Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com
J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(5): 390-394
Received: 22-07-2016
Accepted: 23-08-2016

Zam Wissam
Al-Andalus University for Medical University, Faculty of Pharmacy, Department of Analytical and Food Chemistry

Ali Ali
(a) Al-Andalus University for Medical Sciences, Faculty of Pharmacy, Department of Basic Sciences
(b) Tartous University, Faculty of Technical Engineering, Department of Food Technology

Hasan Rama
Tartous University, Faculty of Technical Engineering, Department of Food Technology

Correspondence
Zam Wissam
Department of Analytical and Food Chemistry, Faculty of Pharmacy, Al-Andalus University for Medical Sciences, Al-Quadmous, Tartous, Syrian Arab Republic

Optimization of extraction conditions for the recovery of phenolic compounds and antioxidants from Syrian olive leaves

Zam Wissam, Ali Ali and Hasan Rama

Abstract
In this study the effect of extraction conditions, solvent (type, composition), time and temperature, on the amount of phenolic compounds extracted from olive leaves obtained from Safita /Syria was investigated and the radical scavenging activity of these extracts was evaluated. The total phenolic content and the radical scavenging activity of olive leaves extracts were determined by Folin-Ciocalteu reagent and scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, respectively. It was found that concentration of solvents (40% ethanol, 40% methanol, 80% acetone, and 6:2: acetone: ethanol: water) gave the highest total phenolic content and possess strong activity against the DPPH radicals (IC_{50} 18.92, 25.01, 33.15, 37.19 µg Gallic acid /ml), respectively. Temperature and time of extraction were also found to have a significant effect on the total phenolic content and IC_{50}. The aqueous solutions of ethanol 40%, extraction temperature of 60 °C and extraction time of 120 min were the most efficient for the extraction of polyphenols from olive leaves.
Therefore, these conditions could be applied for further extraction and isolation of phenolic compounds from olive leaves for their use in the preparation of medicinal products or functional food.

Keywords: Phenolics; natural antioxidants; extraction systems

1. Introduction
It has been known that free radicals generated in vivo can cause oxidative damage to biological macromolecules such as DNA, proteins and lipids by causing oxidative stress which is defined as an imbalance between the formation of free radicals and their elimination by antioxidant defense systems. Most vulnerable to free radical attack is the cell membrane which may undergo enhanced lipid peroxidation, finally producing mutagenic and carcinogenic malondialdehyde and 4-hydroxynonenal and other exocyclic DNA adducts [1]. Antioxidants can significantly delay or prevent oxidation of targeted substrate by scavenging free radicals [2].
Olive tree belongs to the family of *Oleaceae*, which is cultivated for its edible fruits. Olive fruits are consumed as table olives and used for producing olive oil [3]. An important part of Olive mill and olive processing residues is olive tree leaves (usually 5%, but possibly reaching up to 10% of the total olives' weight), which are rich sources of natural antioxidants. For old time olive leaves have been used as animal feed, and recently they could be used for antioxidant or olive-leaf extract production [4, 5].
Olive leaves have been used in the human diet as extracts, herbal teas, and powder and contain several potentially bioactive compounds that may have antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic properties [6].
Studies show that the health benefits of olive leaf extracts are linked to its antioxidant properties due to the bioavailability of some phenolic compounds and their derivatives such as oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol, etc [7-9].
It was also reported that leaf extracts were proven to be good protectors for olive and sunflower oils. Therefore Olive leaves could be a low-cost, renewable and abundant source of phenolic antioxidants, with potent use in fatty foods and natural potential of antioxidants [4].
Many methods for the extraction of antioxidants from olive leaves are available, but solvent extraction is the most common one. Several solvent systems and methods have been used to maximize the polyphenol extraction [10, 11]. It is also reported that an optimum extraction of Polyphenols is usually obtained in polar rather than nonpolar solvents [12]. Therefore, water and organic solvents (methanol, ethanol, acetone and chloroform) are widely used for extraction of plant materials [13].
In fact, there is no single extraction protocol that can be used for extracting phenolic from all types of samples. Also, many parameters such as the type of solvent, composition of solvent, pH, temperature and time can influence the extraction yield [14, 15].

The main objective of this project is to extract antioxidants from olive leaves collected from Syrian coastal region and determine the optimum extraction for further use of this extract in food and pharmaceutical industry.

2. Materials and Methods

2.1. Samples Collection

Olive leaves samples were obtained from trees localized in the sunshine area of Safita/Tartous, Syria. The collection was performed in the middle of June 2016.

2.2 Sample Preparation

Fresh olive leaves were washed and dried by three technical drying methods (in microwave, under vacuum, and in the dark for 8 days) then total phenolic yield was estimated in order to choose the best technical drying method.

Drying in a microwave oven was conducted at 2450 MHZ for 80 sec at maximum power. Whereas, drying under vacuum was performed at -0.5 bar and 55℃ for 24 h.

Olive leaves dried were grinded by blender and their moisture content was evaluated. Then, 0.2 g of dried samples were extracted with ethanol 80% at 60 ℃ for 2 h according to Yateem *et al.* [14]; 2014. The total phenolic content was estimated as by Folin–Ciocalteu reagent method.

2.3 Chemicals

Folin-Ciocalteau (2N) reagent (Sigma-Aldrich, Switzerland), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich, USA), Sodium carbonate (Himedia, India), Gallic acid Sigma-Aldrich, China). Analytical grade acetone, ethanol, methanol were obtained from Sharlau (Spain).

2.4. Extraction Procedures

Four concentrations (20, 40, 60, 80%) of three solvents (Ethanol, Methanol, and Acetone) and three mixtures of acetone/ethanol v/v (20/60, 40/40, 60/20) were used for extracting phenolic compounds from olive leaves.

0.2 g of dried samples were mixed with 10 ml of each solvents at various temperatures (40, 60, 80 ℃) for various times (1, 2, 3) h. The extracts were centrifuged at 5000 rpm for 5 minutes. The supernatant was collected for further experiment. All samples were extracted in duplicates and the total polyphenols concentrations as well as the radical scavenging activity were measured.

2.5 Determination total phenolic content (Folin–Ciocalteu assay)

Total phenolic content was determined using the Folin–Ciocalteu reagent method [16] with little modifications. To 1 mL of diluted extract (250 µl from each extract diluted to 10 ml with distilled water), 0.5 mL of Folin–Ciocalteu (2N) reagent and 4.5 ml of distilled water were added and allowed to stand at room temperature for 3mn. Then 4 mL of Na_2CO_3 7.5% was added. Samples were incubated in bath water for 30 min at 40 °C.

The absorbance was measured at 734 nm and results were expressed as g of Gallic acid equivalents per 100g of dry matter (DM).

2.6 Radical Scavenging activity assay (DPPH assay)

The DPPH radical scavenging activity was evaluated according to the slightly modified procedure described by Brand-Wiliams *et al.* [17]. 200 µl of olive leaves diluted extract at different concentrations was added to 2ml mL of a DPPH solution ($1x10^{-4}$ M in methanol). The reaction mixture was then shaken vigorously and left to stand in the dark at room temperature for 60 min. The absorbance was measured at 520 nm.

The ability to scavenge DPPH radicals was calculated by the following equation:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

Where, $Abs_{control}$ is the absorbance of DPPH radical+ solvent; Abs_{sample} is the absorbance of DPPH radical + sample extract.

The IC_{50} values denote the concentration in ($\mu g.ml^{-1}$) of extraction which is required to scavenge 50% of DPPH free radicals.

3. Results and Discussion

3.1. Sample drying

Table 1 shows that the highest phenolic content is obtained when the olive leaves are dried in a microwave oven at 2450 MHZ for 80 sec at maximum power.

Table 1: Total phenolic content in dried leaves using different technical drying methods.

Total phenolic as g GA/ 100g DM	Technical drying method
6.45	Microwave
3.34	Vacuum
4.57	Dark

3.2 Effect of extraction solvent on total phenolic content and antioxidant activity

Selection of an ideal solvent for extraction is critical, as it determines the amount and type of phenolic compounds extracted because in each plant there are different phenolic compounds of varied chemical characteristics and polarities which may or may not be soluble in a particular solvent [18].

Activity coefficients predicted by the UNICAF model proposed by Galanakis *et al.* [19] indicated that natural phenols possessed a solubility preference to solvents which have intermediate polarity like the alcohols and acetone which were used in this study, rather than more polar (e.g. water) or less-polar solvents (e.g. ethyl acetate). Methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols, whereas aqueous acetone is good for extraction of higher molecular weight flavanols [13].

Table 2: Effect of extraction solvent on total phenolic content and antioxidant activity.

Solvent	Acetone		Methanol		Ethanol	
Concentration	IC_{50} ($\mu g.ml^{-1}$)	Total phenolic (g/100g)	IC_{50} ($\mu g.ml^{-1}$)	Total phenolic (g/100g)	IC_{50} ($\mu g.ml^{-1}$)	Total phenolic (g/100g)
20%	43.68	5.82	187.50	2.68	143.71	4.60
40%	68.99	7.15	**25.01**	7.89	**18.92**	6.63
60%	37.75	7.98	28.39	7.92	53.41	6.67
80%	**33.15**	8.44	34.46	6.19	47.42	7.81
Mixtures	**IC_{50} ($\mu g.ml^{-1}$)**			**Total phenolic (g GA/ 100g DM)**		
Acetone: Ethanol: water 6:2:2	**37.19**			6.06		
Acetone: Ethanol: water 4:4:2	50.06			8.98		

In Table 2, data shows that the total phenol content of extracts varies in response to different solvents due to differences in polarities, which might influence the solubility of various constituents present in olive leaves. Hence, the selection of the appropriate solvent is one of the most relevant steps in optimizing the recovery of plant phenolic compounds. Results indicates that leaves extract exhibited similar phenol content with ethanol: acetone: water mixture 4:4:2 (8.98 g/100g), 80% acetone (8.4 g/100g), 60% methanol (7.9 g/100g), followed by 80% ethanol (7.8 g/100g).

Results presented in table (2) also show the ability of olive leaves extracts to scavenge the DPPH radical measured as IC_{50} varied significantly from 18.9 to 187.5 µg/ml. Dehkharghanian *et al.* [20] also informed that difference of polarity solvent determines a difference of type, composition, and antioxidant activity of phytochemical.

Although ethanol: acetone: water mixture 4:4:2, 80% acetone, 60% methanol and 80% ethanol give similar phenolic yields, the ethanol: acetone: water mixture 4:4:2 (IC_{50}=50.06) extracts of olive leaves show significantly lower antioxidant activity than the other extracts with the best IC_{50} obtained using ethanol 40% (IC_{50}=18.92) as a solvent.

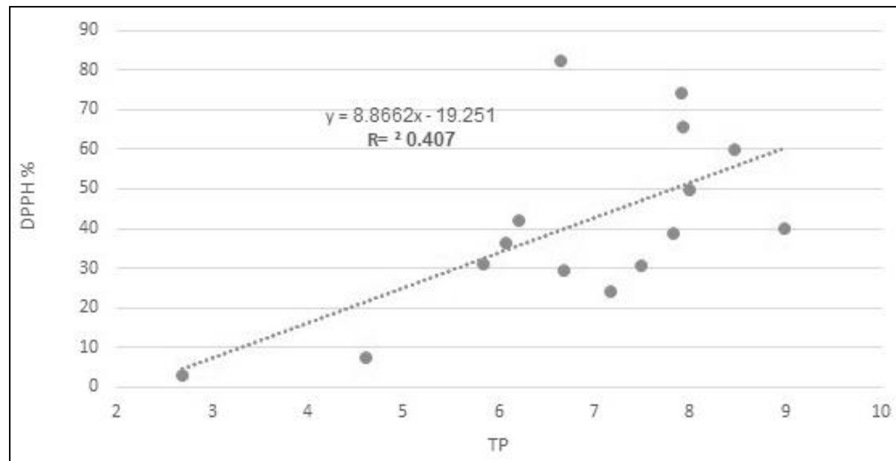

Fig 1: correlation between the amount of total phenolic (TP, expressed as g GA/ 100 g DM) and the DPPH scavenging activity (%).

Our data in figure (1) suggests a weaker correlation between the amount of phenolic compounds and the value of IC_{50}. This can be due to the different polarity of phytochemicals in olive leaves so that they can be extracted by different solvent polarity and every phytochemical can contribute in a different way to the total antioxidant activity. Also, as it is mentioned by Tachakittirungrod *et al.* [21], the antioxidant components existing may possess different predominant mechanisms of action related to their nature and their synergistic effects.

On the other hand and according to Bucić-Kojić *et al.* [22] the weak correlation between the antioxidant capacity and the corresponding total phenolic compounds may imply that phenolic compounds were not the major contributors to the antioxidant capacity of the investigated plant.

Thus 80% acetone, 40% methanol and 40% ethanol were chosen for the determination of extraction time and temperature since they give the best IC_{50}.

3.3 Effect of extraction temperature on phenolic content and antioxidant activity

The phenolics extraction yield and IC_{50} as a function of the extraction temperature is shown in Table 3.

Table 3: Effect of extraction temperature on total phenolic content and antioxidant activity of extracts.

Temperature	Acetone 80%		Methanol 40%		Ethanol 40%	
	IC_{50} (µg.ml^{-1})	Total phenolic g/100g	IC_{50} (µg.ml^{-1})	Total phenolic g/100g	IC_{50} (µg.ml^{-1})	Total phenolic g/100g
40 °C	58.48	8.23	60.19	7.61	56.1	7.32
60 °C	33.15	8.44	25.01	7.89	18.92	6.63
80 °C	33.9	7.59	28.26	8.2	23.65	7.28

Within the investigated temperature interval (60–80 °C), the extractability of phenolic compounds was not significantly different with increasing temperature. Whereas, significant differences in the antioxidant activity expressed by IC_{50} existed among 40 and 60 °C but did not appear between 60 and 80 °C. With the three different used solvents in this study the IC_{50} decreases with increasing temperature of extraction by 2.3±0.6 fold as temperature increased from 40℃ to 60℃.

According to previous researches, the total phenolic content increased as the extraction temperature increases due to the increased solubility and diffusion coefficients of phenolics; decreased solvent viscosity; as well as the enhanced mass transfer and penetration of solvent into the plant matrix [23, 24], thus accelerating the whole extraction. Also according to Shi *et al.* [25], heating might soften the plant tissue and weaken the phenol-protein and phenol polysaccharide interactions in the plant materials. Consequently, more phenolics would transfer to the solvent portion.

On the other hand, denaturation of membranes and a possible degradation of polyphenolic compounds caused by hydrolysis, internal redox reactions and polymerizations which are detrimental to the extraction yield may happen and influence quantification of bioactive compounds [26, 27]. Moreover, it was also reported by Cacace and Mazza [28] that certain phenolic compounds such as flavonoid families are heat sensible, hence an upper limit must be respected to avoid degradation of the thermo-sensitive phenolic compounds.

Most importantly, since acetone, methanol and ethanol have boiling points of 56, 65, 78 °C respectively, a very high extraction temperature may evaporate the solvent from the

aqueous acetone solution and subsequently changes the solvent-to-water ratio. Thus, in practice an extraction temperature of 60 °C could be used, based on the combined effects of the good extraction yield of phenolics, the best IC_{50} and the stability of solvent-to-water ratio.

3.4. Effect of extraction time on Total phenolic Content and Antioxidant activities:

Experimental results show that the extraction time (60, 120 or 180 min) does not uniformly influence the recovery of total phenolic compounds as showed in table 4.

Table 4: Effect of extraction time on total phenolic content and antioxidant activity of extracts.

Time	Acetone 80%		Methanol 40%		Ethanol 40%	
	IC_{50} (µg.ml^{-1})	Total phenolic g/100g	IC_{50} (µg.ml^{-1})	Total phenolic g/100g	IC_{50} (µg.ml^{-1})	Total phenolic g/100g
1 h	70.29	9.64	51.51	9.06	52.22	6.05
2 h	33.15	8.44	25.01	7.89	18.92	6.63
3 h	37.12	9.64	24.39	10.21	21.81	6.05

Results also proved that the high antioxidant activity (IC_{50}) was obtained for 2 h at 60 ℃. This result suggests that at the beginning the fast extraction process could be attributed to the fact that only those phenolic compounds more weakly linked to cell walls and those contained in vacuoles can presumably be easily recovered [29]. After a critical time, the rate of disintegration and oxidation of the phenolic is greater than the rate of extraction, causing a decrease in the antioxidant activity level [15].

Conclusion

This study demonstrates that it is essential to optimize the extraction solvent composition, temperature and time for accurate and reproducible extraction of polyphenols from olive leaves. Our results show that 80% acetone, 40% methanol and 40% ethanol are more efficient in the extraction of polyphenolic compounds. Although high yield was achieved using methanol and acetone for the extraction of phenols from olive leaves, both are not a food grade solvent due to their high toxicity. As a consequence, ethanol was selected as the most appropriate solvent for the extraction of phenolic compounds from olive leaves for production of extracts with high phenol content and high antioxidant activity.

This study confirm that the aqueous solutions of ethanol of 40%, extraction temperature of 60 °C and extraction time of 120 min are the most efficient for the extraction of polyphenols from microwave dried olive leaves.

Olive leaves extracts provided antioxidant rich material which would be helpful as natural alternatives to replace synthetic antioxidants in edible and medicinal products and can be used in function foods.

References

1. Valko M, Jomova K, Rhodes JC, Kuča K, Musílek K. Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. Archives of Toxicology. 2016; 90(1):1-37.
2. Sanchez-moreno C. Review: Methods used to evaluate the free radical scavenging activity in foods and biological system. Food science and technology international. 2002; 8(3):121-137.
3. Soler-Rivas C, Espin JC, Wichers HJ. Oleuropein and related compounds. Journal of the Science of Food and Agriculture. 2000; 80(7):1013-1023.
4. Lafka TI, Lazou AE, Sinanoglou VJ, Lazos ES. Phenolic Extracts from Wild Olive Leaves and Their Potential as Edible Oils Antioxidants. Journal of Foods. 2013; 2(1):18-31.
5. lue H. Extraction of antioxidant compounds from olive (*Olea europaea*) leaf, MASSY university Albany, New Zealand. 2011.
6. Wainstein J, Ganz T, Boaz M, Dayan YB, Dolev E, Kerem Z *et al*. Olive Leaf Extract as a Hypoglycemic Agent in Both Human Diabetic Subjects and in Rats. Journal of Medicinal Food. 2012; 15(7):605-610.
7. Garcia OB, Castillo JL, Ortuno A, Del Rio JA. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. Food Chemistry. 2000; 68(4):457-462.
8. Meirinhos J, Silva BM, Valentão P, Seabra RM, Pereira JA, Dias A *et al*. Analysis and quantification of flavonoidic compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. Natural Product Research. 2005; 19(2):189-195.
9. Savournin C, Baghdikian B, Elias R, Dargouth-Kesraoui F, Boukef K, Balansard G. Rapid high-performance liquid chromatography analysis for the quantitative determination of Oleuropein in *Olea europaea* leaves. Journal of. Agricultural and Food Chemistry. 2001; 49(2):618-621.
10. Buhmann A, Papenbrock J. An economic point of view of secondary compounds in halophytes. Functional Plant Biology. 2013; 40(9):952-967.
11. Falleh H, Trabelsi N, Bonenfant-Magné M, Floch GLe, Abdelly C, Magné C, Ksouri R. Polyphenol content and biological activities of *Mesembryanthemum edule* organs after fractionation. Industrial Crops and Products. 2013; 42:145-152.
12. Liu X, Dong M, Chen X, Jiang M, Lv X, Yan G. Antioxidant activity and phenolics of an endophytic *Xylaria* sp. Ginkgo biloba. Food Chemistry. 2007; 105(2):548-554.
13. Dai J, Mumper RJ. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules. 2010; 15(10):7313-7352.
14. Yateem H, Afaneh I, Al-Rimawi F. Optimum Conditions for Oleuropein Extraction from Olive Leaves. International Journal of Applied Science and Technology. 2014; 4(5):153-157.
15. Candrawinata VI, Golding JB, Roach PD, Stathopoulos CE. Total phenolic content and antioxidant activity of apple pomace aqueous extract: effect of time, temperature and water to pomace ratio. International Food Research Journal. 2014; 21(6):2337-2344.
16. Skerget M, Kotnik P, Hadolin M, Hras AR, Simonic M, Knez Z. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chemistry. 2005; 89(2):191-198.
17. Brand-Williams W, Cuvelier ME, Berset C. Use of free

radical method to evaluate antioxidant activity. Lebensm Wiss Technology. 1995; 28(1):25-30.

18. Chan SW, Lee CY, Yap CF, Wan AWM, Ho CW. Optimisation of extraction conditions for phenolic compounds from limau purut (*Citrus hystrix*) peels. International Food Research Journal. 2009; 16:203-213.
19. Galanakis CM, Goulas V, Tsakona S, Manganaris GA, Gekas V. A knowledge base for the recovery of natural phenols with different solvents. International Journal of Food Properties. 2013; 16(2):382-396.
20. Dehkharghanian M, Adenier H, Vijayalakshmi MA. Analytical methods study of flavonoids in aqueous spinach extract using positive electrospray ionisation tandem quadrupole mass spectrometry. Food Chemistry. 2010; 121(3):863-870.
21. Tachakittirungrod S, Okonogi S, Chowwanapoonpohn S. Study on antioxidant activity of certain plants in Thailand: mechanism of antioxidant action of guava leaf extract. Food Chemistry. 2007; 103(2):381-388.
22. Bucić-Kojić A, Planini M, Toma S, Jokić S, Mujić I, Bilić M *et al*. Effect of Extraction Conditions on the Extractability of Phenolic Compounds from Lyophilised Fig Fruits (*Ficus carica* L.). Polish Journal of Food and Nutrition Sciences. 2011; 61(3):195-199.
23. Al-Farsi MA, Lee CY. Optimization of phenolics and dietary fibre extraction from date seeds. Food Chemistry. 2008; 108(3):977-985.
24. Wang J, Sun BG, Cao Y, Tian Y, Li XH. Optimization of ultrasound-assisted extraction of phenolic compounds from wheat bran. Food Chemistry. 2008; 106(2):804-810.
25. Shi J, Yu JM, Pohorly J, Young C, Bryan M, Wu Y. Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. Food, Agricultural and Environment. 2003; 1(2):42-47.
26. Abad-Garcia B, Berrueta LA, Marquez DML, Ferrer IC, Gallo B, Vicente F. Optimization and validation of methodology based on solvent extraction and liquid chromatography for the simultaneous determination of several polyphenolic families in fruit juices. Journal of Chromatography A. 2007; 1154(1-2):87-96.
27. Alonso-Salces RM, Korta E, Barranco A, Berrueta LA, Gallo B, Vicente F. Pressurized liquid extraction for the determination of polyphenols in apple. Journal of Chromatography A. 2001; 933(1-2):37-43.
28. Cacace JE, Mazza G. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. Journal of Food Science. 2003; 68(1):209-215.
29. Amendola D, de Faveri DM, Spigno G. Grape marc phenolics: Extraction kinetics, quality and stability of extracts. Journal of Food Engineering. 2010; 97(3):384-392.