ORIGINAL ARTICLE

Microwave-assisted efficient extraction of phenolics from *Juglans regia* L.: pellicle; kernel unripe fruits; and leaves in different solvents

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Abstract

Aim: In the present study, microwave-assisted extraction was compared with conventional approaches for the efficient extraction of phenolics from *Juglans regia* unripe fruits and leaves. The main objective of this study was to extract phenolic compounds from Jordanian *J. regia* L. Unripe fruits compared to leaves using microwave and Soxhlet. **Materials and Methods:** The effects of microwave power; part used and solvent type (ethyl acetate; acetonitrile and acetone) on phenolic content, antioxidant activity, and phytochemical profile. **Results and Discussion:** Pellicle was the best place for phenolic compounds using ethyl acetate MW closed system with (18%) while the least amount was also in the pellicle (8%) for acetonitrile Soxhlet method. MW closed system used the lowest solvent to solid ratio, but it gives the best phenol content. Kernel Soxhlet/ethyl acetate shows the best free radical scavenger activity IC_{50} ($2.4 * 10^{-2}$ µg/ml) while Pellicle MW Open/acetone give the lowest activity IC_{50} (1.0 µg/ml) but still better than Juglone IC_{50} (10.21 µg/ml). **Conclusion:** It's worthy to go more through the pharmacological activity for this part as anti-inflammatory wound healing and anticancer activity as secondary metabolite profile for this part from walnut not studied extensively.

Key words: Antioxidant, *Juglans regia* L., microwave, phenolics, unripe fruits

INTRODUCTION

Walnut (*Juglans regia* L.) is a deciduous tree from Juglandaceae family. Leaves have been used in traditional medicine for the treatment of ulcers and skin inflammations and its astringent; antidiarrheal, antiseptic, and anthelmintic activity.[1,2] Flowers; leaves, bark, and green husk of *J. regia* have widespread use in complementary and alternative therapy as antidiarrheal, antimicrobial, anthelmintic, hypoglycemic, and astringent.[3-6] However, there is no scientific report on a phytochemical profile or biological activity of *J. regia* unripe fruits.

Walnuts regular intake could help against oxidative stress-mediated diseases such as due to the antioxidant potential of walnut products including green nuts and/or dry nut and green walnuts mesocarp; leaves; bark, or flowers.[7-13]

Well-known component of walnut is juglone, found in considerable amounts in all green and growing parts of trees and unripe hulls of nut too.[14] Juglone was historically known as a secondary metabolite and allelochemical that is not required for the growth, development, and reproduction of an organism where it is produced, but instead is believed to have a biological effect often on other organisms. This notion has been challenged with a study suggesting that juglone may also play a role in plant development, thus making it a primary metabolite.[15]

Phenolic compounds are secondary metabolites that are widely distributed in plants.[16] They occur as aglycones or

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Received: 19-05-2018
Revised: 25-05-2018
Accepted: 17-08-2018

glycosides, as monomers; highly polymerized structures or as free or matrix-bound. They are not uniformly distributed in the plant, and their stability varies significantly.[17] This significantly obscures their isolation and extraction that is a single standardized procedure cannot be recommended for all phenolics and/or plant materials.[18]

Microwave-assisted extraction (MAE) is an extraction technique that combines traditional solvent and microwave power. This technique has a number of advantages, for example, shorter extraction time, less solvent, higher extraction rate and lower cost, and over traditional method of extraction of compounds from various matrices, especially natural products.

Through the technological developments, it has now become one of the popular and cost-effective extraction methods available today, and several advanced MAE instrumentations and methodologies have become available, for example, pressurized microwave-assisted extraction and solvent-free microwave-assisted extraction.[19]

MAE process is different from those of conventional methods because the extraction occurs as the result of changes caused by electromagnetic waves in the cell structure.[19] This accelerates and harvest high extraction yield possibly will result in a synergistic effect of two transport phenomena: Heat and mass gradients working in the same direction.[19] While, in conventional extractions, the mass transfer occurs from inside to the outside, although the heat transfer occurs from the outside to the inside of the substrate.[20] MAE considered a promising technique for plant extraction because of its use of different physical and chemical phenomena compared to those in conventional extractions, though they need to be optimized.

Up to date, on our knowledge, there are no data about unripe fruits phytochemical constituents. We believe that they might have beneficial use as a source for secondary metabolites differ from leaves. During maturation time the content of phenolic compounds within the green fruits is significantly decreasing,[2] we believe that this will affect the pharmacological activity of fruits husk before and after maturation. Uptodate there is no data available about phenolic content in immature fruits husk (pellicle) and kernel.

In the present work, we evaluated the optimal extract from the unripe fruits of *J. regia* as pellicle and kernel compared to leaves using microwave-assisted extraction technique as well as the conventional Soxhlet method. MAE has been applied to conditions closed (that use assistance of pressure) and open (that use assistance of heat). Their phytochemical analysis and antioxidant activity have been evaluated.

MATERIALS AND METHODS

Materials

J. regia L. unripe fruit and leaves were collected from Shmeisani in Amman-Jordan, in late May all the chemicals and reagents used were of analytical reagent grade and were purchased from Sigma-Aldrich Company.

Plant Material

The immature fruit of walnut plant (*J. regia* L.*)* was picked in May 25st 2016 from the private garden in Shmeisani in Amman, Jordan, and the plant was authenticated by Dr. Fahmi Shatat, Faculty of Agriculture; University of Jordan. The voucher specimen was deposited in a drug discovery laboratory along with a given specimen number R002.

Extract Preparation

The walnut fruits, as well as leaves, were cleaned and stored at −80°C in the deep freezer. Later on, the walnut fruits and leaves were sliced and ground to a fine powder, finally, the samples weighed for the extraction process.

Extraction Methods

The plant samples (50 g from each type: Fruit peel, fruit pulp, and leaves) were extracted in Soxhlet apparatus using various solvents separately according to the gradual increase of polarity; these solvents are: Ethyl acetate, acetonitrile, and acetone. The extraction processes were done for 2–3 h, solid to solvent ratio was 1:5 and the number of extraction cycles using Soxhlet apparatus was considered according to the separation of components using thin-layer chromatography technique.

For microwave-assisted extraction; extraction process was performed using a laboratory scale microwave oven (milestone). For an open system, for each extract, the sample was weighed (50 g) and put with the solvent into the round bottom flask then placed into the oven for open conditions. Whereas for closed conditions: The sample was weighed (15 g), the power was 500 W, the temperature was 50°C, solid to solvent ratio 1:5 for open and 1:2 for closed and the time was 15 min, and these extraction parameters were adjusted using the control panel of the microwave oven.[21] When each extraction was completed, it was filtered under vacuum, followed by evaporation in the fume hood. The yield of the extracted plant was collected, weighed accurately and kept in the dark colored bottles at refrigerator to be used for the analysis.

Preliminary Phytochemical Analysis

Qualitative phytochemical analysis of extracts was tested as follows: Kumar test for flavonoids detection; ferric chloride

test for tannins detection; testing the presence of saponins using the formation of persistent frothing and finally Wagner test for alkaloids detection.

Estimation of Total Phenolics

The Folin–Ciocalteu method[22] was used to determine the total phenolic content of the extracts. This method is based on the principle that phenolic substances reduce Folin–Ciocalteu reagent in the presence of sodium carbonate causing a color change. According to this method, diluted samples were pipetted into test tubes and Folin–Ciocalteu reagent is added and mixed for a minute, then they were allowed to rest for 5 min in a dark place at room temperature for incubation. After that, the relevant amount of sodium carbonate was added. After stirring again, the mixture is kept for 1 h in the dark place at room temperature. The absorbance was measured at 760 nm using a UV/VIS spectrophotometer (Thermo, USA). The results were expressed as mg gallic acid equivalent per (g) of dry material. The calibration curve was prepared using gallic acid solution range from 0.0125 to 0.2 mg/ml.

All of the spectrometric measurements were taken in three replicates, and the average value is used in the calculations of total phenolic content.

Determination of Antioxidant Activity

The antioxidant activity of the walnuts extracts was measured in term of radical scavenging ability using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method.[23] This method is based on the principle that the DPPH radicals are reduced by antioxidants. This reduction causes a color change. According to this method, 0.025 g DPPH reagent, which is a dark purple radical, is dissolved in 1 L methanol. Then, 3.9 mL of this solution is added to 0.1 mL of each sample that was diluted in a pure solvent of extraction at different concentrations.

The mixtures were incubated for 90 min in a dark place at room temperature and the absorbance for each sample was measured at 517 nm using a UV/VIS spectrophotometer.

The antiradical activity was expressed as IC_{50} (µg/mL).

The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $((A_0-A_1)/A_0)*100$

Where A_0 is the absorbance of the control at 90 min, and A_1 is the absorbance of the sample at 90 min.[24]

All samples were analyzed in triplicate.

Statistical Analysis

Statistical analysis was done using GraphPad Prism 7 (free online trail). The statistical significance among groups was determined using one-way ANOVA and paired sample t-test in GraphPad Prism 7. $P < 0.05$ was considered significant.

RESULTS

Extraction Yield

The unripe fruit (pellicle and kernel) and leaves of *J. regia* were extracted in the fresh form by two different methods; Soxhlet and MAE methods (open and closed) using different solvents of increasing polarity, where fruit was divided into two parts: Pellicle and kernel. The yield in each extraction process vary from one sample to another depending on the method, the solvent used or part used as shown in Table 1.

The highest yield was for acetone as solvent and pellicle (6.22 w/w%) with significant difference ($P < 0.05$). Pellicle significantly more yield compared to kernel while it was not significantly different compared to leaves ($P < 0.05$). Moreover, there was a significant difference between Soxhlet and MW (both systems) while it was not within both systems [Table 1].

Preliminary Phytochemical Investigations

All extracts were subjected to semi-quantitative analysis to detect the presence of some important secondary metabolites such as flavonoids, tannins, alkaloids, and saponins and

Table 1: Effects of extracting technique/solvent type on the extract yield (w/w) % *J. regia* unripe fruit (pellicle and kernel) and leaves extraction

Solvent	Part used	Soxhlet	MW open	MW closed
		Yield (%)		
Ethyl acetate	Pellicle	1.25	1.19	1.80
	Leaves	3.33	2.34	1.75
	Kernel	1.11	0.42	0.63
Acetonitrile	Pellicle	4.41	3.63	3.03
	Leaves	2.68	2.07	2.65
	Kernel	3.71	2.49	2.47
Acetone	Pellicle	6.22	5.02	3.69
	Leaves	5.40	4.04	3.97
	Kernel	4.64	3.67	3.29

50 g plant material for microwave open system and Soxhlet; 15 g for MW closed system. For microwave open and closed; 500 W, 15 min and (1:5) open; (1:2) closed solid to solvent ratio were used. For Soxhlet extraction; 1:7 solid to solvent ratio was used. Significance: $P<0.05$. *J. regia: Juglans regia*

the results were shown in Table 2. Ethyl acetate was the best solvent for tannins extraction while they have equal abundance in leaves and unripe fruits in either part: Kernel and pellicle with a slight decrease in the kernel using MW closed system. Flavonoids where absent in unripe fruits whereas available in leaves in all extraction conditions. Saponins where absent with ethyl acetate for all parts, while acetonitrile and acetone were suitable for their extraction as appears in pellicle part, only using MW extraction. We have a different story with alkaloids they are available in pellicle with ethyl acetate more than leaves using Soxhlet and MW open system for both tests Mayers and Wagners, while it appears only in kernel using MW open and closed systems. Moreover, acetone and acetonitrile appear to be suitable for alkaloids extraction for leaves using both tests [Table 2].

Total Phenolic Contents

The total phenolic content of 27 extracts where determined by the Folin–Ciocalteu method as shown in Table 3. All the concentrations were expressed as mg (gallic acid) equivalent per (g) of dry material, and the concentrations were ranged between 8.5 and 15.6 mg/g%. There was no significant difference between MW methods, while MW systems were slightly better than Soxhlet especially when we are talking about pellicle and kernel but it was comparable in case of leaves. For solvent effect, ethyl acetate was better than acetone and acetonitrile whereas acetone and acetonitrile have relatively comparable values ($P < 0.05$) [Table 3]. At the end, pellicle was the best place for phenolic compounds using ethyl acetate MW closed system with (18%) while the least amount was also in the pellicle (8%) for acetonitrile Soxhlet method.

Table 2: Nature and semi-quantitative analysis of phytoconstituents present in ethyl acetate; acetone; and acetonitrile extracts of *J. regia* (unripe fruit kernel; unripe fruit pellicle; and leaves) extracts using Soxhlet ant MW

Solvent	Condition	Part used	Tannins	Alkaloids		Saponins	Flavonoids
				Mayers	Wagners		
Ethyl acetate	Soxhlet	Pellicle	3+	3+	3+	–ve	–ve
		Leaves	3+	1+	1+	–ve	1+
		Kernel	3+	–ve	–ve	–ve	–ve
	MW open	Pellicle	3+	3+	3+	–ve	–ve
		Leaves	3+	1+	1+	–ve	1+
		Kernel	3+	3+	1+	–ve	–ve
	MW closed	Pellicle	3+	3+	3+	–ve	–ve
		Leaves	3+	1+	1+	–ve	1+
		Kernel	2+	–ve	–ve	–ve	–ve
Acetonitrile	Soxhlet	Pellicle	1+	–ve	–ve	–ve	–ve
		Leaves	2+	1+	–ve	–ve	1+
		Kernel	1+	–ve	–ve	–ve	–ve
	MW open	Pellicle	1+	–ve	–ve	1+	–ve
		Leaves	3+	1+	1+	–ve	1+
		Kernel	2+	–ve	–ve	–ve	–ve
	MW closed	Pellicle	2+	–ve	–ve	1+	–ve
		Leaves	1+	1+	1+	–ve	1+
		Kernel	2+	–ve	–ve	–ve	–ve
Acetone	Soxhlet	Pellicle	1+	–ve	–ve	–ve	–ve
		Leaves	1+	1+	1+	–ve	1+
		Kernel	1+	–ve	–ve	–ve	–ve
	MW open	Pellicle	2+	–ve	1+	1+	–ve
		Leaves	2+	1+	1+	–ve	1+
		Kernel	2+	–ve	–ve	–ve	–ve
	MW closed	Pellicle	2+	–ve	–ve	1+	–ve
		Leaves	2+	1+	1+	–ve	1+
		Kernel	1+	–ve	–ve	–ve	–ve

+: Presence, -: Absence, *J. regia: Juglans regia*

The DPPH Free Radical Scavenging Activity

The antioxidant activity of the *J. regia* unripe fruit extracts was assessed using spectrophotometry for the presence of the DPPH radical. Samples with a plant extract concentration range of 0.10–5.0 g/mL were analyzed.

For comparison, we also measured the radical scavenging activity of juglone in the same conditions as shown in Table 4. As seen in Table 4, the radical scavenging activity of the tested extracts and the positive control (juglone) expressed as the percentage of deactivation of the DPPH free radicals. The quality of the antioxidants in the extracts was determined by the IC_{50} values. The DPPH free radical scavenging activity was calculated using the following formula:

DPPH scavenging effect (%) = $((A_0-A_1)/A_0)*100$

Table 3: Total phenolic concentration of *J. regia* (unripe fruit kernel; unripe fruit pellicle; and leaves) extracts using different solvents*

Solvent	Part used	Soxhlet**	MW open**	MW closed**
Ethyl acetate	Pellicle	12.88	14.21	18.09
	Leaves	14.34	14.46	13.15
	Kernel	15.62	15.50	14.24
Acetonitrile	Pellicle	8.52	10.89	10.93
	Leaves	13.05	13.72	10.32
	Kernel	10.83	13.80	13.35
Acetone	Pellicle	9.13	11.77	12.11
	Leaves	10.39	14.20	12.05
	Kernel	10.02	14.34	13.08

*All the concentrations were expressed as mg gallic acid equivalent per (g) % of dry material. **$P<0.05$. *J. regia: Juglans regia*

Where A_0 is the absorbance of the control at 90 min, and A_1 is the absorbance of the sample at 90 min.[24] The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between percentage inhibition and concentrations. Standard and all the extracts showed a dose-dependent inhibition of the DPPH radicals.

Leaves ethyl acetate/Soxhlet extract exhibited flat activity it gives flat activity not concentration dependent activity. Leaves (Soxhlet/acetonitrile) extract has the most potent DPPH activity with IC_{50} ($0.52 * 10^{-02}$ µg/ml) but with insufficient correlation ($R^2 = 0.57$) so taking into consideration correlation factor of more than 0.85 we can conclude the most potent extract with lowest IC_{50} is: Kernel Soxhlet/ethyl acetate ($2.4 * 10^{-02}$); pellicle Soxhlet/ethyl acetate ($2.9 * 10^{-02}$); leaves MW Open/ethyl acetate ($5.4 * 10^{-02}$); leaves MW closed/ethyl acetate($7.7 * 10^{-02}$), and Pellicle MW Open/ethyl acetate($9.0 * 10^{-02}$) [Table 4].

DISCUSSION

Phenolic compounds are complex molecules, and their extraction from a solid matrix requires compatible solvents. They are widely distributed in plant tissues, particularly contributing color, flavor, and astringency to fruits. Their compounds may vary from 0.5 to 5.0 g per 100 g dry weight of plant tissues. A major group of water-soluble phenolic compounds, the anthocyanins, contribute colors to fruits. The greatest concentrations of phenolic compounds in plants are normally present as cinnamic acid derivatives and flavan monomers, dimers, and polymers.[16]

J. regia L. considered an important source for phenolic compounds that have been reported to have pharmacological activity as anti-inflammatory and antioxidant, as well as natural colorants and preservatives.[25] In former studies, the

Table 4: DPPH radical scavenging activity expressed as IC50 values (µg/ml) of various extracts from J. regia

Solvent	Part used	Soxhlet		MW open		MW closed	
		IC_{50}	R^{2**}	IC_{50}	R^{2**}	IC_{50}	R^{2**}
Ethyl acetate	Pellicle	$2.9*10^{-02}$	0.85	$9.0*10^{-02}$	0.91	$8.1*10^{-01}$	0.96
	Leaves	ND***		$5.4*10^{-02}$	0.93	$7.7*10^{-02}$	0.97
	Kernel	$2.4*10^{-02}$	0.96	$3.7*10^{-01}$	0.89	$5.8*10^{-01}$	0.97
Acetonitrile	Pellicle	$7.8*10^{-01}$	0.93	$8.4*10^{-01}$	0.95	$9.5*10^{-01}$	0.92
	Leaves	$0.52*10^{-02}$	0.57	$3.3*10^{-01}$	0.91	$1.6*10^{-01}$	0.89
	Kernel	$3.9*10^{-01}$	0.98	$4.9*10^{-01}$	0.91	$4.8*10^{-01}$	0.93
Acetone	Pellicle	$8.9*10^{-01}$	0.94	$1.0*10^{+00}$	0.97	$9.6*10^{-01}$	0.93
	Leaves	$2.7*10^{-01}$	0.92	$1.4*10^{-01}$	0.99	$4.7*10^{-01}$	0.94
	Kernel	$2.6*10^{-01}$	0.83	$5.2*10^{-01}$	0.88	$6.8*10^{-01}$	0.98

*Juglone IC_{50}: 10.21 µg/ml (R^2: 0.99). **R^2: Correlation factor. ***ND: Not determined (it gives flat activity not concentration dependent activity). *J. regia: Juglans regia*, DPPH: 2,2-diphenyl-1-picrylhydrazyl

phytoextracts obtained from leaves, ripe fruits, bark, and husk of *J. regia* resulted to be characterized by a high content in polyphenols that include several classes mainly identified as tannins, monomeric phenolic acids, flavonoids, stilbenes, and lignans.[6,26,27] The solid-liquid extraction of these compounds is usually carried out using methanol, ethanol, acetone, acetonitrile, and ethyl acetate.[25]

We are interested in the green extraction of phytochemical compounds from walnut unripe fruits. In the present work, we successfully employed the extraction process for leaves; unripe fruits kernel; and unripe fruit pellicle using traditional Soxhlet method compared to MW method. Time, temperature, and solid/liquid ratio (S/L) are important independent variables in microwave extraction as mentioned before the extraction occurs as the result of changes in the cell structure caused by electromagnetic waves.[28]

Soxhlet extraction is the most common and is still used as a standard in all cases. As a result of several secondary metabolites, the development of high performance and rapid extraction methods is an absolute necessity. The new extraction techniques with shortened extraction time reduced solvent consumption, increased pollution prevention, and with special care for thermos labile constituents have gained attention.

During the extraction process, the rate of recovery of the extract is not a linear function of time: The concentration of solute inside the solid varies, leading to unstable condition.

Forces such as the physicochemical interactions that can be exposed during extraction: Dispersion forces, interstitial diffusion, driving forces, and chemical interactions, as well as the persistence, and strength of these phenomena may be closely related to the properties of the solvent (such as polarity, solubilization power, solubility in water, and purity).[29]

The dielectric constant determines how much of the incident energy is reflected at the air sample interface and how much enters the sample, so we tried to use three different solvents with varied dielectric constant(ε′): Ethyl acetate (6.02); acetonitrile (20.7), and acetone (37.5). The degree of microwave absorption usually increases with the dielectric constant. It is important to select a solvent with high extracting power and strong interaction with the matrix and the analyte.

Walnut fruits are rich in phenolic compounds. Their contents depend on many environmental conditions; part used; time of collection; maturation stage; as well as genotype of different cultivars. In Italy and Slovenia traditionally, the green fruits including green husks (before the endocarp hardens,) pickled in vinegar or sliced and steeped into alcohol in preparation of a walnut liqueur.[14,30]

As phenolic are involved in growth and reproduction and provide plants with resistance to pathogens and predators, we believe that during ripening the plant will have high phenols than after hardening of endocarp to protect unripe fruits with a high content of tannins.

Composition in phenols was studied in immature walnut fruits and leaves using MW and Soxhlet extraction methods in addition to three solvents: Ethyl acetate; acetone; and acetonitrile. Soxhlet/acetone was best condition for all parts with highest yield (pellicle: 6.22%; leaves: 5.40; and kernel: 4.64) [Table 1], while ethyl acetate gives the lowest yield in case of pellicle and kernel both MW systems and also for MW but the closed system for leaves. This phenomenon goes in same direction for solvent dielectric constant, acetone is the most polar (37.5), and ethyl acetate is the lowest (6.02), suggesting that bioactive compounds in *J. regia* are easier to extract with solvents that are more polar.

The highest yield was for acetone as solvent and pellicle (6.22 w/w%) which was significantly differ ($P < 0.05$). Pellicle has significant difference compared to kernel while it was not compared to leaves ($P < 0.05$). Moreover, there was significant difference between Soxhlet and MW (both systems) while it was not within both systems [Table 1]. Efficiency of the phenolics extraction depends on the type of the solvent as well on the phenol, which is being isolated; it's a matter of physicochemical properties. As Soxhlet permit higher contact time with plant materiel as well as higher solvent to solid ratio, it gives high extraction yield. In the addition to polar solvent acetone was best solvent it concludes the polarity of extracted constituents in the pellicle.

Table 2 shows semi-quantitative analysis of phytoconstituents. Ethyl acetate was the best solvent for extraction of all constituents for the three systems with the highest yield with slight differences. For tannins they have equal abundance in leaves and unripe fruits in either part: Kernel and pellicle with slight decrease in the kernel using MW closed system. Flavonoids where absent in unripe fruits in all conditions and solvents while available in leaves at all conditions. Saponins were absent with ethyl acetate for all parts while acetonitrile and acetone were suitable for their extraction as appears in pellicle part, only using MW extraction. We have a different story with alkaloids they are available in pellicle with ethyl acetate more than leaves using Soxhlet and MW open system for both tests Mayers and Wagners, while it appears only the kernel in MW open and closed system. Moreover, acetone and acetonitrile appear to be suitable for alkaloids extraction for leaves using both tests [Table 2].

According to Colaric *et al.*[31] they determined: Syringic acid, juglone, and ellagic acid in the kernel and the thin skin of the walnut, termed the pellicle. As well as ferulic and sinapic acid knowing that pellicle is the most important source of walnut phenolic. As seen in Table 3, the highest phenolic contents 18.09% using ethyl acetate MW closed system while pellicle using Soxhlet/acetonitrile system yields the lowest phenolic content (8.52%). There was no significant

difference between MW methods, while MW systems were slightly better than Soxhlet especially when we are talking about pellicle and kernel but it was comparable in case of leaves. Ethyl acetate was better than acetone and acetonitrile while acetone and acetonitrile have relatively comparable values ($P < 0.05$) [Table 3]. At the end, pellicle was the best place for phenolic compounds using ethyl acetate MW closed system with (18%) while the least amount was also in the pellicle (8%) for acetonitrile Soxhlet method.

MW closed system used the lowest solvent to solid ratio but it gives the best phenol content we believe that it's a matter of connecting all suitable parameters altogether, best solvent with closed system that will have low level of solvent loss during extraction and high pressure that will attain more contact between cellular constituents and extraction solvent.

For DPPH radical scavenging activity Table 4 shows that leaves ethyl acetate/Soxhlet extract exhibited flat activity not concentration dependent activity. Leaves (Soxhlet/acetonitrile) extract has the most potent DPPH activity with IC_{50} ($0.52 * 10^{-02}$ µg/ml) but with insufficient correlation ($R^2 = 0.57$) so taking into consideration correlation factor of more than 0.85 we can conclude the most potent extract with lowest IC_{50} is: Kernel Soxhlet/ethyl acetate ($2.4 * 10^{-02}$ µg/ml); pellicle Soxhlet/ethyl acetate ($2.9 * 10^{-02}$ µg/ml); leaves MW open/ethyl acetate ($5.4 * 10^{-02}$ µg/ml); leaves MW closed/ethyl acetate ($7.7 * 10^{-02}$ µg/ml); and pellicle MW open/ethyl acetate ($9.0 * 10^{-02}$ µg/ml) [Table 4].

Pellicle MW open/acetone give the lowest activity (1.0 µg/ml). It is clear that ethyl acetate extracts have the most potent DPPH radical scavenging activity which is the least polar solvent, whereas acetone produce the least active extract which is the most polar. Phytochemical constituents are different as well as the proportional amounts are not the same in all extracts, we believe that it is the case here. There where no noticeable correlation between extract phenolic content and free radical scavenger activity [Figure 1] as free radical scavenger activity is a matter of structural requirements. Possible phenols present in green fruits are gallic, chlorogenic, ellagic, sinapic, and protocatechuic acid, (+)-catechin, and juglone according to Jakopič *et al*. 2009.[32]

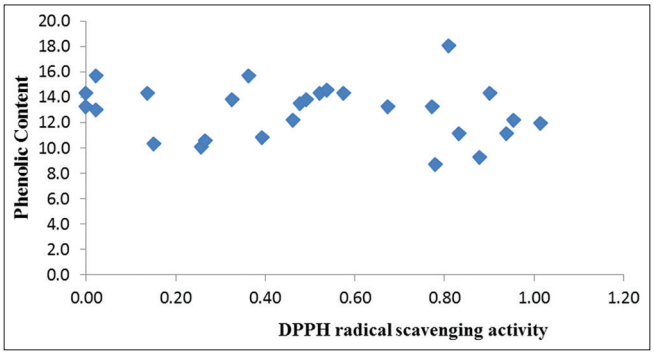

Figure 1: Correlation between the antioxidant capacity and total phenolic content of *Juglans regia* extracts. Antioxidant capacity was measured by 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity and total phenolic contents as gallic acid equivalents (mg/g)

Antioxidant activity of the juglone (as a reference) was tested IC_{50} (10.21 mg/mL), and when compared with all extracts as shown in Table 4, juglone showed weaker activity which indicates the presence of a mixture of phenolic compounds that have synergistic activity.

MW method show superior extraction strategy compared to Soxhlet. Although good recovery rates were obtained with both extraction methods, MW provided advantages with regard to sample handling, cost, analysis time, and solvent consumption. MW closed system showed the best technique even compared to MW open system as it requires less solvent consumption and results high extraction yield. Moreover, unripe fruits can work as important source for polyphenols that have medicinally important value differ from ripe fruits or leaves for a different profile. Further studies are required to identify the active substances from these extracts applying efficient MW methods.

CONCLUSION

J. regia unripe fruits pellicle and kernel have been extracted applying MW compared to Soxhlet using three different solvents with varies dielectric constant. We examined semi-qualitatively the presence of tannins, flavonoids, saponins, and alkaloids, as well as we measured quantitatively the concentration of total phenols yield of the extraction processes. Their antioxidant activity was evaluated.

Pellicle was the best place for phenolic compounds using ethyl acetate MW closed system with 18% while the least amount was also in the pellicle (8%) for acetonitrile Soxhlet method. MW closed system used the lowest solvent to solid ratio, but it gives the best phenol content. Kernel Soxhlet/ethyl acetate shows the best free radical scavenger activity IC_{50} ($2.4 * 10^{-02}$ µg/ml) while pellicle MW open/acetone give the lowest activity IC_{50} (1.0 µg/ml) but still better than Juglone IC_{50} (10.21 µg/ml).

Our results showed a higher efficiency for the microwave extraction especially closed system method over the Soxhlet extraction method in terms of higher yield amount and less time and solvents consuming. Moreover, pellicle shows the best source for phenolic compounds better than leaves and kernel it's worthy to go more through the pharmacological activity for this part as anti-inflammatory wound healing and anticancer activity as secondary metabolite profile for this part from walnut not studied extensively.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Applied Science University, for allowing the use of Milestone microwave oven.

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Source of Support: Nil. **Conflict of Interest:** None declared.