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LC-ESI/LTQOrbitrap/MS/MS^n Analysis Reveals Diarylheptanoids and Flavonol *O*-glycosides in Fresh and Roasted Hazelnut (*Corylus avellana* cultivar "Tonda di Giffoni")

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Hazelnut represents one of the most consumed food, containing a large range of bioactive and health-promoting compounds. Investigation of the fatty component of *C. avellana* nut has been widely carried out, but little is known about its phenolic content. In order to achieve deeper insight into the polar fraction of the *n*-butanol extracts of both fresh and roasted kernels of *C. avellana* cultivar, "Tonda di Giffoni" a PGI (Protected Geographical Indication) product of Campania region (Italy), a phytochemical investigation was carried out.

High performance liquid chromatography coupled to electrospray ionization, multiple-stage linear ion-trap and orbitrap high-resolution mass spectrometry (LC-ESI/LTQOrbitrap/MS/MS^n) profile of the fresh and roasted hazelnut led to the identification of eleven phenolic compounds corresponding to diarylheptanoid derivatives, flavonoid *O*-glycosides and ellagic acid. Moreover, the antioxidant activity of isolated compounds was evaluated by TEAC assay which showed that ellagic acid and flavonoid derivatives possessed the highest free-radical-scavenging activity.

Keywords: Tonda di Giffoni, Fresh and roasted hazelnut, Phenolic compounds, LC-ESI/LTQOrbitrap/MS/MS^n, TEAC assay.

Corylus avellana L. tree, belonging to Betulaceae family, produces edible nuts, known as hazelnuts, on which important commercial and subsistence crops are found in many countries [1]. The edible part of hazelnut is the kernel, a nutritious food consumed raw or preferably roasted, used by the confectionary industry, and displaying a high content of healthy lipids [2]. Food and Drug Administration (FDA) has recognized hazelnut as a "heart-healthy" food due to its nutritional and nutraceutical properties [3].

Hazelnut kernels are rich in fats, proteins, and vitamins and play a relevant role in the agricultural market, mainly in dairy, bakery, confectionery, candy, and chocolate products [4]. In particular, kernels contain unsaturated fatty acids (linoleic, linolenic, oleic acids) essential for human health that have been reported to decrease cholesterol levels in blood and to control adverse effects of hypertension [4, 5]. Moreover, they are reported to contain many phenolic compounds, including flavonoids, phenolic acids and hydrolysable tannins [6, 7].

Italy is the second largest hazelnut producer in the world after Turkey. Currently in Italy there are two hazelnut varieties which have been awarded the Protected Geographical Indication (PGI) label by the European Union: "Nocciola del Piemonte" and "Nocciola di Giffoni". The latter is the product of a *Corylus avellana* cultivar of Campania region ("Tonda di Giffoni") [8]. Previous investigations on phenolic fractions of *C. avellana* cv. "Tonda di Giffoni" byproducts allowed us to isolate cyclic diarylheptanoids and diaryletherheptanoids, named giffonins A-V, some of which highly hydroxylated and able to prevent oxidative damages of human plasma lipids [9-13].

In order to investigate the occurrence of giffonins and phenolic compounds in *n*-butanol extract of fresh hazelnut kernel, without skins, of *C. avellana* cv. "Tonda di Giffoni" (TGF), an analytical approach based on high-performance liquid chromatography coupled to electrospray ionization and multiple-stage linear ion-trap and orbitrap high-resolution mass spectrometry (LC-ESI/LTQOrbitrap/MS/MS^n) was used. By the careful analysis of accurate masses, characteristic fragmentation patterns, chromatographic behavior and literature data, the occurrence of eleven phenolic compounds (Figures 1 and 2), corresponding to diarylheptanoid derivatives (**1**, **7**, **8**, **11**), ellagic acid (**4**), flavonoid *O*-glycosides (**3**, **6**), and flavonoid *O*-deoxyglycosides (**2**, **5**, **9**, **10**), could be identified.

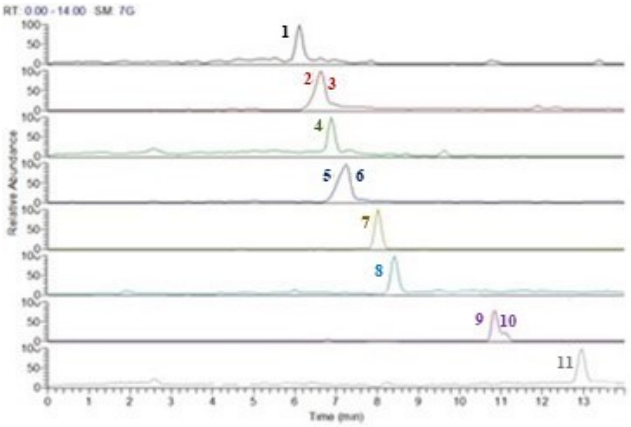

Figure 1: Extracted ion chromatograms of compounds **1**-**11** from the LC-ESI/LTQOrbitrap/MS analysis of *n*-butanol TGF extract.

According to literature data [12], compounds **1**, **7**, **8** and **11** could be promptly identified as diarylheptanoid derivatives, showing typical fragmentation patterns mainly involving the hydroxyl and carbonyl groups located on the heptanoid chain, characterized by the opening of the diarylheptanoid cycle after the dienone-phenol rearrangement of the $[M-H]^-$ pseudomolecular ion. By this way, e.g., the occurrence in the tandem mass spectra of **7** and **8** of main

product ions at *m/z* 269 and 253, respectively, could be explained as related to the neutral loss of a hydroxypropanone molecule (74 Da) deriving from two C-C bond consecutive cleavages (C7-C8 and C10-C11) (Table 1) [12].

On the basis of the molecular formula and of the presence in the tandem mass spectrum of the $[M-H]^-$ ion at *m/z* 300.9984 (t_R 6.89 min) of an intense product ion at *m/z* 284, due to the neutral loss of a radical anion OH^-, along with other characteristic minor product ions, the occurrence of the ellagic acid **4** could be defined (Table 1) [14].

Compounds **2**, **3**, **5** and **6** showed fragmentation pattern typically ascribable to flavonoid glycosides, specifically myricetin, quercetin and kaempferol derivatives. In particular, compounds **3** and **6** showed a principal $[M-H]^-$ ion at *m/z* 463.0867 and 447.0923, respectively, both yielding a tandem mass spectrum in which the main product ion (at *m/z* 301 and 285, respectively) was produced by the neutral loss of 162 Da ascribable to a hexose unit (Table 1) [15]. The MS/MS spectra of compounds **2** and **5** ($[M-H]^-$ ion at *m/z* 463.0870 and 447.0922, respectively) were instead characterized by main product ions at *m/z* 301 and 317, respectively, originated by the neutral loss of 146 Da, corresponding to a deoxyhexose unit [7, 16]. Therefore, compounds **2**, **3**, **5** and **6** were tentatively identified as myricetin-*O*-deoxyglycoside, quercetin-*O*-glycoside, quercetin-*O*-deoxyglycoside and kaempferol-*O*-glycoside, respectively.

Compounds **9** and **10** showed the same $[M-H]^-$ pseudomolecular ion and the same fragmentation pattern, yielding a main product ion at *m/z* 285 by neutral loss of 292 Da (Table 1). Considering their molecular formulae, it could be suggested that the neutral loss was originated by contemporary loss of one deoxyhexose unit and one *p*-coumaroyl moiety, so defining **9** and **10** as kaempferol-*p*-coumaroyl-deoxyglycoside isomers [12].

In order to unambiguously define the molecular structure of **1**-**11**, e.g. discriminating among structural isomers or stereoisomers, LC-ESI/LTQOrbitrap/MS/MS^n analysis of standards, most of which isolated in our previous investigations from different parts of *C. avellana* cv. Tonda di Giffoni [9-13], was performed. By this way, the above mentioned compounds were identified as: giffonin P (**1**), myricetin-3-*O*-α-L-rhamnopyranoside (**2**), quercetin 3-*O*-β-D-glucopyranoside (**3**), ellagic acid (**4**), quercetin 3-*O*-α-L-rhamnopyranoside (**5**), kaempferol-3-*O*-β-D-glucopyranoside (**6**), carpinontriol B (**7**), giffonin M (**8**), kaempferol 3-*O*-(4"-*cis*-*p*-coumaroyl)-α-L-rhamnopyranoside (**9**), kaempferol 3-*O*-(4"-*trans*-*p*-coumaroyl)-α-L-rhamnopyranoside (**10**), and giffonin Q (**11**) (Table 1).

Interestingly, this is the first report describing the occurrence of diarylheptanoids and flavonoids kaempferol 3-*O*-(4"-*cis*-*p*-coumaroyl)-α-L-rhamnopyranoside (**9**), kaempferol 3-*O*-(4"-*trans*-*p*-coumaroyl)-α-L-rhamnopyranoside (**10**), in hazelnut kernels.

Considering that hazelnut kernels are also consumed as roasted product, with the aim to highlight the differences between the phenolic profile of fresh (TGF) and roasted kernels (TGR) of *C. avellana* cv. "Tonda di Giffoni", a LC-ESI/LTQOrbitrap/MS analysis of *n*-butanol TGR extract was carried out. In particular, TGR showed the same compounds of TGF except for the absence of giffonin M (**8**), kaempferol 3-*O*-(4"-*cis*-*p*-coumaroyl)-α-L-rhamnopyranoside (**9**) and kaempferol 3-*O*-(4"-*trans*-*p*-coumaroyl)-α-L-rhamnopyranoside (**10**), allowing us to hypothesize that the thermal treatment of TG hazelnuts by roasting involves the degradation of these compounds.

Table 1: Retention times (R_t), Δppm, molecular formula and $[M-H]^-$ values of compounds occurring in *n*-butanol TGF extract identified by LC-ESI/LTQOrbitrap/MS/MS^n (negative ion mode).

	R_t (min)	Compound	Δppm	Molecular Formula	$[M-H]^-$
1	6.11	Giffonin P	0.34	$C_{19}H_{22}O_7$	361.1278
2	6.54	Myricetin-3-*O*-α-L-rhamnopyranoside	0.07	$C_{21}H_{20}O_{12}$	463.0870
3	6.70	Quercetin 3-*O*- β-D-glucopyranoside	0.11	$C_{21}H_{20}O_{12}$	463.0867
4	6.89	Ellagic acid	0.49	$C_{14}H_6O_8$	300.9984
5	7.09	Quercetin 3-*O*-α-L-rhamnopyranoside	0.08	$C_{21}H_{20}O_{11}$	447.0922
6	7.25	Kaempferol -3-*O*- β-D-glucopyranoside	0.06	$C_{21}H_{20}O_{11}$	447.0923
7	8.03	Carpinontriol B	0.45	$C_{19}H_{20}O_6$	343.1181
8	8.30	Giffonin M	3.42	$C_{19}H_{20}O_5$	327.1261
9	10.82	Kaempferol 3-*O*-(4"-*cis*-*p*-coumaroyl)-α-L-rhamnopyranoside	0.28	$C_{30}H_{26}O_{12}$	577.1337
10	11.08	Kaempferol 3-*O*-(4"-*trans*-*p*-coumaroyl)-α-L-rhamnopyranoside	0.38	$C_{30}H_{26}O_{12}$	577.1339
11	12.98	Giffonin Q	0.29	$C_{19}H_{18}O_3$	293.1175

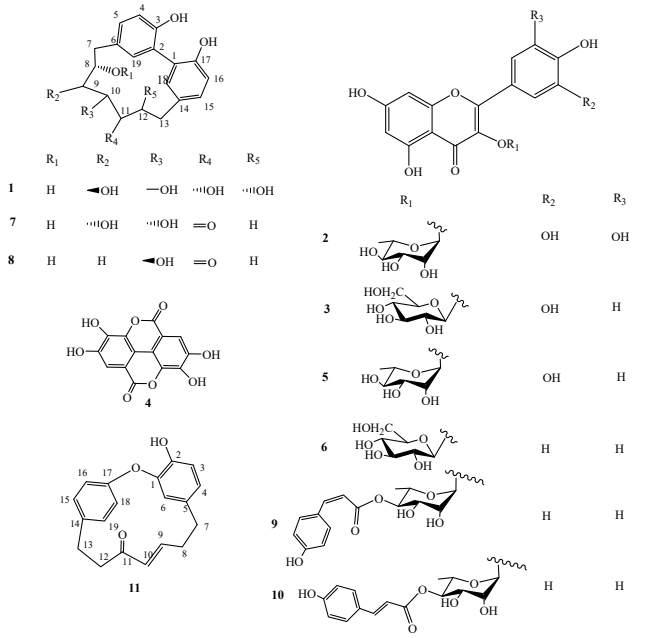

Figure 2: Compounds **1**-**11** identified in *n*-butanol TGF extract.

The antioxidant activity of *n*-butanol extract TGF, TGR and each compound was tested by TEAC assay and expressed as TEAC value, defined as the concentration of Trolox solution with antioxidant potential equivalent to a 1 mM concentration of the test sample; quercetin was used as reference compound.

Among the tested compounds, ellagic acid (**4**) displayed the highest (TEAC value 3.47 mM) activity, while myricetin-3-*O*-α-L-rhamnopyranoside (**2**), quercetin 3-*O*-β-D-glucopyranoside (**3**), quercetin 3-*O*-α-L-rhamnopyranoside (**5**) kaempferol 3-*O*-(4"-*cis*-*p*-coumaroyl)-α-L-rhamnopyranoside (**9**) and kaempferol 3-*O*-(4"-*trans*-*p*-coumaroyl)-α-L-rhamnopyranoside (**10**) showed a free-radical-scavenging activity (TEAC values = 1.86, 1.79, 1.50, 1.44, and 1.46 mM, respectively) comparable to that shown by quercetin (TEAC value 1.82 mM). The diarylheptanoids (**1**, **7**-**8** and **11**), characterized by the presence of two phenolic groups, showed a weak radical-scavenging capacity (Table 2). Despite these results, in our previous investigations carpinontriol B (**7**) and giffonin Q (**11**) reduced both H_2O_2 and H_2O_2/Fe^{2+} induced lipid peroxidation resulting more active than curcumin, the well-known antioxidant belonging to the class of linear diarylheptanoids, used as reference compound [11, 17]. TGF and TGR extracts showed a low activity probably due to the high content of lipids.

This is the first report of flavonoids kaempferol 3-*O*-(4"-*cis*-*p*-coumaroyl)-α-L-rhamnopyranoside (**9**), kaempferol 3-*O*-(4"-*trans*-*p*-coumaroyl)-α-L-rhamnopyranoside (**10**), along with the

diarylheptanoids carpinontriol B, giffonins M, P, and Q in hazelnut kernel. In detail, our previous investigations on different parts of *C. avellana* cv. "Tonda di Giffoni" highlighted the occurrence of carpinontriol B in the green leaf covers [13], giffonin M in the leaves [10], giffonin P in the leaves [10] and hard shells [12], and giffonin Q in the male flowers [11]. To the best of authors' knowledge the phytochemical investigation carried out on *C. avellana* leaves and bark collected in Hungary led to isolation of linear diarylheptanoids [18]. So, this study confirms once again this cultivar labeled with the PGI mark as a source of cyclic diarylheptanoids.

Table 2: Free radical scavenging activities of TGF and TGR extracts and compounds **1**−**11** in the TEAC assay.

Compounds	TEAC value (mM± SD)
1	0.90 ± 0.01
2	1.86 ± 0.02
3	1.79 ± 0.02
4	3.47 ± 0.03
5	1.50 ± 0.03
6	1.40± 0.01
7	0.79± 0.07
8	0.54 ± 0.01
9	1.44± 0.02
10	1.46± 0.01
11	0.56 ± 0.01
quercetin	1.82± 0.02
	TEAC value (mg/mL ± SD)
TGF	0.73± 0.01
TGR	0.70± 0.01

Experimental

General procedures: HRESIMS spectra were carried out by a system of liquid chromatography coupled to electrospray ionization and high resolution mass spectrometry (LC-ESI/HRMS) consisting of a quaternary Accela 600 pump and an Accela autosampler coupled to a LTQOrbitrap XL mass spectrometer (ThermoScientific, San Jose, CA), operating in negative electrospray ionization mode. The Orbitrap mass analyzer was calibrated according to the manufacturer's directions by using a mixture of caffeine, methionine-arginine-phenylalanine-alanine-acetate (MRFA), and Ultramark 1621 in a solution of acetonitrile, methanol (MeOH), and acetic acid. UV measurements were recorded on a UV-visible spectrophotometer (Evolution 201, Thermo Fisher Scientific).

Plant material: *Corylus avellana* L. hazelnuts, cultivar "Tonda di Giffoni PGI", were collected at Giffoni, Salerno (Campania region, Italy), in August 2015, and identified by V. De Feo (Department of Pharmacy, University of Salerno, Italy). A voucher specimen has been deposited in this Department.

Extraction: *C. avellana* kernels were crushed by a knife and stored at room temperature for three days. 480.0 g of hazelnut kernels without skin were defatted with *n*-hexane and chloroform at room temperature (6.4 L × 3 days, three times), and after were extracted with MeOH at room temperature (6.4 L for 3 days, three times). After filtration, the MeOH extract was dried under vacuum and partitioned between n-hexane and MeOH to remove the non polar oily components (mainly triglycerides). The obtained MeOH extract was subjected to *n*-butanol–water partition to remove free sugars. 1.00 Kg of hazelnut kernels without skin were hot air roasted by using a laboratory electric oven at 170°C for 30 min; the roasted hazelnuts were extracted with the same procedure used for the fresh hazelnuts.

LC-ESI/HRMS^n analysis: The *n*-butanol extracts of both fresh and roasted kernels of *C. avellana* cultivar "Tonda di Giffoni" were analyzed by LC-ESI/LTQOrbitrap/MS Data were collected and analyzed by using the software provided by the manufacturer. The analysis were carried out by using an Atlantis T3 (RP-18, 2.1 x 150 mm, 5 μm; Waters, Milford, MA) column, a flow rate of 0.2 mL/min, and a mobile phase consisting of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v). A linear gradient from 15 to 60% B in 9 min, hold at 60% B for 5 min, and from 60 to 100% B in 5 min was used. The autosampler was set to inject 2 μL of extract (0.5 mg/mL). The following experimental conditions for the ESI source were adopted: sheath gas at 15 (arbitrary units), auxiliary gas at 5 (arbitrary units), source voltage at 3.5 kV, capillary temperature at 280 °C, capillary voltage at -48 V and tube lens at -176.47 V. The mass range was from 200 to 1200 *m/z* with a resolution of 30000.

Data Dependent experiments were performed by acquiring MS^2 spectra of the first and the second most intense ions produced during the acquisition using a normalization collision energy at 30%, a minimum signal threshold at 250, and an isolation width at 2.0. Multiple-stage tandem mass (MS^n) experiments on selected product ions were carried out by the same collision energy.

Antioxidant activity (TEAC assay): In vitro antioxidant activities of the *n*-butanol extracts of TGF and TGR were determined by the Trolox Equivalent Antioxidant Capacity (TEAC) assay as previously reported [19, 20]. The TEAC value is based on the ability of the antioxidant to scavenge the radical cation 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) $ABTS^{\bullet+}$ by spectrophotometric analysis. The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. $ABTS^{\bullet+}$ is a blue-green chromogen with a characteristic absorption at 734 nm. The $ABTS^{\bullet+}$ solution was then diluted with PBS (phosphate saline buffer, pH=7.4) to obtain an absorbance of 0.70 at 734 nm and equilibrated at 30 °C.

The extracts were diluted with MeOH to produce solutions of 0.25, 0.5, 0.75, and 1 mg/mL. Samples **1**-**11** and standard were diluted with MeOH to produce solutions of 0.3, 0.5, 1, and 1.5 mM. The reaction was initiated by the addition of 1.5 mL of diluted ABTS to 15 μL of each sample solution. Determinations were repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (MeOH) and was plotted as a function of concentration of compound or standard, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical Co., Gillingham, Dorset, UK). The TEAC value is defined as the concentration of a standard Trolox solution with the same antioxidant capacity as a 1 mg/mL of the tested extract.

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