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Influence of thermal treatment on phenolic compounds and antioxidant properties of oak acorns from Serbia

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Abstract

The aim of the present work was to investigate and compare phenolic compounds and antioxidant activity of methanol extracts of *Quercus robur* and *Quercus cerris* acorn kernels obtained before and after thermal treatment. Content of total phenolics, tannins, non-tannin phenolics and flavonoids was determined spectrophotometrically and content of gallic acid with HPLC. Antioxidant activity of the samples was assayed through FRAP (Ferric Reducing Antioxidant Power), DPPH scavenging test and inhibition of Fe^{2+}/ascorbate induced lipid peroxidation. Extracts of native and thermally treated kernels showed high antioxidant activity, with extracts of thermally treated kernels being more active than extracts of native ones. Hydrolysable tannins and gallic acid were identified in all samples. Non-tannin phenolics, including gallic acid, were present in significantly higher quantities in thermally treated samples, whilst tannin content decreased. This indicates that during thermal treatment hydrolysable tannins were degraded. As the result of this degradation and consequent increase of non-tannin phenolics content, and amongst them especially gallic acid, thermally treated samples possess higher antioxidant activity than do the native ones. The obtained results have provided further grounds for establishing *Q. robur* and *Q. cerris* acorn kernels as a source for functional food preparation.

Keywords: *Quercus robur*; *Quercus cerris*; Acorn kernel; Antioxidant activity; FRAP; DPPH; Lipid peroxidation; Gallic acid

1. Introduction

Usage of acorns of *Quercus* spp. (Fagaceae) in nutrition has a long history. In Europe, oak acorns were especially used in the Mediterranean region, mainly in Italy and Spain, furnishing up to 25% of the food consumed by the poorer classes (Hill, 1937). They were mainly used for making bread or as a substitute for coffee (Fernald & Kinsey, 1943). There are results of using oak acorns in Serbia from 19th century and their beneficial influence on human health (Jevtović, 1980). Oak kernels (*Quercus semen*) were traditionally used in medicine, particularly roasted ones (*Quercus semen tostum*) as astringents, antidiarrhoeals and antidotes (Hoppe, 1958; Tucakov, 1971).

Oak acorns are a rich source of carbohydrates, amino acids, proteins, lipids and various sterols (Hopkins & Chisholm, 1953; Leon-Camacho, Viera-Alcaide, & Vicario, 2004; Lopes & Bernardo-Gil, 2005; Mamedova, Aslanov, & Mirzoev, 1993; Taleb, Mashev, & Vasilev, 1989). Shelled out oak acorns have high energy value and are highly degradable (Saricicek & Kilic, 2004). Kernel oils of *Quercus robur* (English Oak) and *Quercus cerris* (Turkish Oak)

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contain α-linolenic acid, an ω-3 polyunsaturated fatty acid, which is important in eicosanoid synthesis and in the prevention of cardiovascular diseases (Petrović, Šobajić, Rakić, Tomić, & Kukić, 2004).

The acorns of *Q. robur*, beside nutritional components, contain various biologically active compounds (tannins, gallic and ellagic acid, and different galloyl and hexahydroxydiphenoyl derivatives) which possess antioxidant activity (Cantos et al., 2003; Chiou, 1989; Lee, Jeong, & Oh, 1992; Rakić, 2000; Rakić, Maletić, Perunović, & Svrzić, 2004; Rakić, Povrenović, Tešević, Simić, & Maletić, 2006). That is one of the reasons why oak acorns could be used as a functional food. Functional food is a modern concept and encompasses "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Ferrari & Torres, 2003). Antioxidants are considered fundamental in human life and health. Many biologically important processes are strongly influenced by antioxidants and antioxidant systems in the human organism. Hence, dietary antioxidants could be important in affecting some of processes such as ageing and various diseases (atherosclerosis, cancer, diabetes, Alcheimer's disease, etc.) (Cook & Samman, 1996; Pietta, 2000).

In the present study we investigated and compared phenolic compounds of native and thermally treated *Q. robur* and *Q. cerris* acorn kernels, as well as their antioxidant activity using different methods (FRAP, scavenging of DPPH radical and inhibition of lipid peroxidation assays).

2. Materials and methods

2.1. Compounds

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 2,4,6-tris-(2-pyridyl)-*s*-triazine (TPTZ) and indomethacin were obtained from Sigma Chemical Co. (St. Louis, USA); thiobarbituric acid (TBA), polyvinyl-polypirrolidone (PVPP) from Merck (Darmstadt, Germany); trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and L-ascorbic acid from Lachema (Neratovice, Czech Republic); all other reagents used were of analytical grade.

2.2. Plant material

Mature acorns of *Q. robur* (English Oak) were collected in October 2003 in Central Serbia (from the Belgrade outer city area), while those of *Q. cerris* (Turkish Oak) in October 2002 in Western Serbia (20 km southeastern from Bajina Bašta). Acorns were shelled out, the kernels dried at room temperature and powdered (native kernels). One part of these was heated at 200 °C for 10 min (thermally treated kernels).

2.3. Extraction

Both native and thermally treated kernels were extracted twice with methanol for 24 h at room temperature and solvent evaporated under reduced pressure. Obtained dry extracts of native and thermally treated kernels of *Q. robur* (QRN and QRT, respectively) and *Q. cerris* (QCN and QCT, respectively), were used for all investigations.

2.4. Determination of total phenolics

Total phenolics were determined using Folin-Ciocalteu reagent as previously described (Velioglu, Mazza, Gao, & Oomah, 1998), with slight modifications. One-hundred microliters of the extract dissolved in methanol (1 mg/ml) was mixed with 750 μl of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min; 750 μl of Na_2CO_3 (60 g/l) solution was then added to the mixture. After 90 min the absorbance was measured at 725 nm. Results were expressed as gallic acid equivalents.

2.5. Determination of tannins

Tannin content in each sample was determined using insoluble polyvinyl-polypirrolidone (PVPP), which binds tannins. One milliliter of extract dissolved in MeOH (1 mg/ml), in which the total phenolics were determined, was mixed with 100 mg PVPP, vortexed, left for 15 min at 4 °C and then centrifuged for 10 min at 3000 rpm (Makkar, Bluemmel, Borowy, & Becker, 1993). In clear supernatant non-tannin phenolics were determined the same way as the total phenolics (Velioglu et al., 1998). Tannin content was calculated as a difference between total and non-tannin phenolics content.

2.6. Determinations of flavonoids

Flavonoid content was determined spectrophotometrically according to *Crataegi folium et flores* monograph in DAB 10 (1991). One gram of each extract was mixed with acetone and heated with concentrated HCl. Total flavonoid aglycones, released after acid hydrolysis, were extracted with ethyl-acetate and determined using $AlCl_3$ reagent, measuring absorbance at 425 nm. Results were expressed in %, as hyperoside content.

2.7. Identification and determination of gallic acid

Analysis was performed using Agilent HPLC series 1100 with UV detector (DAD), equipped with the ZORBAX Eclipse XDB C18 column (4.6 × 150 mm, 5 μm) at a flow rate of 1 ml/min. Samples were dissolved in a mobile phase and further filtered through a 0.45 μm membrane filter Millex-HV13 (Millipore Corp.). Isocratic elution was performed at 40 °C, using phosphate buffer (pH 4.5):acetonitrile (95:5) solution as eluent. Phenolic compounds were identified at 230 and 272 nm according to their retention times and UV spectra. Quantification was done at 272 nm using external calibration with gallic acid standard. The results were calculated with regard to the dry matter.

2.8. Investigation on antioxidant activity

2.8.1. FRAP assay

Total antioxidant activity (TAA) was investigated using Ferric Reducing Antioxidant Power (FRAP) assay. It depends upon reduction of Fe^{3+}–TPTZ complex, to the blue colored ferrous form, with an increase in absorbance at 593 nm (Luximon-Ramma, Bahorun, Soobrattee, & Aruoma, 2002; Szőllősi & Szőllősi Varga, 2002). FRAP reagent was freshly prepared by mixing 25 ml acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ solution (10 mM TPTZ in 40 mmol/l HCl) and 2.5 ml $FeCl_3$ (20 mM) water solution. One-hundred and fifty microliters of each sample dissolved in methanol was added to 4.5 ml of FRAP reagent, stirred and after 5 min absorbance was measured at 593 nm, using FRAP working solution as blank. Calibration curve of ferrous sulfate (100–1000 μmol/l) was used, and results were expressed in μmol Fe^{2+}/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid.

2.8.2. DPPH radical assay

All samples were dissolved in methanol (5 mg/ml). An aliquot of this solution was mixed with 1 ml of 0.5 mM DPPH in methanol, and the final volume adjusted up to 5 ml, so that final concentrations of the samples were in a range from 2.5 to 100 μg/ml. Mixtures were vigorously shaken and left 30 min in dark. Absorbance was measured at 517 nm using methanol as blank. One milliliter of 0.5 mM DPPH diluted in 4 ml of methanol was used as control (Cuendet, Hostettmann, & Potterat, 1997). Quenching of DPPH radical was calculated using the equation: $I\ (\%) = 100 \times (A_0 - A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the tested sample), and A_s is the absorbance of the tested sample. The IC_{50} value represented the concentration of the extract that caused 50% quenching.

2.8.3. TBA test

Lipid peroxidation (LP) was measured using preparation of liposomes containing 0.03 g lecithin/ml. Liposomes were prepared from the commercial preparation "Lipotech 10", which contains 10% of lecithin, diluted with distilled water in ultrasonic bath for 30 min. Reaction mixture contained 20 μl $FeSO_4$ (0.075 M), 50 μl of liposomes, 10 μl of the sample dissolved in methanol in different concentrations (0.25–10%, w/v), 20 μl of L-ascorbic acid (0.1 M) and phosphate buffer (pH = 7.4, $I = 0.1$) up to a final volume of 4 ml. Samples were incubated 1 h at 37 °C. Then, 0.2 ml of EDTA (0.1 M) and 1.5 ml of TBA reagent (3 g TBA, 120 g TCA and 10.4 ml $HClO_4$ in 800 ml of distilled water) were added in each sample and heated for 15 min at 100 °C. After cooling, samples were centrifuged for 10 min (3000 rpm) and absorbance of supernatant was measured at 532 nm (Afanas'ev, Dorozhko, Brodskii, Kostyuk, & Potapovitch, 1989). Inhibition of LP was calculated the same way as described in DPPH radical assay.

2.9. Statistical analysis

Statistical analysis was performed using Analysis of Variance. Differences were accepted as statistically significant when $p < 0.05$.

3. Results

Content of phenolic compounds in extracts of native and thermally treated kernels of *Q. robur* and *Q. cerris* (samples QRN, QRT, QCN and QCT, respectively) is presented in Table 1. The extracts of thermally treated kernels had slightly higher total phenolics content compared to the extracts of native ones. This difference was more evident when comparing non-tannin phenolics; their content was *ca.* 2.1 times higher in QRT than in QRN (0.040 and 0.019 mg, respectively) and *ca.* 2.6 times higher in QCT than in QCN (0.037 and 0.014 mg, respectively). As for tannin content, it was lower in thermally treated samples. Flavonoids were present in very small quantities in all investigated samples, with higher content in *Q. cerris* kernels. Gallic acid was identified in all samples by means of HPLC. Its content was much higher in thermally treated samples (5.13% and 3.81% in QRT and QCT, respectively), than in native ones (0.46% and 0.30% in QRN and QCN, respectively).

Total antioxidant activity (TAA) of the samples was shown in Table 2. In this case, FRAP values for the extracts of thermally treated kernels were also higher than

Table 1
Content of total and non-tannin phenolics, tannins, gallic acid and flavonoids in extracts of native and thermally treated *Q. robur* and *Q. cerris* acorn kernels

Sample	Phenolics content[a]			Gallic acid[b]	Flavonoids[c]
	Total	Non-tannin	Tannins		
QRN	0.223 ± 0.004	0.019 ± 0.002	0.204 ± 0.003	0.46	0.023
QRT	0.237 ± 0.006	0.040 ± 0.002	0.197 ± 0.008	5.13	0.033
QCN	0.229 ± 0.011	0.014 ± 0.002	0.218 ± 0.010	0.30	0.051
QCT	0.247 ± 0.001	0.037 ± 0.001	0.211 ± 0.001	3.81	0.077

Values are expressed as mean of three replications ± S.D.
[a] Expressed as mg gallic acid equivalent/mg dry weight extract.
[b] HPLC determination, in %.
[c] Expressed in %, as hyperoside equivalents.

Table 2
Total antioxidant activity (TAA) of extracts of native and thermally treated *Q. robur* and *Q. cerris* acorn kernels

Sample	FRAP value[a]
QRN	2.44 ± 0.03
QRT	3.76 ± 0.00
QCN	2.58 ± 0.03
QCT	3.58 ± 0.36

Values are expressed as mean of three replications ± S.D.
[a] In units μmol Fe^{2+}/mg dry weight extract.

for extracts of native ones (3.76 and 2.44 μmol Fe^{2+}/mg for QRT and QRN, and 3.58 and 2.58 μmol Fe^{2+}/mg for QCT and QCN, respectively). TAA was strongly correlated with non-tannin phenolics content. TAA of L-ascorbic acid and quercetin used as standards were 7.41 and 7.69 μmol Fe^{2+}/mg, respectively.

As seen from Table 3, all samples exhibited strong, concentration dependent DPPH radical scavenging activity. IC_{50} values were below 10 μg/ml and were comparable with the activity of L-ascorbic acid (IC_{50} = 4.09 μg/ml) and quercetin (IC_{50} = 2.75 μg/ml). The extracts of thermally treated kernels also showed higher "scavenging" activity than the extracts of native ones.

As for lipid peroxidation (LP), results shown in Table 4. indicate significant activity of all investigated extracts. Extract of thermally treated kernels of *Q. robur* was more active compared to native (IC_{50} = 16.39 and 32.13 μg/ml, respectively). The same was observed for the extracts of *Q. cerris* (IC_{50} = 41.75 and 51.25 μg/ml, for QCT and QCN, respectively). Quercetin used as standard had IC_{50} value at 0.75 μg/ml.

Table 3
Scavenging effect of extracts of native and thermally treated *Q. robur* and *Q. cerris* acorn kernels on DPPH radical

Concentration (μg/ml)	Quenching (%)			
	QRN	QRT	QCN	QCT
2.5	16.84 ± 0.75	25.85 ± 3.22	15.33 ± 0.24	22.55 ± 0.40
5	33.20 ± 0.55	43.17 ± 0.14	29.76 ± 0.16	43.63 ± 0.56
10	59.92 ± 1.64	81.61 ± 1.03	55.24 ± 0.48	72.83 ± 0.00
20	93.03 ± 0.21	94.38 ± 0.27	93.35 ± 0.16	94.25 ± 0.16
50	94.97 ± 0.00	94.29 ± 0.14	94.93 ± 0.08	94.36 ± 0.32
100	94.77 ± 0.48	93.61 ± 0.14	95.26 ± 0.16	94.02 ± 0.00
IC_{50}	8.04 ± 0.35	5.77 ± 0.01	8.88 ± 0.03	5.93 ± 0.18

Values are expressed as mean of three replications ± S.D.

Table 4
Influence of extracts of native and thermally treated *Q. robur* and *Q. cerris* acorn kernels on Fe^{2+}/ascorbate induced lipid peroxidation in liposomes

Concentration (μg/ml)	Inhibition (%)			
	QRN	QRT	QCN	QCT
12.5	10.76 ± 2.58	33.77 ± 0.47	33.71 ± 0.22	32.53 ± 0.26
25.0	39.40 ± 4.68	70.53 ± 2.81	39.22 ± 6.10	40.78 ± 5.54
62.5	81.95 ± 1.64	82.12 ± 1.19	54.90 ± 3.22	61.35 ± 0.72
125.0	89.96 ± 1.06	81.12 ± 2.33	62.98 ± 4.16	80.49 ± 0.23
250.0	82.88 ± 7.70	69.65 ± 0.83	77.80 ± 1.73	74.20 ± 0.61
IC_{50}	32.13 ± 3.01	16.39 ± 0.44	51.25 ± 7.07	41.75 ± 7.35

Values are expressed as mean of three replications ± S.D.

4. Discussion

All extracts have shown significant antioxidant activity. The extracts of thermally treated kernels of both investigated species, *Q. robur* and *Q. cerris*, exhibited higher antioxidant activity than extracts of native ones. As expected, these activities were correlated with phenolics content in investigated extracts. This refers particularly to non-tannin phenolics, since their content was highly correlated with total antioxidant and DPPH radical scavenging activities (r = 0.9685 and −0.9966, respectively). Such correlation in the case of LP inhibition was not substantial (r = −0.6415).

Presented results also pointed out the fact that thermal treatment of investigated Oak kernels significantly changed content and composition of phenolic compounds. Thermally treated samples (QRT and QCT) had lower total phenolics and tannin content than native ones (QRN and QCN), but content of non-tannin phenolics, including gallic acid, was much higher.

This is explained by the presence of thermally degradable hydrolysable tannins in native Oak kernels. Their presence was confirmed using a preliminary $FeCl_3$ test, while a formaldehyde test showed the absence of condensed tannins. Hydrolysable tannins present in extracts of native kernels (samples QRN and QCN) were degraded under high temperature, causing increase of non-tannin phenolics and gallic acid content and consequently increase in antioxidant activity of thermally treated samples.

This is in agreement with the results of some previous investigations concerning type of *Quercus* acorns polyphenols and their antioxidant activity. Gallic acid, digallic acid and gallotannin were identified in the ethyl-acetate fraction of *Q. acutissima* acorns and caused its high antioxidant efficacy (Lee et al., 1992). Cantos et al. (2003) showed that methanol extracts of acorns of three different *Quercus* species (*Q. ilex*, *Q. rotundifolia*, and *Q. suber*) contain hydrolysable tannins. All of them were gallic acid derivatives. These extracts showed antioxidant activity similar to BHA and Trolox (Cantos et al., 2003). The acorns of *Q. robur* were also suggested to be convenient nutritional components, with significant antioxidant effects. Antioxidant activity of the aqueous extract of thermally treated *Q. robur* kernels (0.04%) in the Schall-oven test was comparable with the activity of 0.02% BHA (Rakić et al., 2006).

Based on the results obtained in this work, it is reasonable to believe that presence of gallic acid and its low molecular mass derivatives caused the potent antioxidant activity of the investigated samples. Although correlation between the phenolics content, especially non-tannin phenolics and the antioxidant activity became evident, the presented results also indicated that not only the amount, but also the type of phenolic compounds present in samples influenced observed antioxidant activity. Because of better

organoleptic properties and higher antioxidant and radical "scavenging" activity, thermally treated kernels are more adequate for employment. The obtained results on antioxidant activity of English and Turkish Oak kernels provided as well further grounds for their establishing as a functional food and their applicability.

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