JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2012. Vol. **6**(2), p. 583-588

Chemical Composition and *in vitro* Antimicrobial Activity of Walnut (*Juglans regia*) Green Husks and Leaves from West Anatolia

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(Received: 04 September 2011; accepted: 26 October 2011)

***Juglans regia* L. (Juglandeaceae) is used medicinally for centuries, but it also is used in pharmaceutical and cosmetic industries. The extract of walnut green husks and walnut leaves *Juglans regia*L. (*Juglandeaceae*) were screened *in vitro* for their antimicrobial activity against Gram-positive, Gram-negative bacteria and two yeasts. The chemical composition of walnut leaves and green husks were analyzed. Disk diffusion method was used for antimicrobial activity. GC/MS analysis was tested for chemical composition of walnut green husks and walnut leaves extract. The aqueous extract of green husks showed antimicrobial activity against five of the seven Gram-positive bacteria and six of the nine Gram-negative bacteria. Contents of the aqueous extract of walnut green husks were ethylene oxide (83.67%), cyclotrisiloxane hexamethyl (5.04%), and walnut leaves were ethylene oxide (14.74%), cyclotrisiloxane hexamethyl (17.89%). The aqueous extract of the walnut leaves was effective against four Gram-positive and one Gram-negative organism. Both walnut green husks and leaves extracts exhibited antifungal activity. The results of the present in vitro work indicate that aqueous extract of walnut leaves could be used as natural antimicrobial agents in the food preservation and human health for pathogenic and/or antibiotic resistant bacteria because of contain minimum percentage of ethylene oxide (14.74%) compared to walnut green husks.**

Key Words: *Juglans regia* L, Green husk, Chemical composition, GC/MS analysis, Antimicrobial activity.

Herbs and spices are known for their antimicrobial and antioxidative properties. Generally, essential oils of spices possess strong antibacterial properties against foodborne pathogens and contain high concentration of phenolic compounds[1,2]. These compounds exhibit a wide range of biological effects, including antioxidant properties. The content of phenolic compounds depends on many environmental conditions, as well as genotype of differ-ent cultivars[3] and on the geographical location, on climatic conditions[4]. The concentrations of phenols depend on developmental stages of nuts[3]. As regards to correlation between content and time period, the highest content of phenolic compounds was found in May and July[5]. Therefore, we collected samples in June.

The walnut (*Juglans regia* L. Juglandaceae) plant has a high nutritional value and high-quality wood[5]. Because walnut is a valuable crop, the nut is very popular and largely consumed in Turkey, this species is widely spread

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throughout the country[6]. In addition, walnuts have significant economical value and medicinal importance for human health because of their biochemical composition of polyunsaturated fatty acids, especially 18:2 and 18:3 and protein value[5]. Not only dried fruit (nuts) have been used, but also green walnuts, shells, kernels, bark, green walnut husks (epikarp) and leaves have been used in both cosmetic and pharmaceutical industry[6]. Dryied walnut leaves are boiled then used for as a tea in Turkey. Since aqueous extracts of walnut leaves and green husks were tested in the present study. Therefore, our study aims to test antimicrobial activity against microorganisms of aqueous extract, obtained from walnut green husks and leaves, and their chemical compositions. Chemical compositions were accessed by GC/MS analysis. We also demonstrate for the first time, as far as we know, the antimicrobial activity of aquoeous walnut green husks and leaves extracts from West Anatolia studying their antimicrobial capacity against some bacteria and two yeasts.

MATERIALS AND METHODS

Samples

Walnuts' green husks and leaves were obtained from Çine that were collected on 1st June, West Anatolian of Turkey (N:37-32, 30.1E: 28º 08' 35.6 altitude: 520m). The orchard has a planting density of 5.5x9m.The trees are twenty-five years old. They are pruned when necessary. No phytosanitary treatments were applied.

Preparation of samples

Walnut green husks

Before each kind of analyses (antimicrobial activities assays) the walnut green husks were extracted in 250 mL of boiling water for 45 min, and filtered through Whatman no. 4 paper (for each cultivar, three powdered subsamples, 5g, 20 mesh). The aqueous extracts were frozen, lyophilized (using CHRIST Alpha 1-4 lyophilizer) and redissolved in water at concentrations of 100 mg/mL for antimicrobial activity assay [7].

Walnut leaves

For each sample, about 100 g of walnut leaves were manually collected from the middle third of branches exposed to sunlight, dried in a stove at 30 °C for five days and stored in paper bags in order to protect them from light. The walnut leaves were extracted with 250 mL of boiling water for 45 min, and filtered through Whatman no. 4 paper (for each cultivar, three powdered subsamples 5g,20 mesh). The aqueous extracts were frozen, lyophilized (using CHRIST Alpha 1-4 lyophilizer) and redissolved in water at concentrations of 100 mg/mL for antimicrobial activity assay [7].

Antimicrobial activity

Test microorganisms

In this study, the following microorganisms were used seven Gram-positive bacteria strains; *Staphylococcus aureus* ATCC 6538P, *S.aureus* ATCC 43300 methicillin-oxacillin resistant, *S.aureus* MU 40 methicillin-oxacillin resistant, *Micrococcus luteus* NRRLB-4375, *S.faecalis* ATCC 4083, *B.subtilis* ATCC 6633, *Bacillus cereus* CCM 99, nine Gram-negative bacteria strains; *Escherichia coli* ATCC 29998, *E.coli* ATCC 35218, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 13047, *Salmonella typhimurium* CCM 3819, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC12843, two specific pathogenic strains (hemorrhagic *E.coli* O157:H7 RSSK 04054, and *Klebsiella pneumoniae* CCM 2318).Yeasts; *Candida albicans* ATCC 10259 and *Candida tropicalis* ATCC 750.

Bacteria were cultured on Trypticasein Soya Agar (TSA), and yeasts on Yeast Extract Agar (YEA). All stock strains were kept at 4°C.

Disk diffusion method

Antimicrobial activity was measured by the disk diffusion method [8]. 30 µL/disc walnut husks and leaves extract (1.0 mg/disc) were applied to per sterile 6 mm diameter filter paper discs (Schleicher and Schüll, No. 2668, Germany). The suspensions of microorganisms were initially adjusted with sterile distilled water to a density equivalent to the 0.5 McFarland standards. 0.2 ml of a 24 h-broth culture (10^6 cfu/ml) of the microorganism species were spread on the surface of gelled sterile Mueller-Hinton Agar plates. The extract of walnut husks and leaves were prepared and then adsorbed onto the sterile discs (30 µL). The paper discs containing the extract were air-dried and placed on the surface of each plate. The antimicrobial activity of the extract against the test microorganisms was indicated by the growth-free "zone of inhibition" near the respective disc. All

tests were performed under sterile conditions in duplicate and repeated two times. Eritromycin discs (Oxoid, 10 µg/disc), penicilin discs (Oxoid, 10U/disc) and nystatin discs (Oxoid, 30 µg/disc) were used as positive reference standards and water was used as negative control to determine the sensitivity of the tested strains.

GC/MS analysis

The steam-distilled components were analysed by GC/MS. A HP 6890 gas chromatograpph equipped with a HP-PTV and a 0.32mX0.60m HP-Innowax capillary column (0.5µm coating) was employed for the GC analysis. GC/MS analysis was performed on a HP-5973 mass selective detector coupled with a 6890 gas chromatograph, equipped with a HP 6890 gas chromatograph, equipped with HP-1capillary column. Identification of the individual components was performed by comparison of mass spectra with literature data and by a comparison of their retention indices (RI) relative to a $C_{8-}C_{32}$ *n*-alkanes mixture [9]. A computerized search was carried out using the Wiley 7n.l GC/MS library and ARGEFAR GC/MS library created with authentic samples.

RESULTS AND DISCUSSION

The results indicate that aqueous extracts of walnut green husks and leaves showed antimicrobial activity in table 1. The results revealed that aqueous extracts of walnut green leaves showed antimicrobial activity against *C.albicans* ATCC 10259 and *C.tropicalis* ATCC 750, *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633 and *K.pneumoniae* CCM 2318, *S.aureus* MU 40 methicillin-oxacillin resistant, *S.aureus* ATCC 43300 methicillin-oxacillin resistant in *table 1*. All extracts studied in this work showed antimicrobial activity against some of the test microorganisms with the exception of *M. luteus* NRRL B-4375, *S.faecalis* ATCC 4083, *E. aerogenes* ATCC 13048, *E. cloacae* ATCC 13047, *S. typhimurium* CCM 3819 which showed no such activity. Also, both aqueous

Table 1. Antimicrobial activity of walnut green husks and walnut leaves extracts.

Microorganisms	Gr	Inhibition zone (mm)* (30µL) discs		Standarts antibiotics		
		Walnut green husks	Walnut leaves	Ery	Pen	Nys
S.aureus ATCC 6538P	+	11	13	21	20	nt
M.luteus NRRL B-4375	+	-	-	-	-	nt
S.faecalis ATCC 4083	+	-	-	8	-	nt
B.subtilis ATCC 6633	+	10	13	22	21	nt
B.cereus CCM 99	+	8	-	12	-	nt
*S.aureus*ATCC 43300 met-oxa res	+	10	17	22	10	nt
S.aureus MU 40 met-oxa res	+	11	14	24	13	nt
E.coli ATCC 29998	-	10	-	9	11	nt
E.coli ATCC 35218	-	10	-	9	14	nt
E.coli O157:H7 RSSK 04054	-	9	-	12	10	nt
E.aerogenes ATCC 13048	-	-	-	11	11	nt
E.cloacae ATCC 13047	-	-	-	9	10	nt
S.typhimurium CCM 3819	-	-	-	11	14	nt
P.aeruginosa ATCC 27853	-	8	-	-	-	nt
*P.fluorescens*ATCC12843	-	15	-	22	11	nt
K.pneumoniae CCM 2318	-	12	13	19	23	nt
C.albicans ATCC 10259	Y	12	9	Nt	nt	18
C.tropicalis ATCC 750	Y	14	9	Nt	nt	19

*Zone of inhibition, including the diameter of the filter paper disc (6 mm); mean value of two independent experiments; Ery, eritromycin (10 µg/disc); Pen, penicilin (10U/disc) ;Nys, nystatin (30 µg/disc); nt, not tested; Gr, gram reaction; –, no activity, Y, yeast.

extracts of walnut green husks and aqueous extracts of walnut leaves shown antimicrobial activity against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *S.aureus* ATCC 43300 methicillin-oxacillin resistant, *K.pneumoniae* CCM 2318. The aqueous extracts of walnut green husks (72%,13/18) showed antimicrobial activity against microorganisms species, whereas aqueous extracts of walnut leaves (38%, 7/18) showed antimicrobial activity.

In Portugal, Pereira and his colleaques reported that five different aqueous extracts of different cultivars walnut (*J.regia* L.) green husks showed antimicrobial activity on *B.cereus*, *B.subtilis* and *S.aureus*, but the extracts didn't inhibit the growth of *E.coli*, *P.aeruginosa* and *K.pneumoniae*[10]. In our study aqueous extracts of green husks inhibited *P.aeruginosa* ATCC 27853, *K.pneumoniae* CCM 2318, *E. coli* ATCC 29998, *E.coli* ATCC 35218. Their results are not in accordance with ours. In their study gram negative bacteria and fungi were resistant to the extracts of 100mg/mL. It may be affect geographical location and genotype of different cultivars.

Alkhawajah (1997) reported that aqueous extract of *J.regia* L. bark showed antimicrobial activity *S.aureus*, *S.mutans*, *E.coli*, *P.aeruginosa* and *C.albicans* like ours[11] . Also Qa'dan *et al* (2005) reported that *J.regia* leaves extracts with antimicrobial activity isolated from acne lesion, *Propionibacterium acnes*, *S.aureus* and *S.epidermidis*[12] . Their results were similar to ours. Also, Mehrabian *et al*. (2000), reported that methanolic extracts of *J.regia* had more antimicrobial effects than aqueous their extracts because most of their effective compounds are from the quinone family and are alcohol soluble[13].

In our study, *S.aureus* ATCC 43300 methicillin-oxacillin resistant, *S.aureus* MU 40 methicillin-oxacillin resistant and *E. coli* O157:H7 have been tested for the first time. Aqueous extracts of walnut green husk's showed antimicrobial activity on *E. coli* O157:H7 (9 mm inhibition zone) and most of the foodborne bacteria. But walnut green leaves didn't. Methicillin resistant *S.aureus* (MRSA) is a major cause of morbidity and mortality around the world and has been the most common cause of nosocomial infection since 1970s[14]. *S.aureus* ATCC 43300 methicillin-oxacillin resistant, *S.aureus* MU 40 methicillin-oxacillin resistant were also tested in our work. It is important that these bacteria were inhibited by the extracts of walnut leaves and walnut green walnut husks harvested in the Western part of Anatolia, Turkey.

In Turkey,Yigit *et al* (2009) reported that aqueous and methanol extracts of the walnut green husks and leaves showed anticandidal activity especially *C.albicans, Candida glabrata, C.tropicalis* and *C.kefyr* [15]. Also Oliveria *et al*(2008) aqueous extract of walnut leaves showed antifungal activity for *C.albicans, Crytococcus neoforans*[6]. In our study both walnut leaves and green husks showed anticandidal activity as before reported studies.

The main constituents of the walnut leaves extract were cyclotrisiloxane hexamethyl (17.89%) and ethylene oxide (14.74%) (Table 2) and the main constituents of the walnut green husks extract were ethylene oxide (83.67%) and cyclotrisiloxane hexamethyl (5.04%) (Table 3). However ethylene oxide has a high percentage in walnut green husks extract, it has a small percentage in walnut leaves extract. There is cyclotrisiloxane hexamethyl in walnut leaves extract more than aproximately three times in walnut green husks extract. In different works reported that chemical composition of walnut leaves and green husks extracts using by different methods and extracts[4,5,10,16]. Other researchers used ether extract of walnut leaves and green husks[17,18]. Buttery *et al*.,(1986) identified caryophyllene, â ocimene, â pinene and limonene in walnut leaves[17]. Also Buttery *et al*., (2000) identified (E)-4,8-dimethyl-1,3,7-nonatriene, pinocarvone, pinocarveol, myrtenal, myrtenol, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, caryophyllene epoxide, verbenol, verbenone, and terpinolene in walnut green husks[18]. Cosmulescu *et al*.,(2010) reported that six compouds (ferulic acid, vanillic acid, coumaric acid, syringic acid, myricetin and juglone) were identified in all cultivars by using reverse phase-high performance liquid chromatography (HPLC-RP)[16]. Also Amaral *et al*.,(2004) showed that two extractive procedures were assayed and best results were obtained using acidified water and a solid phase extraction column purification step. Qualitative analysis was performed by HPLC-DAD/MS and, in all samples, seven phenolic compounds were identified (3-caffeoylquinic,3-*p*-coumaroyl-quinicand 4-*p*-coumaroylquinic acids, quercetin3-

Table 2. Volatile compounds of walnut leaves extract (GC/MS analysis)

Compound*	Area (%)	Retention time (min)
Ethylene oxide	14.74	3.55
Cyclohexane	4.15	4.13
Carbonic acid	3.58	4.56
Cyclotrisiloxane,hexamethyl	17.89	4.62
Acetic acid,ethyl ester	3.31	5.14
Ethanol	9.92	5.64
Cyclotetrasixane,octamethyl	10.88	6.11
2-Pentanone	8.71	6.52
Methane	3.41	7.20
Spiro(cyclopenta)	0.70	8.10
Cyclopentasiloxane,decamethyl	7.56	13.07
6-Aza-5,7,12,14-Tetrathiapentacene	1.17	13.20
2,4-di(trimethylsiloxy)	5.43	17.69
Acetic acid	0.74	19.68
1-Pentene,1,3diphenyl-1	2.48	19.76
Methoxycarbonyl	1.22	20.12
Benzaldehyde,2,4-bis	0.9	20.22
Dimethylamine	1.06	20.47
Benzoic acid	2.08	21.20
Total	99.98	

* Compounds listed in order of elution from the column.

Table 3. Volatile compounds of walnut green husk's extracts (GC/MS analysis)

Compound*	Area (%)	Retention time (min)
Ethylene oxide	83.67	3.71
2-Formylhistamine	0.31	4.05
Cyclohexane	0.79	4.14
Acetanone	1.11	4.56
Cyclotrisiloxane,hexamethyl	5.04	4.83
N-ethyl-1,3-dithiosoindoline	0.30	5.15
Cyclotetrasixane,octamethyl	2.33	5.97
N-methyl-propylamine	0.06	6.11
Cyclopentaasiloxane,decamethyl	2.62	6.21
2,4-di(trimethylsiloxy)-6,7-(methyl lenedioxy)	1.64	17.68
11H-Dibenzo	1.04	19.76
p-Meth-1-en-3,1,semicarbazone	0.11	20.12
Pentadecone	0.08	20.22
6-Aza-5,7,12,14-tetrathiapentacene	0.43	21.19
Total	99.53	

* Compounds listed in order of elution from the column.

galactoside, quercetin 3-arabinoside, quercetin3-xyloside, quercetin3-rhamnoside) and two other partially identified phenolics (quercetin3-pentoside and kaempferol 3-pentoside derivatives) were also detected. In their study revealed that quercetin 3-galactoside was always the major compound while 4-*p*- coumaroylquinic acid was the minor one[4]. Pereira *et al* (2007) reported that phenolics analysis was performed by reverse-phaseHPLC-DAD and 10 compounds were identified and quantified: 3 and 5 caffeoylquinic acids, 3 and 4-*p*-coumaroylquinic acids, *p*-coumaric acid, quercetin 3-rhamnoside[10]. Mishra and Sree (2007) reported that the GC-MS analyses of different extracts analysis revealed the presence of different chemicals as major constituents. Although, chloroform extract is found to contain Urs-12-ene(27.44%) as major constituents, hexan extract is found to contain 1,2-benzenedicarboxyclic acid, dioctyl ester (10.22%) as major constiuents. FAME of Folch extract is found to contain hexadecanoic acid/palmitic acid (54.65%) as major constiuents in their study[19]. In our study, we used aqueous extracts of walnut leaves and green husks. As far as we know, this is the first report that GC-MS analysis was tested for chemical composition of walnut green husks and walnut leaves extract. Different extracts and methods may be affected main components of walnut leaves and green husks.

Ethylene oxide and cyclotrisiloxane hexamethyl are well known chemicals with their pronounced antimicrobial potentials [20,21,22.] Ethylene oxide is the most commonly used form of sterilization.Ethylene oxide is also classified as a carcinogen, mutagenic and thus, exposure should be kept to minimum.Antimicrobial activities of unsaturated cyclohexane have also been previously reported by Hinou *et al*.,(1989)[23].

CONCLUSIONS

The results of the present *in vitro* work indicate that although aqueous extracts of walnut leaves and green husks have antimicrobial activity, aqueous extracts of walnut leaves could be used as natural antimicrobial agents in the food preservation and human health for pathogenic and/or antibiotic resistant bacteria, because of contain minimum presence of ethylene oxide (14.74%)

compared to aqueous extracts walnut green husks (83.67%). Further research is needed in order to obtain information regarding the practical effectiveness of extracts to prevent the growth of food borne and spoiling microorganisms under the specific application conditions. The use of some antibiotics no longer recommended because of the potency of the widespread resistance to them. Thus, herbs and plants can be used instead of antibiotics.

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