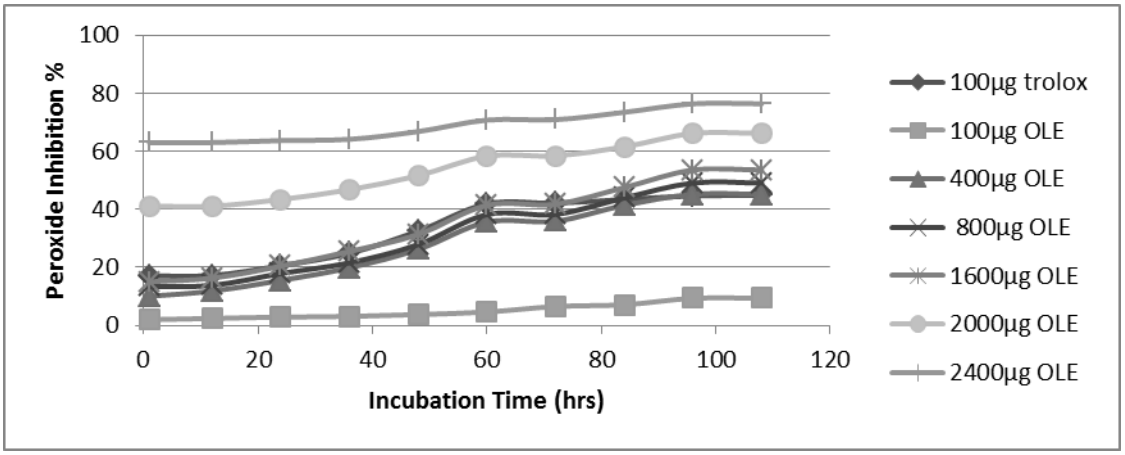

Fig. 2. Antioxidant activity in linoleic acid system of studied OLE at different concentrations. Results are mean and standard deviation of triplicates. Significant differences at $P<0.05$.

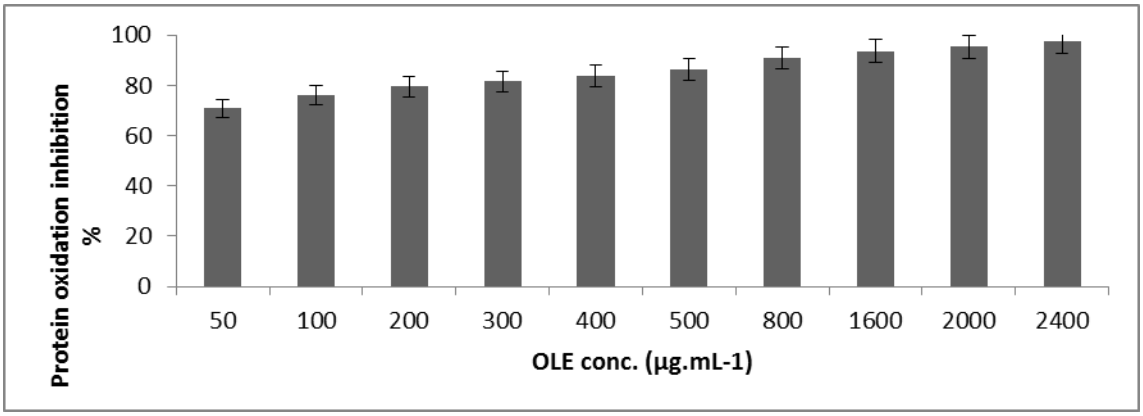

Fig. 3. Antioxidant activity of different OLE concentrations against protein oxidation. Results are mean and standard deviation of triplicates. Significant differences at $P<0.05$.

Table 1. Total polyphenols, total flavonoids, and total tannins extracted from olive leaves using different solvents.

Solvents	Total polyphenols ($mg.g^{-1}$ extract)	Total flavonoids ($mg.g^{-1}$ extract)	Total tannins ($mg.g^{-1}$ extract)
Methanol	392 [a]*	71 [a]	18 [a]
Ethanol	335 [b]	54 [b]	14.5 [b]
Acetone	196 [c]	22 [c]	10 [c]
Water	158 [d]	11 [d]	8 [c]

* Means within the same column with different superscript letters have significant differences using LSD ($p \leq 0.05$).

It can be observed from the results in Figure 2 that the samples with 400, 800 and 1600 $\mu g.ml^{-1}$ of OLE and the control sample followed the same kinetic profile. It is also noted that the reference sample and that with 400 $\mu g.ml^{-1}$ of OLE have reached to a maximal value of peroxide inhibition of 45.18 % after 96 hrs. The highest peroxide inhibition was obtained with 2400 $\mu g.ml^{-1}$ of OLE in methanol after 96 hrs and then followed by OLE at a concentration of 2000 $\mu g.ml^{-1}$ of OLE in methanol. However, the peroxide inhibition of OLE at concentration 100 $\mu g.ml^{-1}$ of OLE in methanol was less effective than that of trolox and the other concentrations of OLE.

Baladi olive leaf extracts contained appreciable amounts of polyphenols, total flavonoids, and total tannins extracted (Table 1) which may increase its total properties such as antioxidant, DPPH radical scavenging and retardation of lipid oxidation. The results of this study indicate that OLE from Nabali Baladi variety can remarkably restrain the peroxidation of linoleic acid and lower the hydroperoxide development; this suggests that these plant extracts are potent natural antioxidants due to the presence of bioactive phenolic compounds. The results of Maqsood and Benjakul, (2010) support the findings of this study. They studied the role of various phenolic compounds in the prevention of lipid oxidation and fishy odor developments in fish and fish products; they found that tannic acid shown the highest DPPH radical scavenging capacity and retardation of lipid oxidation among the tested phenolic compounds.

Inhibition of protein oxidation

Proteins and lipids oxidations are the main cause of chemical deterioration in food. During food processing and handling, free radical mediated oxidation of proteins and lipids result of reactive oxygen species (ROS) are generated (Davies *et al.,* 1995; Stadtman and Levine, 2003). Oxidation of protein results in the amino acids loss, texture modification, solubility, changing in the function of protein and can even stimulate the formation of toxic compounds (Karel *et al.,* 1975; Rice-Evans and Burdon, 1993). The results in Figure 3, presents the antioxidant activity of the OLE against protein oxidation. It can be noted that the extent of lipid oxidation inhibition increased progressively with rising concentrations of the OLE.

The percentage of inhibition raised to from 70.80 to 97.33 % when the concentration of OLE increased from 50 to 2400 $\mu g.ml^{-1}$, respectively. Consistent with the findings of this study, other researchers mentioned that polyphenols possess an inhibitory effect against the oxidation of protein, while the formation of protein carbonyls in cured pork was found to be inhibited by vitamin E supplementation. (Ventanas *et al.,* 2006; Vuorela *et al.,* 2005). Botsoglou *et al.,* (2014b) have recently demonstrated that a supplementation of olive leaves extract at 200 mg $GAE.kg^{-1}$ decrease lipids and proteins oxidation. Because of inhibition of oxidation process improvement in sensory properties has appeared. Solar-Cantero *et al.,* (2012) have mentioned that a food supplied with extract rich in phenolic compounds reduced proteins oxidation of plasma and lipid deterioration.

CONCLUSION

In the light of the above results, it is concluded that the addition of olive leaves extract of Nabali Baladi variety exhibited a good antioxidant activity for delaying lipid and protein oxidation, as well as the formation of free radicals. These results add to the other findings that support that these extracts have a prominent effect on health and should be seen as an important element of the health and longevity program. Understanding the correlation between polyphenolic substances from one side and proteins and lipids from the other side may result in the elaboration of novel, effective and multifunctional antioxidant policies which might be applied in the different industrial sectors such as food, cosmetic and pharmaceutical.

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(*Accepted for publication March 2016*)