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Full Length Research Paper

Selection studies on high quality walnut types and their antibacterial properties

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This study was conducted to determine high quality walnut types and their antimicrobial properties in the Hasankeyf District of Batman province in Turkey between 2008 and 2009. This research is very important because no studies have been performed in the district about walnut trees until now. At the end of the study, it was determined that average fruit weight, kernel weight and kernel ratio of the selected types varied from 11.32 to 15.33 g, from 6.24 to 7.77 g and from 49.78 to 57.17 g, respectively. In addition, it was found in the antibacterial activities of extracts in ethanol and water of the walnut types. Microorganisms were inhibited by the walnut types in different ratios.

Key words: Walnut, selection, antibacterial activity, water and ethanol.

INTRODUCTION

Walnut is found throughout the world such as in Japan, China, Southern Asia from India and Turkey, in South Eastern Europe to the Carpathian Mountains of Poland, in the eastern and southern parts of the United States, in Mexico and Central America from Colombia to Argentina (Granahan and Leslie, 1990). Turkey, with its various eco-geographical regions, is one of the major centres for the Persian walnut diversity. Native walnut populations are widely present in the country (Jay-Allemand et al.,1996) and are found as scattered individuals or groups of several trees in the borders of agricultural lands, orchards or by the rivers, usually close to human settlements (Fernandez-lopez et al., 2003).

The Juglans genus (family Juglandaceae) comprises several species and is widely distributed throughout the world. The walnut tree (*Juglans regia L.*), its well-known member, is constitute an important species of deciduous trees, found primarily in temperate areas and cultivated commercially throughout southern Europe, Northern Africa, eastern Asia, United States and western South America. The nuts are very popular and are largely consumed as a part of the Mediterranean diet. Nevertheless, not only seeds are used but also shells, bark, green husks and leaves in the cosmetic and pharmaceutical industries (Oliveira et al., 2008).

Several studies suggest that regular consumption of walnut types and cultivars may have useful effects against oxidative stress mediated diseases such as cardiovascular diseases and cancer. Walnut contains several phenolic compounds which have contributed to their biological properties. The present study reports the total phenolic contents and antioxidant properties of methanolic and petroleum ether extracts were obtained from seed, green husk and leaf of walnut (Carvalho al., 2010). Various studies have been carried out by researchers about a lot of fruit species in some regions of Turkey (Sen, 1980; Akca and Sen, 1994; Askin and Gun, 1995; Kuden et al., 1995; Akca and Ayhan, 1996; Akca and Osmanoglu, 1996; Akca and Muratoglu, 1996; Karadeniz and Sahinbas, 1996; Beyhan, 2005; Oguz and Askin, 2007; Simsek, 2009; Beyhan et al., 2010; Demirkiran and Cengiz, 2010; Simsek, 2010; Simsek and Osmanoglu, 2010). The Southeast Anatolia region of Turkey generally faces arid and hot summers as well as cold and rainy winters. It was observed that Walnut leaves selectively inhibited the growth of Gram positive

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and Gram negative bacteria. The antimicrobial effect was screened against Gram positive (*B. cereus, B. subtilis, Staphylococcus aureus*) and Gram negative bacteria *(Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae)* (Pereira et al., 2007). It was inhibited the growth of several species of pathogenic micro-organisms representing Gram-positive bacteria (*S. aureus* and *Streptococcus mutans*) and Gram-negative bacteria (*E. coli and P. aeruginosa)* by walnut types (Alkhawajah, 1997). Antiradical and antibacterial activities have also been recently described for different *J. regia* cultivars (Almeida et al., 2008). The objective of this study is to determine to antibacterial activity and significant fruit properties of some walnut types which have high quality.

MATERIALS AND METHODS

The present study was carried out in the Hasankeyf District of Batman Province in Turkey during the years 2008 and 2009.

Selection of superior walnut types

In this study, the first 50 walnut trees were marked and evaluated. Among these walnut trees, 25 types were eliminated because the fruit weight, kernel weight and kernel ratio of them were less than 11.00 g, less than 6.00 g and less than 49.00%, respectively. Finally, 5 superior walnut types were selected according to Weight Ranked Method of Sen (1980) as shown in Table 1.

Microorganisms

Strains of microorganisms used in the study were taken from Laboratory of Microbiology Culture Collection in Department of Biological Sciences of Arts and Sciences Faculty in Kahramanmaras Sutcu Imam University. In this department, *Klebsiella oxytoca, K. pneumoniae* FMC 5, *P. aeruginosa* DSM 50071, *Acinobacter boumonii, Brucella melitensis, Bacillus megaterium* DSM 32, *S. aureus* 6538, *Bacillus cereus, Micrococcus luteus* LA, Bacillus. brevis FMC 3 species of bacteria were used in the investigation.

Preparation of antimicrobial disks from the walnut extracts

Samples were brought to the laboratory and dried in shadow. They were broken into pieces under sterile conditions. Then, 20 g from each of the samples was dipped in 150 ml ethanol and 150 ml water extracts, respectively and then placed as separated to Soxhlet device. The extraction in this device took 24 h (Khan et al., 1988; Özçelik, 1992). This extracts were impregnated to sterile empty antibiotic discs in 6 mm diameter by 10 and 20 µl micropipettes. Ethanol or water impregnated disks were used as control.

Standard antibiotic discs (Ampicillin, Aztreonam Gentamicin, Fox, Penicillin, Erythromycin, Teicoplanin, Vancomycin) used for comparison were provided from Microbiology Laboratory of Bingol State Hospital.

Preparation of microorganism cultures

Bacterial strains were vaccined to Nutrient Broth and incubated at 37 ± 0.5°C for 18 h. In addition, Müller Hinton Agar in erlenmayer cups were cooled down to 45 to 50°C. 10^6-10^7CFU/ml bacteria were immunized into this agar.

Then, 15 ml Müller Hinton Agar was transferred to each Petri dishes in 9.0 cm diameter and incubated for about 18 h. Extract-impregnated discs were placed by pressing gently to solidified agar. To ensure prediffusion, the bacteria inoculated plates were incubated at 37 ± 0.5°C for 18 to 24 h after waiting one hour at 4°C (Collins and Lyne, 1987; David and Mccuen, 1988; Bradshaw, 1992). Finally, inhibited zones in these Petries were evaluated as millimeter.

RESULTS AND DISCUSSION

Some physical properties of the selected walnut types (average of years 2008 to 2009) were given in Table 2. Fruit and kernel weights of walnut types and cultivars are two significant physical properties for their breeding objectives. Other significant properties for the selected walnut types and cultivars are kernel ratio, shell thickness, shell colour and shell roughness.

It was determined that the fruit weight, the fruit length, the fruit width, the fruit height, the shell thickness, the kernel weight and the kernel ratio of the selected walnut types changed from 11.52 to 15.33 g, 25.37 to 39.64 mm, 30.86 to 35.02 mm, 31.08 to 33.52 mm, 1.24 to 1.85 mm, 6.24 to 7.77 g and 49.78 to 57.17%, respectively. Additionally, it was observed that all the selected types had light inward colour, light peel colour, easy shell removal, about 90% fullness ratio of kernel, about 90% wholeness ratio of kernel, about 90% ratio of non-shrivelling kernel, weak shell adhesion and smooth shell roughness. Akca and Sen (2001) determined that the fruit weight, the kernel weight, the shell thickness, the fruit width and the fruit length changed between 7.49 to 13.93 g, 2.61 to 5.73 g, 1.32 to 2.45 mm, 22.30 to 32.26 mm and 32.90 to 49.25 mm, respectively. Simsek and Osmanoglu (2010) determined that fruit weight, fruit length, fruit width, shell thickness, kernel weight and kernel ratio of promising walnut genotypes varied from 10.29 to 14.55 g, 35.64 to 42.02 mm, 29.78 to 34.46 mm, 1.27 to 1.90 mm, 5.55 to 7.22 g and 43.58 to 63.10%, respectively. Simsek (2010) determined that the average fruit weight, the fruit length, the kernel weight, and the kernel ratio of the walnut types changed between 10.19 to 15.22 g, 34.42 to 42.89 mm, 5.26 to 7.33 g and 48.24 to 59.75%, respectively.

Beyhan and Ozatar (2007) determined to be fair or smooth of shell roughness, dark or light of peel colour, light yellow, yellow, yellow brown and brown of kernel colour and the higher than 90% of internal ratio of non-shrink of the walnut types. Kernel colour and peel colour walnut types and cultivars can change according to the genetic properties and light density. The results with respect to physical properties of the walnut types selected in this study were partly different from each Other and those of the other researchers. The findings about antibacterial activity properties of the walnut types are given in Tables 3, 4, 5 and 6, respectively. As shown

Table 1. Evaluations according to the weighted ranked method of the superior walnut types.

Characteristics	Weighting factor (coeficient)	Classifications and points		Characteristics	Weighting factor (coeficient)	Classifications and points	
Fruit weight	25	17 g < 15-17 g <15 g	25 20 15	Kernel ratio	20	50%< 45-50% <45%	20 15 10
Shell roughness	15	Smooth Medium Roughness	15 10 5	Peel Colour	15	Light Dark Brown	15 10 5
Fruit width	5	35 mm< 30-35 mm	5 3	Shell adhesion	5	Weak Strong	5 3
Fullness ratio of kernel	5	90-100% 80-90% <80%	5 3 1	Wholeness ratio of kernel	5	90-100% 80-90% <80%	5 3 1
Shell thickness	5	<1.2 mm 1.2-1.5 mm 1.5 mm <	5 3 1				
Kernel weight	25	8.0 g < 7-8 g <7.0 g	25 20 15	Kernel ratio	20	50%< 45-50% <45%	20 15 10
Inward colour	20	Light Dark yellow Brown	20 15 10	Shell removal	15	Easy Medium Hard	15 10 5
Fullness ratio of kernel	5	90-100% 80 -90% <80%	5 3 1	Wholeness ratio of kernel	5	90-100% 80-90% <80%	5 3 1
Ratio of non-shrivelling kernel	5	90-100% 80-90% <80%	5 3 1				

Table 2. Some physical properties of the selected walnut types (average of the years 2008 to 2009).

Type no.	Fruit weight (g)	Fruit length (mm)	Fruit width (mm)	Fruit height (mm)	Shell thickness (mm)	Kernel weight (g)	Kernel ratio (%)
HK-1	12.09±1.02	25.37±1.52	30.86±0.39	31.24±0.30	1.54±0.38	6.89±0.16	57.17±4.00
HK-2	11.32±0.19	38.07±0.57	32.68±0.58	31.08±0.45	1.24±0.32	6.42±0.15	56.71±1.89
HK-3	14.62±0.19	39.64±0.82	34.06±0.12	31.83±0.41	1.76±0.26	7.77±0.21	53.14±1.50
HK-4	11.52±0.13	39.49±0.95	31.88±1.19	33.05±0.64	1.85±0.15	6.24±0.30	54.12±1.96
HK-5	15.33±2.06	39.26±0.39	35.02±0.79	33.52±1.50	1.63±0.20	7.56±0.34	49.78±5.67
LSD	2.72	23.76	1.67	2.14	0.81	0.67	8.93±

in these tables, it was determined that the extracts in ethanol of the kernel portion had an antibacterial effect on bacteria named *K. oxytoca, K. pneumoniae* FMC 5, *P. aeruginosa* DSM 50 071, *A. boumonii, B. melitensis, B. megaterium* DSM 32, *S. aureus* 6538, *B. cereus*, *M. luteus* LA, *Bacillus brevis* FMC 3 bacteria (7 to 18 mm inhibition zone). However, it was assessed that water extracts were less effective than ethanol extracts. No antibacterial effects have been shown in water and ethanol of husks of HK-8. Shell extracts of HK-2 and HK-3

Table 3. Antibacterial susceptibility of kernel and shell of walnut in water and etanol of HK-1 and HK-2.

Bacteria	HK-1								HK-2							
	SW	SW	SE	SE	KW	KW	KE	KE	SW	SW	SE	SE	KW	KW	KE	KE
	2 mg/disc	4 mg/disc	2 mg/disc	4 mg/disc	2 mg/disc	4 mg/disc	2 mg/disc	4 mg/disc	2 g/disc	4 mg/disc	2 mg/disc	4 mg/disc	2 mg/disc	4 mg/disc	2 mg/disc	4 mg/disc
Klebsiella oxycota	-	8	-	7	7	9	10	13	7	8	7	8	-	8	11	14
Klebsiella pneumonia	-	-	7	7	7	8	9	12		-	8	8	-	-	8	15
Pseudomonas aeroginosa DSM 50071	7	8	7	8	7	8	9	12		7	7	8	-	-	10	14
Acinobacter boumonii	-	-	7	8	-	-	10	12		7	7	8	-	-	8	12
Brucella melitentis	-	7	-	-	-	7	8	12		7	-	-	-	-	9	11
Bacillus megaterium DSM 32	7	8	7	8	7	9	8	11	7	8	7	8	-	-	8	12
Staphylococcus aureus 6538	-	7	7	8	8	10	8	10		7	7	7	-	-	9	11
Bacillus cereus	7	8	-	7	7	9	9	12	7	9	7	8	-	7	9	11
Micrococcus luteus LA 2971	-	7	-	7	7	9	8	12		7	-	7	-	-	10	12
Bacillus brevis FMC 3	7	8	7	8	8	10	9	11		7	7	7	-	-	12	11

SW = Shell in Water, SE = Shell in Ethanol, KW = Kernel in Water, KE = Kernel in Ethanol.

Table 4. Antibacterial sussestibility of kernel and shell of walnut in water and etanol of HK-3 and HK-4.

Bacteria	HK-3								HK-4							
	SW	SW	SE	SE	KW	KW	KE	KE	SW	SW	SE	SE	KW	KW	KE	KE
	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc	2 µg/disc	4 µg/disc
Klebsiella oxycota	7	9	7	8	-	-	10	14	-	-	-	-	7	9	10	13
Klebsiella pneumonia	-	-	7	8	-	-	9	14	-	-	-	-	7	8	9	12
Pseudomonas aeroginosa DSM 50071	-	7	7	8	-	-	8	13	-	-	-	7	7	8	9	12
Acinobacter boumonii	-	7	-	-	-	-	8	9	-	-	-	-	-	-	10	12
Brucella melitentis	-	7	-	7	-	-	9	10	-	7	-	7	-	7	8	12
Bacillus megaterium DSM 32	7	8	7	8	-	-	8	9	-	7	-	-	7	9	8	11
Staphylococcus aureus 6538	-	7	7	8	-	-	9	12	-	7	-	-	8	10	8	10
Bacillus cereus	7	9	-	7	-	-	8	12	-	7	-	7	7	9	9	12
Micrococcus luteus LA 2971	-	8	7	8	-	-	8	10	-	-	-	-	-	9	8	12
Bacillus brevis FMC 3	7	8	-	7	-	-	8	11	-	-	7	-	7	10	9	11

SW = Shell in Water, SE = Shell in Ethanol, KW = Kernel in Water, KE = Kernel in Ethanol.

Table 5. Antibacterial susceptibility of Kernel and Shell Walnut in water and Etanol of HK-5.

Bacteria	SW 2 µ g/di	SW 4 µ g/di	SE 2 µ g/di	SE 4 µg/di	KW 2 µg/di	KW 4 µg/di	KE 2 µg/di	KE 4 µg/di
Klebsiella oxycota	-	-	-	-	7	9	10	13
Klebsiella pneumonia	-	-	-	-	8	13	12	15
Pseudomonas aeroginosa DSM 50071	-	-	-	-	8	9	10	14
Acinobacter boumonii	-	-	-	-	8	11	12	14
Brucella melitentis	-	7	-	7	8	10	12	15
Bacillus megaterium DSM 32	-	-	-	-	7	9	12	18
Staphylococcus aureus 6538	-	-	-	-	7	9	12	15
Bacillus cereus	-	-	-	7	8	11	11	15
Micrococcus luteus LA 2971	-	-	-	7	7	10	11	15
Bacillus brevis FMC 3	-	-	-	-	8	11	12	16

SW = Shell in Water, SE = Shell in Ethanol, KW = Kernel in Water, KE = Kernel in Ethanol.

Table 6. Strains of bacteria compared with standart antibiotics for susceptibility of inhibition zone mm.

Bacteria	P (10 µg)	VA (30 µg)	E (15 µg)	CN (10 µg)	AM (10 µg)	CAZ (10 µg)
Klebsiella oxycota	-	13	28	25	7	-
Klebsiella pneumonia	7	15	27	24	9	7
Pseudomonas aeroginosa DSM 50071	-	14	26	25	9	7
Acinobacter boumonii	-	18	26	26	-	-
Brucella melitentis	8	16	21	25	7	-
Bacillus megaterium DSM 32	-	13	26	25	8	-
Staphylococcus aureus 6538	-	13	26	25	8	-
Bacillus cereus	-	15	30	26	8	-
Micrococcus luteus LA 2971	-	13	27	25	7	-
Bacillus brevis FMC 3	7	15	20	25	10	7

P = Penicillin, VA = Vancomicin, E = Eritromicin, CN = Gentamicin, AM = Amicacin and CZ= Ceftazidim.

in water and ethanol showed weak inhibition zone in 7 to 9 mm diameter. As expected, the 4 µg/disc inhibited more than the 2 µg/disc concentration. Ethanol extract of kernel of HK-2 inhibited growth of bacteria at different rates. In addition, it was observed that ethanol extract of kernel of HK-3 had maximum antibacterial effect with 9 to 15 mm versus *K. oxytoca, K. pneumoniae* FMC 5 and *P. aeruginosa* DSM 50071 bacteria. In the same way, water extract of shell of HK-2 was similar to that of HK-3 with 7 to 9 mm inhibition zone, and *K. pneumoniae* FMC 5 showed resistance against these extracts. It was identified that the water extract of shell of HK-2 did not show antibacterial effect against *K. pneumoniae* FMC 5 and *A. boumonii* bacteria. The 2 µg/disc showed weak inhibition with 7 mm zone diameter versus *P. aeruginosa* DSM 50 071, *B. megaterium* DSM 32, *B. cereus* and *B. brevis* FMC 3, but it was not observed in the antibacterial activity against other bacteria. The 4 µg/disc prepared from

water extract of shell of HK-3 was measured as 7 to 8 mm inhibition zone against other strains except *K. pneumoniae* FMC 5. Shell extracts in ethanol of HK-2 did not show activity against *B. melitensis*, but they were measured as 7 to 8 mm zone diameter against other bacteria. *A. boumonii* showed resistance to water extract of kernel of HK-2. The 4 µg/disc prepared from water extract of kernel of HK-3 were measured as 7 to 8 mm inhibition zone against *K. oxytoca* and *B. cereus* but no activity was shown against other bacteria. *K. pneumoniae* FMC 5, *K. oxytoca* and *P. aeruginosa* DSM 50071 bacteria were mostly blocked by the 4 µg/disc prepared from kernel extracts in ethanol of HK-3. The best-known active ingredient in green young leaves is juglon matter which contains very powerful antioxidant and antimicrobial substances (Clark et al., 1990). In addition, especially tree bark, leaves, green fruit bark and juglon substance of walnut species have shown antimicrobial activity (Oliveira et al., 2008). There is an extended interest in using of natural antimicrobial compounds as the consumer makes pressure on the food industry to avoid from chemical preservatives and to increase resistance against antibiotics (Oliveira et al., 2007; Cowan, 1999). Studies have demonstrated the antimicrobial activity of walnut products particularly the bark (Alkhawajah, 1997).

Conclusion

In this study, five walnut types appear promising with respect to some properties. These walnut types can be suitable for such regions as the Southeast Anatolia. These walnut types should be adapted to the same ecological conditions with standard walnut types and cultivars. As a result of adaptation, the best walnut types and cultivars can be produced and positively contribute to the economy of our country. In addition, because of the antibacterial effects, some walnut types can be used as alternative against some antibiotics because they can be preservation against bacterial diseases. The kernel extracts in ethanol which has 4µg/disc have shown similar activities to vancomisin used in staphylococcal infections. Finally, we believe if the production and growing processes of these walnut types are controlled scientifically, they can be more practicable. In addition, it was found that kernel samples in ethanol showed much more activities. This study is an important source for the next researches.

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