

## Original Research Paper

**Phytochemicals from *Castanea* spp. buds and green extraction technologies: the Finnover project****Dario Donno<sup>1,4\*</sup>, Federica Turrini<sup>2</sup>, Raffaella Boggia<sup>2</sup>, Maddalena Guido<sup>3</sup>, Maria Gabriella Mellano<sup>1,4</sup>, Gabriele L. Beccaro<sup>1,4</sup>**<sup>1</sup> Department of Agriculture, Forestry and Food Science – DISAFA, University of Torino, Italy; [gabriella.mellano@unito.it](mailto:gabriella.mellano@unito.it); [gabriele.beccaro@unito.it](mailto:gabriele.beccaro@unito.it)<sup>2</sup> Department of Pharmacy – DIFAR, University of Genoa, Italy; [raffa@difar.unige.it](mailto:raffa@difar.unige.it); [turrini@difar.unige.it](mailto:turrini@difar.unige.it)<sup>3</sup> Azienda Agricola Geal Pharma, Bricherasio (TO), Italy; [info@gealpharma.it](mailto:info@gealpharma.it)<sup>4</sup> Chestnut R&D Center, Chiusa Pesio (CN), Italy.\* Corresponding author: [dario.donno@unito.it](mailto:dario.donno@unito.it); Tel.: +39-011-670-8751

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**Abstract:** Green economy is a sustainable development tool based on the valorization of economic, natural and social resources. It is recognized as a tool to be applied to all the production sectors (goods and services), as well as for the conservation and sustainable use of natural resources. The FINNOVER project (“Innovative strategies for the development of cross-border green supply chains”) proposes a technical-economic path for the creation and development of new supply chains for the eco-sustainable extraction and use of natural bioactive compounds. In this study, an innovative extraction and re-use strategy to obtain value-added products from botanical by-products was developed as an alternative to waste incineration or composting. It was applied to *Castanea sativa* bud extract production as a case study, but it could be analogously applied for other herbal preparations. *Castanea* spp. and their preparations have been widely used for hundreds of years as medicinal plants in composite formulae. Bioactive compounds (botanicals) are quite variable in the plant material, according to genotype (intraspecific chemodiversity), different collection stages, pedoclimatic conditions of sampling sites (wild or cultivation zones), agrotechniques, and post-harvest handling. This research aimed to compare the bioactive compound pattern of *Castanea* spp. bud preparations (herbal preparations derived from embryonic fresh plant tissues as buds and sprouts) with the composition of extracts derived from the bud-waste management process. Molecules were extracted by the encoded traditional method (maceration in hydroglyceroalcoholic solution) and by green extraction technologies (Pulsed Ultrasound-Assisted Extraction). HPLC methods were used to identify and quantify the main bioactive compounds, and to obtain a specific profile to assess the contribution of every single bioactive class to the total phytocomplex. The established protocol was simple, sensitive and reliable and it could be used for the evaluation and quality control of natural products and relative eco-sustainable extracts. The valorization of bud marcs, which remain after the bud-preparation production, could have a significant economic impact for the commercial producers, representing an important innovation in this sector.

**Keywords:** chestnut; bud-preparations; processing waste; eco-sustainable extraction; botanicals.

## 1. Introduction

The genus *Castanea* is widespread in boreal regions and includes 13 species. Centuries of co-evolution between *Castanea* spp. and human populations have resulted in the spread of a rich and varied genetic diversity of chestnut throughout most of the world, especially in mountainous and forested regions. Its plasticity and adaptability to different pedoclimates and the wide genetic variability of the species determined the spread of many different ecotypes and varieties in the wild (Mellano et al., 2018; Mattioni et al., 2017). Throughout the centuries, man has used, selected and preserved these different genotypes – cloning them by grafting – for many applications, from fresh consumption to production of flour, from animal nutrition to timber production. However, due to their particular chemical composition, leaves, bark and buds are also traditionally used in some applications as a raw material for herbal industries (Braga et al., 2015).

In recent years, traditional and complementary medicine has attracted the attention of both consumers and medical professionals. In particular, bud derivatives are typical products used widely in European countries, even though they are still poorly studied to date. Bud extracts or bud preparations are generally obtained exclusively from fresh buds and young sprouts (meristematic fresh plant tissues) macerated and extracted with hydro-glycerol and hydro-alcoholic mixtures. These herbal products form a new category of plant product, being used in homoeopathy as well as in modern phytotherapy, also known as gemmotherapy (Donno et al., 2015).

Herbal preparations contain many different biologically active substances (botanicals). Many active substances with largely different therapeutic activities (*e.g.* amino acids, alkaloids, cardiac glycosides, mono-, di-, tri- and sesquiterpenes, phenolic compounds, antrachinons, coumarins, and enzymes) have been identified in – and isolated from – a large number of common botanical species (Vasanthi et al., 2012). In addition to the above-mentioned therapeutic substances, other biologically active compounds are known to be present in plants and herbal preparations, including organic acids, vitamins, minerals and other nutrient molecules with physiological effects in humans and which are prone to variation due to genetic and environmental factors and manufacturing conditions (Donno et al., 2015). Commercial companies, including familiar producers, promote their products by emphasizing their healthier properties and higher convenience as compared to other similar products on the market (Nicoletti, 2012). Despite the appeal of these products, research on these plant preparations is very limited or completely absent to date. With regards to the preparations obtained from the buds of *Castanea* spp., no systematic chemical investigation has been reported on the occurrence of significant amounts of bioactive compounds, even though these products are promoted on the market for their positive effects on vascular circulation, and for their properties against cystitis and cardiovascular diseases (Boggia et al., 2017).

In most EU countries, bud-derivatives are classified as plant food supplements and their production is quite expensive compared to other botanical extracts since the availability of buds or sprouts is extremely limited during each growing season. Consequently, the valorizations of their by-products could have a significant economic impact on the producers, and it could be an important innovation in this field. Food waste valorization and re-use strategies, rather than conventional food waste processing (*i.e.* incineration or composting), are becoming more and more popular (Turrini et al., 2019a). These strategies are particularly interesting for processing companies, but also for small-scale processes (*i.e.* herbal supplements production), where wastes can represent an important source of botanicals to be valorized. The use of sustainable extraction strategies could become particularly important for the extraction of bioactive compounds from herbal preparation production waste. Among these extraction strategies, the use of ultrasounds has recently emerged because of the many advantages shown in the processing (*i.e.* filtration, degassing, cutting), preservation (*i.e.*

enzyme and microorganism inactivation) and extraction of a natural product (Chemat et al., 2012). In particular, ultrasound-assisted-extraction (UAE) is an efficient, relatively low-cost, green, and sustainable methodology, used both on a small and large scale, which presents several advantages if compared to conventional extractions (Turrini et al., 2019b). When UAE is used in pulsed mode (PUAE), the ultrasound processor is intermittently turned on (active time) and off (inactive time) during the extraction process. This technical approach allows preserving the heat-sensitive biomolecules from degradation, giving the lower heat generation as compared to continuous sonication (Chemat et al., 2017).

FINNOVER (Innovative strategies for the development of cross border green supply chains) is the name of an Interreg ALCOTRA Italy/France trans-frontier project started in 2017 to innovate agro-industrial production chains in a perspective of green circular economy. One of FINNOVER targets is the management of agricultural waste (Turrini et al., 2019c). Within the framework of this project, this paper deals with the evaluation of a novel pulsed ultrasound-assisted-extraction method as an innovative tool for the management and valorization of wastes derived from the traditional herbal preparation from chestnut buds.

## 2. Materials and Methods

### 2.1. Plant material

Chestnut buds were collected from plants spontaneously grown in the Chisone, Pellice, Germanasca, Bronda, and Varaita valleys (Turin Province, Italy) during March 2018. The fresh plant material was immediately used by an Italian food supplement commercial company (Geal Pharma, Bricherasio, Turin) for the formulation of the corresponding bud derivatives according to the European Pharmacopeia (8<sup>th</sup> edition, 2014) and following the procedure described in the French Pharmacopeia (Ordre National des Pharmaciens, 1965). The waste material derived from the herbal medicine production was used for the following extraction steps assisted by PUAE.

### 2.2. Solvents and Chemicals

Maceration and extraction solvents (ethanol and glycerol), analytical HPLC grade solvents (acetonitrile, methanol, and formic acid), reagents for HPLC buffer (potassium dihydrogen phosphate and phosphoric acid) were purchased from Fluka Biochemika (Buchs, Switzerland) and Sigma–Aldrich (St Louis, MO, USA). Cetyltrimethylammonium bromide (cetrimide) was purchased from Extrasynthèse (Genay, France), while 1,2-phenylenediamine dihydrochloride (OPDA) was purchased from Sigma–Aldrich.

All polyphenolic standards (caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, hyperoside, isoquercitrin, quercetin, quercitrin, rutin, ellagic acid, gallic acid, catechin, epicatechin, castalagin, vescalagin) were purchased from Sigma–Aldrich, while organic acids (citric acid, malic acid, oxalic acid, quinic acid, succinic acid, and tartaric acid) were purchased from Fluka Biochemika. Ascorbic acid and dehydroascorbic acid were purchased from Extrasynthèse. Milli-Q ultrapure water was produced by Sartorius Stedim Biotech mod. Arium (Sartorius, Göttingen, Germany).

### 2.3. Preparation of commercial bud-derivatives

The extraction solution was prepared based on the protocol of bud preparations detailed in the monograph ‘Homeopathic preparations’, quoted in the French Pharmacopoeia, 8<sup>th</sup> edition, 1965 (Ordre National des Pharmaciens, 1965). The mother bud extracts were prepared using one part of the fresh material (calculated as dried weight) in 20 parts of glycerol–ethanol solution (1:1 ratio). Bioactive compounds were extracted through a cold maceration process for 21 days, in a solution of ethanol (95%) and glycerol, followed by a first filtration (Whatman filter paper, hardened ashless circles, 185 mm diameter), a manual

pressing and, after two days of decanting, a second filtration with the Whatman filter paper. Macerated samples were stored in dark bottles at normal atmosphere (N.A.), at 4 °C and 95% relative humidity until analysis. At the same time, the wet marcs obtained after the 2<sup>nd</sup> filtration, which represent the solid by-products, were stored frozen at  $-20 \pm 2^\circ\text{C}$  until further treatments.

#### *2.4. Waste management by Pulsed Ultrasound-Assisted Extraction (PUAE)*

The frozen marcs were carefully homogenized by grinding in a blender (Grindomix GM200, Retsch, Haan, Germany) for 20 s at 5000 rpm and further sieved (150  $\mu\text{m}$  mesh size). Their moisture content (relative humidity) was determined to be  $52.0\% \pm 0.3$  by a Sartorius moisture analyzer (Massachusetts, USA). All measurements were made in triplicate and mean values ( $\pm$  standard deviations) were reported.

The marcs extraction operations were carried out directly under the pulsed mode, keeping the temperature always below  $70 \pm 1^\circ\text{C}$ . The extraction solvent was the same solvent used in the traditional protocol to produce commercial products. PUAE was directly performed using a sonicator (Hielscher Ultrasonics UP200 St, Germany) with an operating frequency of 26 kHz, effective output of 200 W, equipped with a titanium (7 mm i.d.) sonotrode suitable for the used solvent volumes (Santos et al., 2009). The pulse duration and pulse interval refer to “ON” time and “OFF” time of the sonochemical reactor, respectively. The total time of a pulse duration period plus a pulse interval period is the cycle time. The duty cycle (expressed as % and related to a second in steps of 0.1 s) is the proportion of the pulse duration period with respect to the cycle time. The process conditions of the PUAE have been optimized by using a  $2^{4-1}$  fractional factorial design. Four process variables, which were the amplitude level (30%, 40%, 50%), the duty cycle (20%, 50%, 80%), the extraction time (5 min, 10 min, 15 min), and the sample/solvent ratio, at two levels (first time: 1/40, 1/50, 1/60; second time: 1/20, 1/15 and 1/10), were investigated. Final experimental conditions of extraction were developed as follows: a duty cycle of 80%, an extraction time of 15 minutes, and a solvent/ratio of 1/10.

#### *2.5. HPLC sample preparation and storage*

Macerated bud preparations and PUAE extracts were filtered with circular pre-injection filters (0.45  $\mu\text{m}$ , polytetrafluoroethylene membrane, PTFE) and then stored for a few days at N.A., 4 °C and 95% relative humidity. All samples were analysed as such without dilution. For vitamin C analysis, 250  $\mu\text{L}$  of OPDA solution (18.8  $\text{mmol L}^{-1}$ ) was added to 750  $\mu\text{L}$  of extracted samples for dehydroascorbic acid derivatisation into the fluorophore 3-(1,2-dihydroxy ethyl)furo(3,4-b)quinoxaline-1-one (DFQ). After 37 min in the dark, samples were analysed with a high-performance liquid chromatograph (HPLC) coupled to a diode array detector (DAD).

#### *2.6. Preparation of HPLC standards and chromatographic analysis*

Stock solutions of cinnamic acids and flavonols with a concentration of  $1.0 \text{ mg mL}^{-1}$  were prepared in methanol. From these solutions, four calibration standards (1000 ppm, 50 ppm, 250 ppm, 125 ppm) were prepared by dilution with methanol; stock solutions of benzoic acids, tannins, and catechins with a concentration of  $1.0 \text{ mg mL}^{-1}$  were prepared in a solution of 95% methanol and 5% water. From these solutions, four calibration standards were prepared by dilution with 50% methanol-water. Stock solutions of organic acids with a concentration of  $1.0 \text{ mg mL}^{-1}$  were prepared in ultrapure water. From these solutions, four calibration standards were prepared by dilution with water. Finally, stock solutions of ascorbic and dehydroascorbic acids with a concentration of  $1.0 \text{ mg mL}^{-1}$  were prepared in methanol. From these solutions, four calibration standards were prepared by dilution with methanol.

Separation and identification of compounds were performed by HPLC analysis, using an Agilent 1200 HPLC - UV-Vis Diode Array Detector (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a Kinetex C18 column (4.6 x 150 mm, 5 µm, Phenomenex, Torrance, CA, USA). Different chromatographic conditions were utilised to analyse extracts according to the methods described and previously validated by Donno et al. (2016). This information is reported in Table 1.

**Table 1.** Chromatographic conditions of the used HPLC methods.

Method	Classes of Interest	Mobile Phase <sup>1</sup>	Wavelength (nm)
A	cinnamic acids, flavonols	A: 10 mM KH <sub>2</sub> PO <sub>4</sub> /H <sub>3</sub> PO <sub>4</sub> , pH = 2.8; B: CH <sub>3</sub> CN	330
B	benzoic acids, catechins, tannins	A: H <sub>2</sub> O/CH <sub>3</sub> OH/HCOOH (5:95:0.1 v/v/v), pH = 2.5; B: CH <sub>3</sub> OH/HCOOH (100:0.1 v/v)	280
C	organic acids	A: 10 mM KH <sub>2</sub> PO <sub>4</sub> /H <sub>3</sub> PO <sub>4</sub> , pH = 2.8; B: CH <sub>3</sub> CN	214
D	vitamins	A: 5 mM C <sub>16</sub> H <sub>33</sub> N(CH <sub>3</sub> ) <sub>3</sub> Br/50 mM KH <sub>2</sub> PO <sub>4</sub> , pH = 2.5; B: CH <sub>3</sub> OH	261; 348

<sup>1</sup>Elution conditions

A. gradient analysis: 5%B to 21%B in 17 min + 21%B in 3 min (2 min conditioning time); flow: 1.5 mL min<sup>-1</sup>

B. gradient analysis: 3%B to 85%B in 22 min + 85%B in 1 min (2 min conditioning time); flow: 0.6 mL min<sup>-1</sup>

C. gradient analysis: 5%B to 14%B in 10 min + 14%B in 3 min (2 min conditioning time); flow: 0.6 mL min<sup>-1</sup>

D. isocratic analysis: ratio of phase A and B: 95:5 in 10 min (5 min conditioning time); flow: 0.9 mL min<sup>-1</sup>

Stationary phase: KINETEX – C18 column (4.6 × 150 mm, 5 µm)

Different bioactive compounds were used for the phytocomplex evaluation, both for commercial bud-derivatives and PUAE extracts. Phytocomplex was intended as the sum of biomarkers, selected for their demonstrated health-promoting activity and identified comparing retention times and spectroscopic data with authentic standards using the same chromatographic conditions. Quantitative determinations were performed using an external standard method. Calibration curves with good linearity for a four-point plot were used to determine the concentration of bioactive compounds in bud-preparations and PUAE extracts. The linearity for each compound was established by plotting the peak area (y) versus the concentration (x) of each analyte. The “multi-marker approach” by Mok et al. (2006) was followed as a strategy for the evaluation of the phytochemical composition. Measuring the concentration of one or very few markers or active components (“marker approach”) is the most important technique used for the characterisation of plant material. The “multi-marker approach” used in this research is the natural extension of the “marker approach” since it uses many, or even all, identified substances (chemical profile of the considered sample) to represent the whole sample. Thanks to the multi-marker approach, the phytocomplex is intended as the sum of the most important compounds with biological activity (Cuadros-

Rodríguez et al., 2016; Liu et al., 2012; Shi et al., 2014). In this case, five phenolic classes (15 markers) were selected for the evaluation. Levels of vitamin C (two markers) and organic acids (six markers) were studied as well. Bioactive compound contents were expressed as mg/100 g of fresh weight (FW).

### 2.7. Statistical analysis

Student's t-test was used to detect significant differences in the phytochemical composition between commercial bud-preparations and PUAE extracts. Differences with  $P < 0.05$  were considered statistically significant ( $N = 3$ ).

## 3. Results and discussion

HPLC analysis of botanicals is today a common characterisation tool and several analytical reports are available in the literature (Hakimzadeh et al., 2014; Harkey et al., 2001; Nunes et al., 2017). In this study, a preliminary phytochemical fingerprint was obtained by HPLC/DAD analyses. UV-visible absorption spectra, chromatographic retention times, and literature data were used for compounds identification. In total 23 compounds were selected and quantified as biomarkers for fingerprinting because of their known health-effective activity on humans (Table 2). Phytochemicals selected as health-promoting markers were grouped into different classes for both traditional bud-derivative and marc extract to compare their contents (expressed as mg/100 g FW). The HPLC analysis allowed also to obtain a specific profile of PUAE extracts and to assess the contribution of every single bioactive class to the total phytocomplex. This was achieved through a relatively simple, sensitive and reliable protocol that could be also used for the evaluation and quality control of other different natural products and relative eco-sustainable extracts.

For each bioactive class, a percentage comparison between phytochemical amounts in the original bud-derivatives and PUAE extracts has been calculated. About the 13% of the *C. sativa* bud-derivative phytochemical content was preserved in the marc extracts and it could be recovered for further products (Table 3). PUAE allowed to rapidly extract a total phytochemical content of 160.4 mg/100 g FW, that represents a good amount when compared to the bioactive content of the corresponding commercial product (1276.17 mg/100 g FW).

Concerning the classes of phytochemical compounds, the total concentration (in mg/100 g FW) in both the commercial product (bud derivative) and the PUAE extract are reported in Figure 1. Cinnamic acids, vitamin C and flavonols resulted as the most preserved classes of compounds in the marc extract after PUAE application.

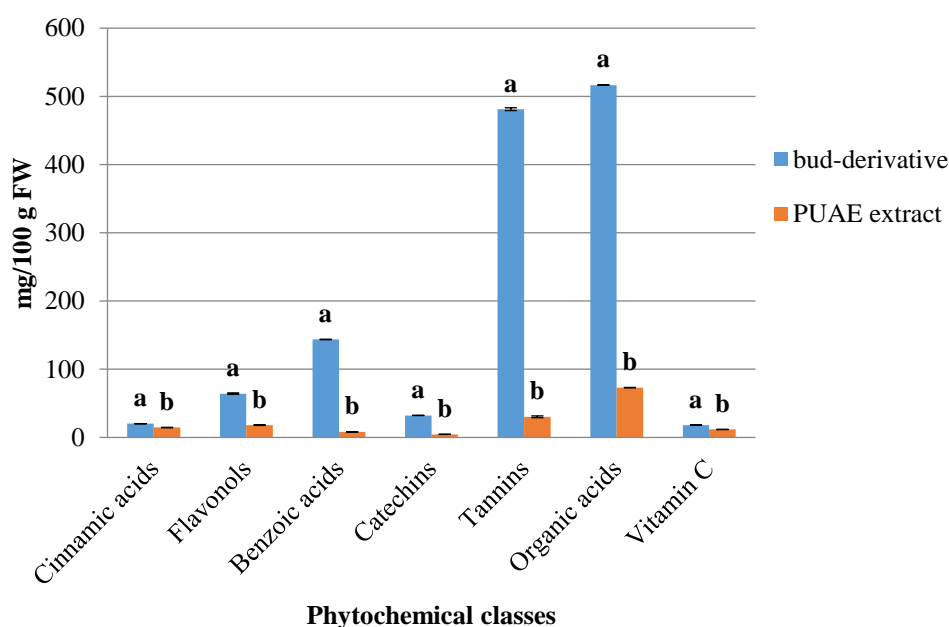
The described chromatographic phytochemical pattern resulted comparable with those of other studies (Turrini et al., 2019c). It is important to underline that HPLC-DAD generally does not allow to identify all the bioactive substances that may be included in the phytocomplex due to very high number of potential molecules (Donno et al., 2019). Giving the relevance of the synergistic effect of several bioactive substances for the overall health-promoting value of a product, the addition of numerous bioactive markers in the described analysis could be considered as an important achievement of this study, which could represent a further step toward the full identification of the active compounds characterising the complex herbal preparation (Donno et al., 2019).

**Table 2.** HPLC-fingerprint of the chestnut bud-preparations and extracts obtained by PUAE from the corresponding marcs.

Chemical class	Bioactive compound	Sample	Mean (mg/100 g FW)	Student's t-test (p < 0.05; N = 3)
Cinnamic acids	caffeic acid	bud-derivative	1.61±0.02	a
		PUAE extract	1.49±0.02	b
	chlorogenic acid	bud-derivative	14.23±0.29	a
		PUAE extract	11.10±0.06	b
	coumaric acid	bud-derivative	n.d.	
		PUAE extract	n.d.	
Flavonols	ferulic acid	bud-derivative	4.34±0.22	a
		PUAE extract	1.93±0.30	b
	hyperoside	bud-derivative	3.03±0.10	a
		PUAE extract	1.19±0.07	b
	isoquercitrin	bud-derivative	n.d.	
		PUAE extract	n.d.	
	quercetin	bud-derivative	30.64±0.23	a
		PUAE extract	15.39±0.22	b
	quercitrin	bud-derivative	29.01±0.82	a
		PUAE extract	1.24±0.46	b
Benzoic acids	rutin	bud-derivative	1.54±0.12	a
		PUAE extract	0.29±0.14	b
	ellagic acid	bud-derivative	48.95±0.13	a
		PUAE extract	5.87±0.10	b
Catechins	gallic acid	bud-derivative	94.71±0.24	a
		PUAE extract	2.36±0.11	b
	catechin	bud-derivative	1.28±0.32	a
		PUAE extract	0.39±0.12	b
Tannins	epicatechin	bud-derivative	31.02±0.17	a
		PUAE extract	4.14±0.10	b
	castalagin	bud-derivative	327.61±1.64	a
		PUAE extract	23.37±0.81	b
Organic acids	vescalagin	bud-derivative	153.61±0.40	a
		PUAE extract	6.90±0.46	b
	citric acid	bud-derivative	185.97±0.49	a
		PUAE extract	9.19±0.45	b
	malic acid	bud-derivative	97.21±0.25	a
		PUAE extract	5.01±0.02	b
	oxalic acid	bud-derivative	58.34±0.25	a
		PUAE extract	18.97±0.06	b
	quinic acid	bud-derivative	53.47±0.40	a
		PUAE extract	9.98±0.18	b
Vitamin C	succinic acid	bud-derivative	31.84±0.68	a
		PUAE extract	2.78±0.14	b
	tartaric acid	bud-derivative	89.69±0.15	a
		PUAE extract	27.14±0.15	b
	ascorbic acid	bud-derivative	15.79±0.12	a
		PUAE extract	10.49±0.04	b
	dehydroascorbic acid	bud-derivative	2.29±0.14	a
		PUAE extract	1.22±0.13	b

**Table 3.** The recovering percentage for each class of bioactive compounds in PUAE extracts.

Bioactive class	Recovering percentage
Cinnamic acids	71.92%
Flavonols	28.19%
Benzoic acids	5.73%
Catechins	14.04%
Tannins	6.29%
Organic acids	14.15%
Vitamin C	64.76%
Total bioactive compound content	12.57%



**Figure 1.** Bioactive class contents of *C. sativa* commercial products and PUAE extracts. Results are reported as mg/100 g of fresh weight (FW) of buds/marcs. Mean values and standard deviation bars are reported. Significant statistical differences ( $P < 0.05$ ) are indicated by different letters.

For several years, the protective effects of polyphenols and vitamin C have been mainly ascribed to their antioxidant and anti-inflammatory capacities. Studies have shown that phenolics may engage with cellular signalling flow, controlling transcription factor actions and subsequently affecting the expression of those genes involved in cellular metabolism and cellular survival (De Biaggi et al., 2020). In particular, flavonols and chlorogenic acid may be considered the main phenolics responsible for *in vitro* anti-cancer activity (e.g., against liver, colon, breast, and lung cancer), as recently reported by Li et al. (2013). In this research, cinnamic acids, mainly represented by chlorogenic acid, and vitamin C in the marc extracts, were respectively the 71.92% and 64.76% of the correspondent *C. sativa* bud-derivative content. As also reported by Turrini et al. (2019c), the other classes of bioactive compounds, as benzoic and organic acids, catechins, and tannins were identified and quantified in the marc extracts, but they showed lower values (about 5–15%) than the relative commercial products (Figure 1).



## 4. Conclusions

Results obtained in this study showed that waste extracts derived from the ultrasound extraction of a chestnut bud-preparation are characterized by a valuable amount of bioactive compounds, with a phytochemical profile that resulted similar to the one of the original bud-derivatives. This new extraction technology (PUAE) should also be considered favorably, being a valid and ecological alternative to waste management based on incineration or composting. It is believed that the outcomes of this research could be considered by the nutraceutical, phytotherapeutic, and phytopharmaceutical industries for the development of innovative products for the market.

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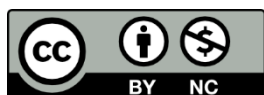
**Conflicts of Interest:** The authors declare no conflict of interest.

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