In vitro antioxidant and antibacterial activity of various extracts from exocarps and endocarps of walnut

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Abstract: *Juglans regia* seed has been used in traditional medicines as antimicrobial, antihelmintic and anti-diarrhoeal. In the present study, the antibacterial capabilities dichloromethane, ethyl acetate, methanol and aqueous extracts of endocarp and exocarp of walnut were determined against two Gram-positive bacteria and one Gram-negative bacteria. The antioxidant activity was screened by 2, 2□-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing antioxidant power (FRAP) assays. The highest antioxidant activity was observed for methanol extract of endocarp in both methods and it was stronger than positive control butylated hydroxy toluene (BHT). The total phenolic contents were ranging between 34.59 to 68.34mg GAE/g DW. The results revealed that all extracts had antibacterial activity against selected bacteria except aqueous extract. The methanol extract of endocarp presented the highest zone of inhibition against tested pathogens (9-21mm). From the results it is conclude that the methanol extract from endocarp of walnut could be used as a natural preservative ingredient in food and pharmaceutical industries.

Keywords: *Juglans regia*, endocarp and exocarps, antioxidant, antibacterial.

INTRODUCTION

The Juglans genus belongs to Juglandaceae family and it consists of 21 species (Anonimous, 1999). All parts of the plant; stem, bark, leaves, fruits, seeds, seed oils are a source of phytochemicals (Cosmulescu *et al*., 2011). The seed of this plant is known as walnut (Anonimous, 1999). Recently, walnut has been considered as natural functional food due to its nutritional and medicinal benefits (Bouabdallah *et al.,* 2014). Walnuts, shells, kernels, bark and leaves have been used in the pharmaceutical and cosmetic industries (Stampar *et al*., 2006). There are three major walnut species: *J. regia L.*, *Juglans cinerea L.* and *Juglans nigra L.* (Gharibzahedi *et al*., 2013). Among these species, *J. regia* seed was used as plant material in our study. *Juglans regia* have been used in traditional medicines as antimicrobial, antihelmintic, astringent, antidiarrhoeal, hypoglycaemic, depurative, and carminative (Vaidyaratnam, 2005). Antiradicalar and antibacterial activities have been described for *J. regia* (Pereira *et al.,* 2007). In *Juglans regia*, naphthoquinones and flavonoids are major phenolic compounds, and several phenolic compounds such as pyrogallol, p-hydroxybenzoic acid, vanillic acid, protocatechuic acid, gallic acid, tannins, adenosine and adenine isolated from *J. regia*, (Fukuda *et al.,* 2003). The walnuts contain useful compounds, such as polyunsaturated fatty acids, proteins and minerals (Rabrenovic *et al.*, 2008) and they have been used in the treatment of diabetes (Kendall *et al.,* 2011) and cardiovascular diseases (Banel and Hu, 2009). Juglone is present in all parts of walnut and in walnut mesocarp, while the content of juglone in endocarp is very low or absent (Jakopic *et al.*, 2008).The husk is one of the major waste products of the walnut production and it is the basic material for the walnut liqueur and bioactive compounds. These compounds are capable of killing prostate carcinoma cells by inducing apoptosis (Stampar *et al.,* 2006). It is reported that leaves of *J. regia* L. contain monoterpenes and sesquiterpenes and the bark shows presence of juglone, regiolone, sterols and flavonoids (Inbaraj and Chignell, 2005). The bark, branches and exocarp of the fruit of this plant have been used to treat gastric, liver and lung cancer (Liu *et al.,* 2004). Aqueous extract of husk is a natural source of phenolic compounds with low cost and it has antiradicalar, antioxidant and antimicrobial activities (Oliveira *et al.,* 2008, Carvalho *et al.*, 2010). The antioxidant activity from extracts of walnut kernels, husks and leaves showed strong antioxidant activity and methanol extracts from seed, husk and leaf of *J. regia* showed growth inhibition against human renal cancer cell lines (Carvalho *et al.,* 2010). The walnuts mesocarp has high antimicrobial and antioxidant activity (Oliveira *et al.,* 2008). Numerous studies have demonstrated the antioxidant potential of walnut products; nuts and walnuts mesocarp (Ghasemi *et al.,* 2011; Oliveira *et al.,* 2008), leaves (Pereira *et al.* 2007), bark (Noumi *et al.*, 2011). So in this work, the antioxidant and antibacterial activity from various extracts of endocarp and exocarp in walnuts was studied.

MATERIALS AND METHODS

Plant material

Walnuts collected in Jun 2014 from the North Khorasan Province of Iran and were peeled. The exocarps and endocarps were then removed.

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Preparation of the extracts

The shade-dried (500g) powders of the exocarps and endocarps were suspended in methanol at room temperature for 24h. The whole extracts were filtered through a paper filter and the solvent was evaporated under a vacuum at 45˚C, to yield crude extracts. The extracts were resolved in methanol 95% and successively partitioned with dichloromethane (CH2Cl2), EtOAc and finally, water. The CH2Cl2, EtOAc and water fractions were evaporated under a vacuum. The extracts were stored at 4˚C until analysis (Boozari, 2015).

Total phenolic determination

The total phenolic content was determined using Folin-Ciocalteu method (Hayouni *et al*., 2007). 100µL from each extract (1000mg/L) was added with Folin-Ciocalteu reagent that was diluted with water (1/10, 500µL). After 1 min of reaction, Sodium carbonate (Na_2CO_3) (20%, 1.5 mL) was added to each tube, then tubes were vortexed and incubated for 120min at room temperature. The absorbance of samples was read at 760nm. The analyses were done in triplicates. The standard curve was prepared using 50 to 500mg/L solutions of Gallic acid in methanol. Total phenol values were expressed as Gallic acid equivalents (mg Gallic acid: (GA) per dry weight of extract (mg GAE/g DW).

DPPH radical scavenging assay

2, 2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging ability of the extracts was evaluated by the method of Sharma *et al*. (2009). DPPH was prepared to 0.16mM with methanol. Extracts were dissolved in methanol to several concentrations (8, 4, 2, 1, 0.5, 0.25 mg/ml) and 0.1mL of the solution and 0.1 ml of DPPH were mixed and were kept in the dark for 30 min and the absorbance was read at 517 nm. The experiment was carried out in triplicate. The percentage of radical scavenging activity was calculated using Eq.1.

$$\%\text{DPPH scavenging activity} = \frac{\text{A517 of control - A517 of sample}}{\text{A517 of control}} \quad (1)$$

Methanol was used as control, ascorbic acid and butylated hydroxy toluene (BHT) used as positive controls. AI was calculated as IC_{50} values were calculated using Graph Pad Prism software, version 5.01.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) of the extracts was measured by method of Xu *et al*., (2010). The FRAP reagent was contained from 1mL of 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10mM in 40mM HCl), 1mL of $FeCl_3.6H_2O$ (20mM) and 10mL of acetate buffer with pH 3.6 (0.3M). Three mL from FRAP reagent mixed with 100µl of each sample and then they were incubated at 37^{o}C for 10min in a water bath. After incubation, the absorbance was measured at 593 nm. Aqueous solutions of $FeSO4.7H_2O$ in the range of 0-1 mM, were used for calibration curve. FRAP values were expressed as mean ± standard error (SE) mmol Fe (II) per gram.

Microbial cultures and inoculation conditions

Methanol, dichloromethane, ethyl acetate and aqueous extracts from endocarp and exocarp of walnuts were assayed for antimicrobial activity against three species of bacteria. The bacterial strains included Gram-positive *Staphylococcus Aureus* (PTCC 1431) and *Bacillus cereus* (PTCC 1015); Gram-negative *Escherichia coli* (PTCC 1399). All microorganisms were clinical isolates, obtained from the Persian Type Culture Collection (PTCC). Mueller-Hinton broth was applied for growing and diluting the microorganism suspensions. Bacterial strains were grown in Mueller-Hinton broth at 37°C for 18h and adjusted to a final density of 10^8 CFU/ml by diluting fresh cultures and comparing with McFarland density. In order to prepare the McFarland standard 0.5ml of 0.048M $BaCl_2$ (Merck, Germany) was added to 99.5ml of 0.18M H_2SO_4. In this study, antibacterial activity was assayed by three methods such as Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC), Disk and well diffusion methods.

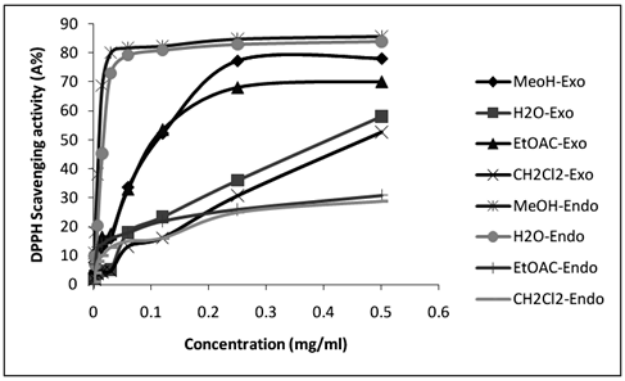

Fig. 1: The effect of concentration on DPPH scavenging

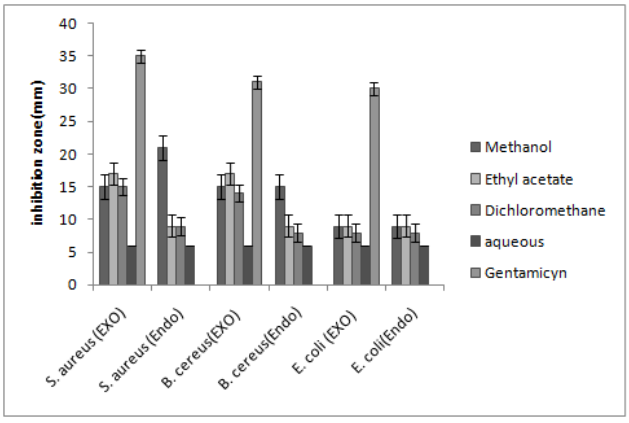

Fig. 2: Inhibition zone of various extracts via Well diffusion

Disk-diffusion method

The discs with 6 mm diameter were placed on the plates with 80 mm diameters containing Mueller Hinton agar (MHA) with the 1.5×10^8 bacterial cells. The extracts were dissolved in dimethyl Sulfoxide (DMSO) to give 500 mg ml^{-1} concentration of them. 20µl of samples placed on the

discs and then they were incubated at 37°C for 24h. Antimicrobial activity was evaluated by measuring the zone of inhibition surrounding the discs. Gentamycin was positive control and DMSO used as negative control (Selim *et al.,* 2014).

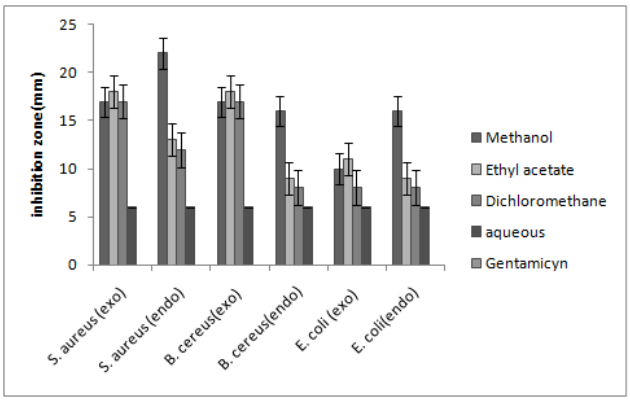

Fig. 3: Inhibition zone of various extracts via Disc diffusion

Well diffusion method

Wells with 6 mm diameter were punched with the help of sterilized cork borer (6 mm), into the agar plates of the appropriate media, which had been surface spread with bacteria at a 10^8 CFU/ml density. Stock solution of plant extract was prepared at a concentration of 500 mg/ml and then 50µl of them were added sterile syringe into the wells and allowed to diffuse at room temperature for 2h. The plates were incubated at 37°C for 24h. Antibacterial activity was evaluated by measuring the zone of inhibition (mm) (Firdaus *et al.,* 2011).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC was determined by micro-dilution method using serially diluted plant extracts according to the National Committee for Clinical Laboratory Standards. MIC was determined by the broth micro-dilution method in a 96-wells micro-plate. The final concentration of microorganism inoculum was 1.5×10^6 CFU ml^{-1} (Coccia *et al.,* 2012). The extracts were diluted in dimethyl Sulfoxide (DMSO) and Mueller Hinton Broth (MHB) to give a concentration of 100, 50, 25, 12.5, 6.25 and 3.125 mg ml^{-1} (Dhiman *et al.,* 2011). Then, 100µl of each concentration was added in a well into 96-well micro plate containing of 1.5×10^6 CFU ml^{-1}. Two wells of each row were designated to be as positive (without the extract) and negative (without the organism) controls. The micro plate was incubated at 37°C for 24 h. After an overnight incubation at 37°C, 20µl of 2,3,5 triphenyltetrazolium chloride (TTC) (5 mg/ml) was added to each well as a colorimetric indicator of bacterial growth and incubated for 60 min at 37°C. The MIC was determined as the lowest concentration of the extracts that showed no color change (Umer *et al.,* 2013). To determine MBC, 10µl was taken from each well and inoculated in MHB for 24 h at 37ºC. The highest dilution that yielded no signal bacterial colony on the solid medium was taken as MBC (Umer *et al.,* 2013).

RESULTS

The yield of extract from endocarps and exocarps of walnut, by four different solvents with varying polarities (dichloromethane, ethyl acetate, methanol and aqueous) is reported in table 1. It was shown from this work that the highest extraction yield was obtained from aqueous extract of exocarp. In this study, phenolic contents in various extracts from endocarp and exocarp of walnut was determined by Folin-Ciocalteu method. The regression equation for determination of total phenolic contents was: $Y=3.742X+0.061$ ($R^2=0.985$) and gallic acid was used for standard curve. The total phenolic contents of extracts are shown in table 1. The total phenolic content of exocarps and endocarps ranged from 47.86 to 58.66 and 34.59 to 68.34 (mg Gallic acid equivalents /g of dry extract) respectively. Two different methods; DPPH and FRAP, were used for the determination of antioxidant activities, it was found that exocarps and endocarps of walnut extracts possess antioxidant properties (table 1). As shown in fig 1, the scavenging activities of all samples were concentration-dependent and results of DPPH reduction are shown in table 1. Equation of FRAP for standard solutions was: $y= 0.350x+ 0.012$ ($R^2 =0.992$). The results of the FRAP assay are reported in table 1. The various extracts of endocarp and exocarp from walnut were screened for their antimicrobial properties against *B. cereus*, *S. aureus* (Gram + bacteria) and *E. coli* (Gram- bacteria). The antibacterial activity determined by diameters of inhibition zones and minimum inhibition concentration of extracts. The response for each microorganism was different. The results of antibacterial activity indicated that the diameters of inhibition zones varied from 6-21mm and 30-35mm for the various extracts and gentamycin respectively. Among the four extracts, the aqueous extract of endocarp and exocarp has no antimicrobial effect against the all bacteria tested. On the other hand the methanolic extract from endocarp of walnut with 21mm inhibition zone had substantial antimicrobial activity against *S. aureus*. The MIC and MBC values of extracts summarized in table 2 and results show that the methanol extract of endocarp and ethyl acetate extract of exocarp were able to prevent the growth of *S. aureus* and *B. cereus*, within the concentration of 25 to 100 mg ml^{-1} the tested bacteria.

DISCUSSION

The antioxidant activity of extracts may be due to the active components and Phenolic compounds (Babbar *et al.,* 2011). Several phenolic compounds such as pyrogallol, p-hydroxybenzoic acid, vanillic acid, ethyl gallate, protocatechuic acid, gallic acid, tannins, glansrins, adenosine and adenine have been isolated from *J. regia*

(Islam Shah *et al.,* 2014). The phenolic compounds such as p-coumaric acid, quercetin glycosides of walnut leaves are responsible for the antibacterial activities of this plant (Stampar *et al.,* 2006). As shown in the table 1, the extract that displayed the highest concentration of total phenols was methanol extract from endocarp. Also in another research, the results showed that the highest content of polyphenols was recorded in endocarp, followed by exocarp and mesocarp of walnut (Nour *et al.,* 2014). The recovery of phenolic compounds from plants is influenced by the solubility of them in the solvent used for the extraction and in other studies, the methanol was better solvent than the others in extracting phenolic compounds due to their polarity and good solubility of them, Therefore, solvent polarity will play an important role in extraction of phenolic compounds (Naczk and Shahidi, 2006). So, the polar extracts had more phenolics than the non-polar extracts (Hernandez-Hernandez *et al.,* 2009). Also the results indicate that the phenolic compounds presented on walnut husk had a moderately polar characteristic, (Chew *et al.,* 2011). The antioxidant activity of phenolic compounds is mainly due to their redox properties and chemical structure, which allow them to act as reducing agents, hydrogen donors and singlet quenchers (Babbar *et al.,* 2011). DPPH is a free radical and stable at room temperature and it can accept an electron or hydrogen radical to become a stable molecule, and free radical scavenging is frequently was done with DPPH method. This method is an easy way to evaluate antioxidant activity. An alcoholic solution of DPPH has a UV–Vis absorption maximum at 517 nm. It loses this absorption when accepting one electron and the result is color variation from purple to yellow, and the degree of discoloration indicates the antioxidant activity (Silva *et al.,* 2005). In this method, IC_{50} was evaluated and it was the concentration of the sample necessary to decrease initial concentration of DPPH and it is well-known that the lower IC_{50} has the higher antiradical activity (Magalhães *et al.,* 2008). The result obtained in this study clearly demonstrated that the extracts of endocarps and exocarps of walnut are powerful antioxidant. In the current investigation, vitamin C had the highest radical scavenging activities. Among the tested extracts, methanol extract from endocarps of walnut exhibited the strongest activity (IC_{50}= 10.3µg/mL) compared to other extracts at all tested concentrations and this extract was stronger than BHT. Radical scavenging activity is related to the nature of phytochemicals and their hydrogen donating activity (Khan *et al.,* 2003). The antioxidant activity is generally attributed to phenolic compounds in plant extracts (Nour *et al.,* 2014). The FRAP assay is a simple and inexpensive method for measuring the antioxidant activity in a sample by oxidation-reduction potential. In this method the ferric reducing ability of plasma was measures and in this method, the antioxidants react with the ferric tripyridyltriazine complex and produce the intense blue color ferrous tripyridyltriazine complex (Gülçin, 2012). The antioxidant activities were expressed as the concentrations of antioxidant having a ferric reducing ability equivalent to that of 1mM of $FeSO_4$. Antioxidants in the samples reduce Fe (III)-TPTZ to form a blue colored Fe (II)-TPTZ complex with increase in the absorbance at 593nm (Gülçin, 2012). As it was shown in table 1, all the plant extracts had antioxidant activity, while methanol extract of endocarp showed significantly the highest results and dichloromethane extract showed the lowest power. In one study, among of shell, leaf, stem, defatted walnut kernel and husk, the walnut shell extract showed the highest phenolic content and it has the best antioxidant activity (Wang *et al.*, 2015). In another research, from different extracts of Walnut Shell, the methanol extract shows the greatest total antioxidant activity and reducing power (Yang *et al.,* 2014). As shown in figs 2,3 the extracts had antimicrobial effect against Gram negative bacteria *E. coli*, but their activity on this microorganism was less than Gram positive bacteria, because the Gram negative bacteria have a lipo-polysaccharide outer membrane and the transfer of molecules is achieved through the cell membrane. The antimicrobial effect is related with the ability of the compounds to penetrate the outer membrane and reach its site of action, influenced by their size and shape (Kavak *et al.,* 2010). And in this study, the compounds present in aqueous extract from walnut probably couldn't pass through the cell membrane. In this study, the results showed in endocarp of walnut, methanol extract has higher amount of phenolic compounds and also this extract had highest antibacterial activity on three tested microorganisms. On the other hand, in exocarp of walnut, ethyl acetate extract with high level of phenolic content, had highest antibacterial activity, so we can say the phenolic compounds can attributed in antibacterial activity. In another study, the antibacterial activity of *J. regia* hull extracts was determined and it exhibits good antibacterial activity against all the bacterial species (Sharma 2013). Antimicrobial activity of aqueous walnut husk extracts against *B. cereus*, *S. aureus* and *B. subtilis* was previously tested and it found considerable lower values for the MIC (Oliveira *et al*., 2008). In one study, the minimum inhibitory concentrations (MIC) for ethanol extract of walnut leaves ranged between 15.6 and 187.5 mg/mL and minimum bactericidal concentrations (MBC) ranged between 31.25 and 250mg/mL (Sharafati-Chaleshtori, and Rafieian 2011). According to another study on hull of *Juglans regia*, the maximum antibacterial activity was observed in ethanol extract when compared to other extract (Sharma 2013). The differences found in the antimicrobial activity of the different extracts could be attributable to the different phenolic composition of the extracts (Alanon *et al*., 2011). Antimicrobial activity seems to be dependent on the phenolic structure and the phenolic compounds influence the growth of microorganisms.

Table 1: Extraction yield, total phenolic compounds, antioxidant potential by DPPH and FRAP from exocarps and endocarps of green walnut from exocarps and endocarps of green walnut

				Exocarp	Endocarp			
Extracts	Extraction yield (%)	Total phenolic (Gallic acid equivalents mg/g of dry extract)	IC_{50} (µg/mL) in DPPH assay	FRAP value (mmol $Fe^{2+/}$g dry extract)	Extraction yield (%)	Total phenolic (Gallic acid equivalents mg/g of dry extract)	IC_{50} (µg/mL) in DPPH assay	FRAP value (mmol $Fe^{2+/}$g dry extract)
CH_2CL_2	0.896%±0.9	42.69±0.12	600±0.3	535±0.8	1.12%±0.1	51.32±0.2	7090±0.7	482.75±0.8
ETOAC	0.63%±0.2	58.66±0.37	114±1.4	705±1.2	2.03%±0.7	34.59±0.9	960±1.4	1114.94±1.2
MEOH	3.328%±1.9	37.86±0.41	117±0.7	1220±0.6	4.43%±1.6	68.34±5.9	10.3±0.8	2202.29±0.5
H_2O	16.21%±0.6	54.42±1.68	420±0.5	485±2.9	2.3%±2.3	58.91±3.1	16±2.8	2020.68±0.7
BHT			13±0.2				13±0.2	
Vit C			3±0.9				3±0.9	

Table 2: Minimum inhibitory concentrations (MIC, mg/ml) and Minimum bactericidal concentrations (MBC, mg/ml) from exocarps and endocarps of walnut various extracts

		Exocarp				Endocarp			
Extract		MEOH	ETOAC	CH_2CL_2	H_2O	MEOH	ETOAC	CH_2CL_2	H_2O
S. aureus	MIC	50	25	50	℘ 100	25	100	100	℘ 100
	MBC	℘100	100	℘100	℘100	100	℘100	℘100	℘100
B. cereus	MIC	50	25	50	℘100	50	100	℘100	℘100
	MBC	℘100	100	℘100	℘100	100	℘100	℘100	℘100
E. coli	MIC	100	50	100	℘100	100	℘100	℘100	℘100
	MBC	℘100	℘100	℘100	℘100	℘100	℘100	℘100	℘100

CONCLUSION

The data obtained in this study showed that the methanol extract from endocarp of *Juglans regia L.* was a rich phenolics and flavonoids and possessed significant free radical scavenging activity which was comparable to a synthetic antioxidant compound (i.e. BHT). Based on results it can be concluded that methanol extract from endocarp of *Juglans regia L.* may prove effective in treating various diseases that are caused by free radicals produced in body as a result of extreme oxidative stress, thus can provide an alternative source of various synthetic antioxidant agents e.g. BHT.

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