Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(3):171-177

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Antioxidant activity of olive mill wastewater extracts and its use as an effective antioxidant in olive oil; kinetic approach

Asma Yangui[1,2], Mohamed Hedi Abessi[1,2], Manef Abderrabba[1]

[1]Laboratory of Materials Molecules and Applications, Preparatory Institute for Scientific and Technical Studies, Carthage University, B.P:51, La Marsa, 2075 Tunis, Tunisia
[2]Chemistry Department, University of Sciences of Tunis, El Manar University, B.P 248 El Manar II, 2092 Tunis, Tunisia

---

ABSTRACT

The production of olive oil generates huge quantities of by-product called olive mill wastewater (OMW), which poses serious environmental problems. This effluent contains several polyphenols. In this work, we have studied the liquid-liquid extraction on olive mill wastewater using ethyl acetate. By a colorimetric assay, we 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. To conclude, our data 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.

Keywords: Olive Mill Wastewater OMW, phenolic compounds, antioxidant activity, kinetic behavior, DPPH method.

INTRODUCTION

Virgin olive oil, extracted from fresh and mature olives without any refining process, conserves natural constituents ensuring a great biological interest. Therefore, the consumption of olive oil is not limited, as known, on the Mediterranean regions, but it is expanding to other countries worldwide [1-3].

To produce Olive oil of high quality, traditional discontinuous press or a continuous centrifugation (two phase and three-phase systems) are used. In both methods, the process generates two residues: a solid (pomace) and a liquid (Olive Mill Wastewater OMW) wastes. OMW once thrown into rivers and sewers without any treatments poses serious environmental problems. Their harmful effects are largely derived from their content of phenolic compounds, which may inhibit the growth of microorganisms [4]. Pursue the big interest for the environment; it is consequently conceivable to recover phenolic compounds without distortion, to develop this potential resource [5]. In fact, biophenols [6] in olive mill wastewater highlighted a great protection for health resulting from their vegetable origin [7, 8]. They are characterized by a big role as antioxidants revealed from their hydroxyl substituents and aromatic structure; what gives them the property to scavenge free radicals, which are the reason of oxidative damage of biomolecules such as nucleic acids, proteins and lipids [9-12].

Given the complexity of the oxidation process and the diverse nature of antioxidants, there is not a universal method by which the antioxidant activity can be measured quantitatively in a specific way. From the methodological point of view, the test free radical DPPH• is an easy method for the determination of antioxidant activity, this reagent is

available in the commerce, stable and can be used and stored easily. It reacts with a hydrogen or an electron donor compound present in the solution. This method is based on the measurement of the absorbance [13] that decreases with the increase of quantity of antioxidants in the solution. This technique is very employed for the determination of antioxidant activity of some foodsrich in phenolic compounds such as virgin olive oil [14] and extracts such as cocoa and rosemary [15], tea [16,17], coffee [16], olive leaves [17], olive mill wastewater [18-20]. The objectives of this paper were to verify the kinetic approach of the antioxidant activity of extracts obtained from OMW as a source of natural antioxidants added with different proportion to olive oil.

EXPERIMENTAL SESSION

2.1. Materials

The 2,2-diphenyl-1-picrylhydrazyl DPPH•, hexane, methanol and ethyl acetate were purchased of high purity from Sigma Aldrich. Ascorbic acid, butyl-hydroxy-toluene (BHT), Sodium carbonate anhydrous Na2CO3 and Folin Ciocalteu's phenol reagent were bought from Fluka.

The Olive Mill Wastewater OMW was engendered by an oil factory using three-phase centrifugation processes from Tunisia. It was collected directly after pressing, filtered using GF/F filter paper Buchner funnel, and stored at 4°C for analyses. Commercially virgin olive oil was used for this study.

UV–VIS spectrophotometer (Aquarius CECIL CE 7400) characterized by high accuracy and stability with time was used for measuring the absorbance for all experiments.

2.2. Extraction of phenolic compounds from OMW

A liquid-liquid extraction of the OMW was performed with ethyl acetate. Briefly, a sample of OMW was acidified with concentrated HCl to pH 2 and then was extracted with hexane (1:1) twice for an hour to remove fat. The extraction of phenolic compounds was performed five times with a ratio of 1:2 (v/v) with ethyl acetate [20]. The phenolic extracts were reserved at 4°C for further analysis.

2.3. Total phenolic contents in OMW fractions

The total phenolic compounds were determined using the method of Folin & Ciocalteu [21]. Solutions of Gallic Acid (GA) were prepared (60, 120, 180, 240 and 300 mg L^{-1}) for the calibration curve. The results were expressed as g of GA equivalents per liter of olive mill wastewater. Briefly, 100 µL of diluted extracts or standards were mixed with 6 mL of distilled water, 500 µL of Folin & Ciocalteu's reagent and 1.5 mL of Na_2CO_3 (20% in water), the solution was then adjusted to 10 mL with distilled water and stirred vigorously , after two hours of incubation, the absorbance was measured at 760 nm.

2.4. Determination of antioxidant activity

For the evaluation of the antioxidant activity of samples, The DPPH• method was used according to reference [22]. Five concentrations of DPPH• radical were daily prepared in order to check the linearity of response and a linear regression (R^2=0.997) was established.

For antioxidant activity of samples and standards, 25 mg L^{-1} solution of DPPH• was freshly prepared in methanol and protected from light. A series of diluted fractions of OMW with known concentrations were prepared and 0.1 mL of each solution was added to 3.9 mL of DPPH• solution. The mixtures were incubated 30 min and the absorbance was recorded at 517 nm using Ascorbic acid and BHT as references. The absorbance of DPPH (25 mg L^{-1}) in methanol was, according to our experiments, in all the cases close to 0.700 nm. The concentration EC_{50} required to scavenge 50% of DPPH solution was expressed in the following equation as;

$$\text{Scavenging Activity (\%)} = (A_{control} - A_{sample}) / A_{control} \times 100$$

$A_{control}$ is the absorbance of the control at t = 0 min and A_{sample} is the absorbance of the antioxidant at t = 30 min. EC_{50} is expressed as gram of antioxidants needed to scavenge one Kg of DPPH• (g of antioxidants / Kg of DPPH•) in the assay medium.

Reactions Kinetics of Ascorbic acid and OMW extracts were registered at 0, 1, 2, 3, 5, 10, 15, 20, 25 and 30 minutes. The percentage of DPPH• remaining was expressed in the following equation as;

$$\text{\% of DPPH• remaining} = A_f / A_0 \times 100$$

A_0 and A_f are the absorbance at 517 nm of the radical at the beginning and at any instant t, respectively.

2.5. Ultrasonic enrichment of olive oil with OMW extracts

To investigate the effect of phenolic additives, a series of olive oil enriched with phenolic fractions of OMW was made in the proportion of 1%, 0.1% and 0.01%. We proceed first to weigh in a dry bottle OMW extracts, then we supplement with the appropriate volume of olive oil. All samples were well shaken and placed in an ultrasonic bath until complete dissolution of the extract.

2.6. Antioxidant activity in olive oil

Usually, methanol is the comment solvent for the determination of the antioxidant activity of DPPH• method in polar fraction. Moreover, ethyl acetate is used in the case of non-polar fraction such as vegetable oils [23]. Hence, the same experiments of section 2.4 were repeated using ethyl acetate as solvent for the determination of the antioxidant activity of olive oil and olive oil enriched with phenolic extracts with different proportion (1%, 0.1% and 0.01%). The EC_{50} values were expressed as g of antioxidants / g of DPPH• in the assay medium.

2.7. Data analysis

Results were expressed as Mean ± Standard Deviation (SD) of three experiments for each antioxidant; EC_{50} values were expressed as 95% confidence interval. Data analysis was performed using a statistical program, Graph Pad Prism, version 6.04. Briefly, values were log-transformed and normalized, and nonlinear regression analysis was used to generate a sigmoidal dose–response curve. This program was selected the most efficient program to calculate the percentage of scavenging activity in a recent study [24].

RESULTS AND DISCUSSION

3.1. Content of phenolic compounds

Olive mill wastewater was acidified objectify to increase the solubility of phenolic compounds in organic solvents to allow the extraction of the maximum quantity of polyphenols [6] and to promote the hydrolysis of long chains of phenolic compounds to phenolic monomers [4]. Crude olive mill wastewater contains 8.21 g L^{-1} of total phenolic compounds. Brown red viscous liquid resulting from the extraction process was recovered. Liquid-liquid extraction using ethyl acetate as solvent provides 2.85 g GA equivalents/L of OMW. The phenolic yields attained by using ethyl acetate are about 34.7 %. Our results were found to be in agreement with those reported byresearchers [21]. The solvent used in the liquid extraction process, the initial pH and the extraction time make the comparison of the total phenol composition of olive mill wastewater very varied in the literature. Other factors are also responsible for this variation (1) the olive variety, the method of production of olive oil (continuous or discontinuous process), (2) the climatic conditions, (3) the use of fertilizers and pesticides and the time of picking and ripening of olives [25]. Content of polyphenols in OMW reported in previous studies are summarized in Table 1.

Table 1-The content of Polyphenols (g L^{-1}) of OMW according to some authors

Polyphenols (gL^{-1})	References
2.5	[4]
10.7	[26]
0.98	[27]
3.8	[28]
1.6	[39]
7.8	[30]
8.6	[31]
3.22	[32]

3.2. Antioxidant activity

DPPH• assay is a consistent method to determine the scavenging activity of biological compounds. The EC_{50} is a characteristically employed parameter to define the antioxidant activity and to compare the capacity of different compounds to scavenge free radicals. In this study, a specific computer program GraphPad Prism analysis was performed to calculate this parameter.

Several researchers have been interested to determine the antioxidant activity of phenolic compounds by DPPH• radical scavenging method and expressed their results as the depletion of free radical to 50% with different units. In some studies, the inhibitory concentration EC_{50} was expressed as a function of the initial concentration of antioxidant and in other studies as the concentration of the antioxidant responsible to scavenge free radicals when introduced in the assay medium. Because of our attention to distinguish the inhibitory concentration by 50% for different antioxidants, we have made a review of literature and observed several procedures using the DPPH radical assay. The comparison between values reported in previous studies will lighten the efficiency of olive mill waste compared to known antioxidants. We have considered maintaining the values of EC_{50} reported in literature to the

same unit (g of antioxidant / kg of DPPH). For instance, volume, initial concentration of DPPH radical and original units were taken into account (Table 2).

Table 2- List of antioxidants with EC50 concentrations obtained from various reported studies

Ant	EC_{50}		References
	Original unit	Reported values[b]	
BHT	15.2±1.1g ant/Kg DPPH•	131.66	[19]
	0.89 µg/mL	7.52	[33]
	0.0129±0.0005 mg/mL	11.28	[34]
	36.1 µM	201.73	[37]
	0.89 µg/mL	7.52	[39]
	-	141.4±8.818[a]	This study
Ascorbic Acid	158±14.3g ant/Kg DPPH•	12.66	[19]
	121g ant/Kg DPPH•	121	[22]
	11.8±0.2 10^{-6} mol/L	2.13	[35]
	76±7g ant/Kg DPPH˙	76 ± 7	[36]
	10.2 µM	46.89	[37]
	0.216mol ant/mol DPPH•	96.47	[38]
	-	109.4±5.895[a]	This study
Phenolic Extracts	169±5.2 µg/mL	140.83	[19]
	1.2 µg/mL	10.4	[39]
	-	124.2±3.987[a]	This study

[a] *Values expressed as mean ± standard deviation of three measurements; ant – antioxidants.*
[b] *Values are expressed in g ant/Kg DPPH• in the assay medium.*

EC50 is contrariwise correlated with the scavenging activity of a compound, as it defines the amount of antioxidant required to decrease the radical concentration by 50%. The inhibitory concentration by 50% values are particularly diversified, actually, the phenolic extract obtained from olive mill wastewater is the potent scavenging one 124.2 ± 3.987 g antioxidants / kg DPPH• compared with BHT 141.4 ± 8.818 g antioxidants / kg DPPH• in the assay medium (Fig.1). The result obtained for the extract in this work is in accordance with some authors [19].

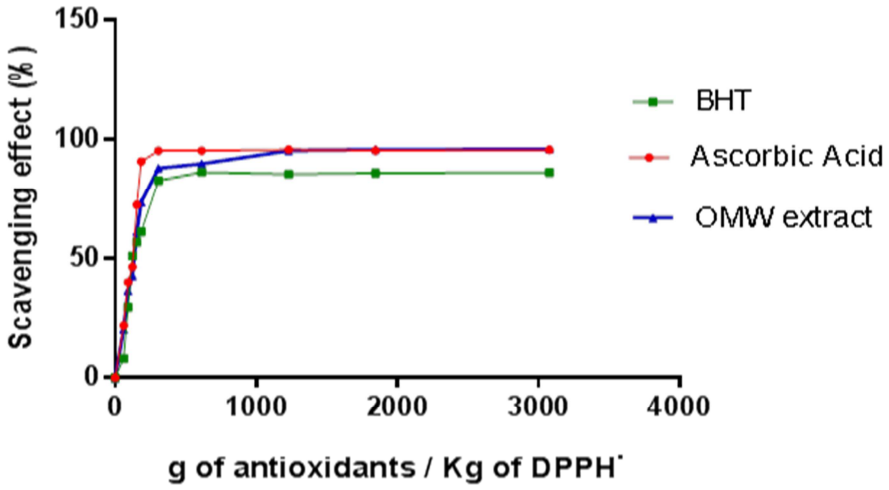

Figure 1- Influence of the concentration of antioxidants on the scavenging effect (%)

3.3. Kinetic study

DPPH• reacts with phenolic compounds ArOH trough two mechanisms [40];

1) A direct abstraction of a hydrogen H from phenol:

ArOH + DPPH• → ArO• + H-DPPH

2) An electron transfer process

ArO• + DPPH• → products

Generally, in non-polar solvent, the first reaction is predominant, but in polar solvent such as methanol, capable to form strong hydrogen bond, the second reaction becomes of wide interest [35]. The evolution of the different reaction kinetics depends on the nature of the antioxidant being tested. Three types of behavior were reported by [22] a rapid, an intermediate and a slow kinetic behavior. In our study, for standard antioxidant and OMW extracts, the reaction is instantaneous and fast. The change of color from purple to yellow, which indicates the passage of DPPH form radical (DPPH •) to (DPPH-H) takes place in an extremely short time where the equilibrium state is

reached immediately and reduction is almost complete. Ascorbic acid and OMW extracts own a rapid kinetic behavior. In fact, the reactions present two phases, with a fast decrease in absorbance at the beginning of reaction, followed by a slower step up to equilibrium. The absorbance versus time plots show a fast initial phase expressed the decrease of the DPPH• absorbance followed by a slow phase expressed the disappearance of DPPH• (Fig 2, 3). The steady state was fast reached for Ascorbic Acid and OMW extracts and not requires a long time to reach equilibrium (1-3).

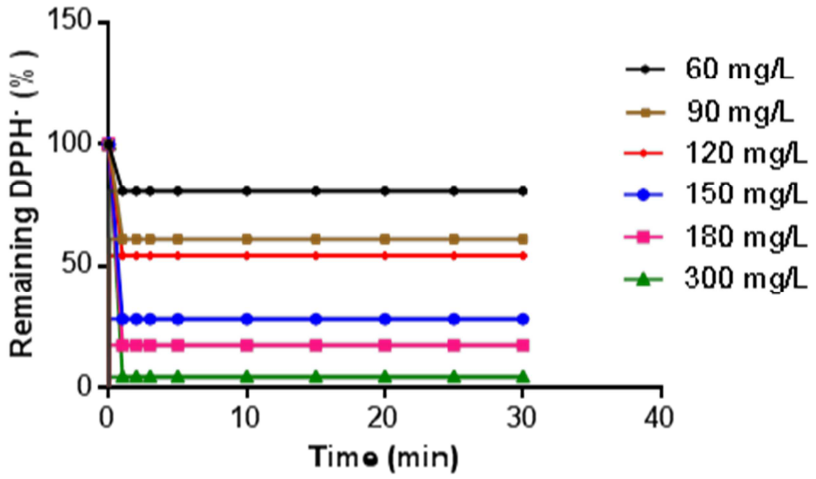

Figure 2-Spectrophotometric recording for the remaining DPPH• in the presence of Ascorbic acid

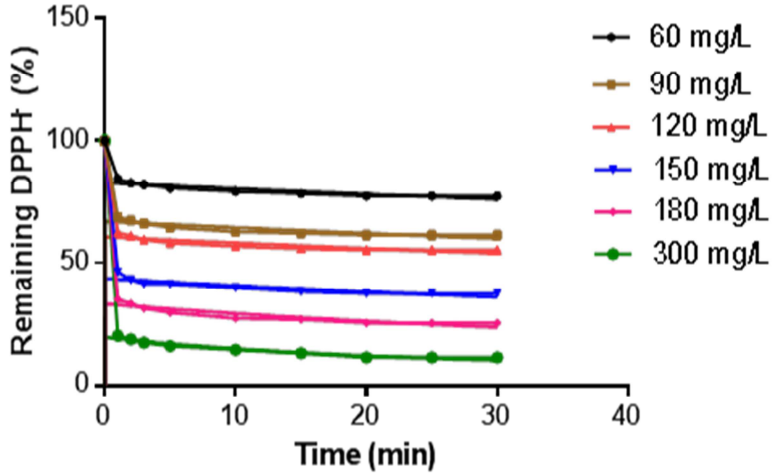

Figure 3-Spectrophotometric recording for the remaining DPPH• in the presence of OMW extracts

3.4. Enrichment of olive oil

Olive oil, an elementary ingredient in the Mediterranean diet, offers potentially beneficial effects, preventing certain diseases and enhancing general human health due to the presence of antioxidants. The inhibitory concentration of virgin olive oil (439.7 ± 8.4 g antioxidants /g DPPH•) reported in this study is slightly lower than values reported by [12] consequently it promotes a higher antioxidant ability. This difference can maybe due to variety of olives and origin of oil. The inhibitory concentrations of olive oil decrease regularly with the addition of phenolic extract (Fig. 4). The exploitation of reported results (Table 3) shows that if the proportion of a phenolic extract increases, the inhibitory concentration decreases in all experiments. Therefore, it is established that OMW extract is able to stabilize olive oil and can replace frequently used synthetic antioxidants [33]. Per consequence, incorporation of extracts derived from olive mill wastewater in food industry mainly in olive oil may contribute health benefits and protect the human body from oxidation damages.

Free radicals generated by metabolic processes in the living systems can damage biomolecules and modify their functions, which can lead to cellular degeneration. Natural antioxidants can reduce and prevent the bad effects of free radicals in cells.

Nowadays, there is a growing interest in the natural antioxidant compound versus synthetic ones because they can cause detrimental effects on human health. Antioxidant compounds extracted from OMW are demonstrated to have a great antioxidant activity. This originality is very interesting because they can be added to foods and oils to prevent the formation of toxic complexes by donating a hydrogen atom to the lipid radical. The autoxidation of foods through free radical reaction received important attention and the introduction of antioxidant can protect and extend the duration of life of foods.

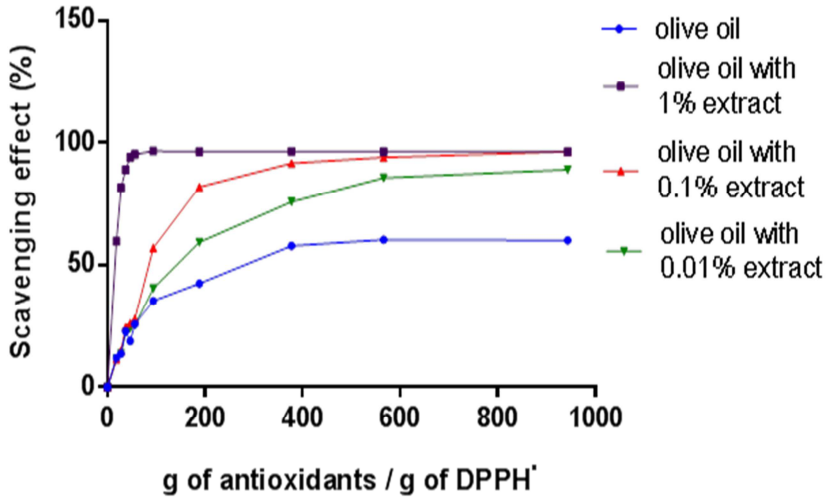

Figure 4-Influence of the concentration of additive antioxidants on the scavenging effect (%) of olive oil

Table 3-list of EC_{50} for enriched olive oil

Antioxidants	EC_{50} (g antioxidants / g DPPH•)
Olive oil	316.4 ± 3.777
Olive oil with 0.01% of extract	133.1 ± 1.577
Olive oil with 0.1 % of extract	84.09 ± 3.058
Olive oil with 1 % of extract	15.93 ± 2.445

CONCLUSION

The results reported in this study attest that the extracts obtained from olive mill wastewater contain potent phenolic compounds which own a high antioxidant activity to scavenge free radical and a fast kinetic behavior similar to Ascorbic acid (Vitamin C) illustrated by DPPH• test.

Antioxidants have an important role in human nutrition as preventive agents against various diseases and for tissue protecting of the body against oxidative stress. The extraction of these compounds from vegetation water can lead to a natural source of growing interest in pharmaceutical and food industries. Therefore, data reported for the enrichment of olive oil with OMW extracts prove that antioxidants from OMW can stabilize oil from autoxidation up to a greater amount than employed synthetic antioxidant and improve the ability of the Olive oil as preventive care for many diseases related to oxidative stress, supported by several research and clinical studies [41]. To conclude, this work confirms the interest of olive oil residues as a natural and cheap source of natural antioxidant, which can stop or limit the damage caused by free radicals and by synthetic antioxidants used in food industries.

Acknowledgement
We thank the support of the Higher Education Ministry, Carthage and El Manar Universities.

REFERENCES

[1] FD Gustone. Production and trade of vegetable oils, In F. D. Gustone (Ed.),Blackwell Publishing, USA,**2002**, 11.
[2] D Boskou, Culinary applications of olive oil-Minor constituents and cooking, In D. Boskou (Ed.), CRC PressINC, USA, **2009**,1-4.
[3] DL García-González; R Aparicio-Ruiz; R Aparicio,*Eur. J. Lipid Sci. Technol*.,**2008**, 110, 602-607.
[4] E Marco; M Savarese; A Paduano; R Sacchi,Food Chem.,**2007**, 104, 858-867.
[5] W Dhifi; F Angerosa; A Serraiocco; I Oumar; I Hamrouni; B Marzouk,*Food Chem*.,**2005,** 93, 697-701.
[6] HK Obied; MSAllen; DR Bedgood; PD Prenzler; K Robards; R Stockmann,*J. Agr. Food Chem*.,**2005**, 53, 823-837.

[7] LA Bazzano; J He; LGOgden; CM Loria; SVupputuri; L Myers; PK Whelton,*Am. J. Clin. Nut.*,**2002**, 76(1), 93-109.
[8]L Le Marchand; SP Murphy; JH Hankin; LR Wilkens;LN Kolonel,*J. Natl. Cancer Inst.***2000**, 92, 154-160.
[9] OI Aruoma,*J. Am. Oil Chem. Soc.*,**1998**, 75, 199-212.
[10] MF Ramadan;JT Moersel,*J. Food Comp. Anal.*,**2006**, 19, 838-842, 2006.
[11] E Tripoli, M Giammanco, G Tabacchi, D Di Majo, S Giammanco, M La Guardia,*Nutr. Res. Rev.*,**2005**, 18, 98-112.
[12] S Arranz; R Cert; J Pérez-Jiménez; A Cert; F Saura-Calixto,*Food Chem.*,**2008**, 110, 985-990.
[13] RL Prior; X Wu; K Schaich,*J. Agr. Food Chem.*,**2005**, 53, 4290-4302.
[14] C Samaniego-Sánchez; AM Troncoso-González; MC García-Parrillab; JJ Quesada-Granados; H López García de la Serrana; MC López Martínez,*Anal. Chim. Acta*.,**2005**, 593, 103-107.
[15] M Bubonja-Sonje; J Giacometti; M Abram,*Food Chem.*,**2011,** 127(4), 1821-1827.
[16] J Anissi; M El Hassouni; A Ouardaoui; K Sendide,*Food Chem*., **2014,** 150, 438-447.
[17] R Malheiro; N Rodrigues; G Manzke; A Bento; JA Pereira; S Casal,*Ind. Crops Prod*.,**2013**, 44, 37-43.
[18] J He; M Alister-Briggs; T de Lyster; GP Jones,*Food Chem.*,**2012,** 131, 1312-1321.
[19] A El-Abbassi; H Kiai; A Hafidi,*Food Chem.*,***2012***, 132, 406-412.
[20] H Azaizeh; F Halahlih; N Najami; D Brunner; M Faulstich; A Tafesh,*Food Chem*.,**2012**, 134, 2226-2234.
[21] VL Singleton; R Orthofer; RM Lamuela-Raventos, *Meth. Enzymol*.,**1999**, 299, 152-178.
[22] W Brand-Williams; ME Cuvelier; C Berset,*Food Sci. Technol*.,**1995**, 28, 25-30.
[23] JC Espín; C Soler-Rivas; HJ Wichers,*J. Agr. Food Chem.*,**2000**, 48, 648-656.
[24] Z Chen; R Bertin; G Froldi, *Food Chem.*,**2013**, 138(1), 414-420.
[25] AS Erses; HV Oral; TT Onay; O Yenigün,*Food Chem.*,**2012,** 60, 64-71.
[26] AG Vlyssides; M Loizides; PK Karlis,*J. Clean. Prod*.,**2004**, 12, 603-611.
[27] S Filidei; G Masciandaro; B Ceccanti,*Water Air Soil Poll*., 145,**2003,**79-94.
[28] ES Aktas; S Imre; L Ersoym,*Water Res*.,**2001,** 35 (9), 2336-2340.
[29] CI Piperidou ; CI Chaidou ; CD Stalikas ; K Soulti ; GA Pilidis ; C Balis,*J. Agr.Food Chem.*,**2000,** 48, 1941-1948.
[30] C Paredes; J Cegarra; A Roig; MA Sánchez-Monedero; MP Bernal,*Biores. Technol.*,**1999**, 67, 111-115.
[31] G Iamarino; MA Rao; L Gianfreda,*Chemosphere*, **2009**, 74, 216-223.
[32] A Mekki; A Dhouib; F Aloui; S Sayadi, *Agro. Sustain. Dev*.,**2006**, 26, 61-67.
[33] M Bouaziz; I Fki; H Jemai; M Ayadi; S Sayadi, *Food Chem*.,**2008**, 108(1), 253-262.
[34] P Terpinc; M Bezjak; H Abramovič,*Food Chem*.,**2009**, 115, 740-744.
[35] D Villaņ̃o; MS Fernàndez-Pachón; ML Moyá; AM Troncoso; MC García-Parrilla,*Talanta*,**2007**, 71, 230-235.
[36] C Sánchez-Moreno; JA Larrauri; F Saura-Calixto,*J. Sci. Food Agr*.,**1998**, 76, 270-276.
[37] K Mishra; H Ojha; NK Chaudhury,*Food Chem*.,**2012**, 130, 1036-1043.
[38] R Lo Scalzo, *Food Chem*.,**2008,**107, 40-43.
[39] I Fki; N Allouche; S Sayadi,*Food Chem*.,**2005**, 93, 197-204.
[40] MC Foti; C Daquino; C Geraci, *J. Org. Chem*.,**2004,** 69, 2309-2314.
[41] MI Covas, *Pharmacol Res*.,**2007**, 55, 175-186.