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Research article

Hydrolysable Tannins from Sweet Chestnut (*Castanea sativa* Mill.) to improve Tobacco and Food/Feed Quality. Note 1: Fraction characterization, and Tobacco biostimulant effect for gall-nematode resistance

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Abstract: Hydrolysable tannins, water-extracted from sweet chestnut (*Castanea sativa* Mill.) biomass (CHT) and membrane concentrated, have several remarkable effects as antioxidant, antimicrobial, and metal complexing agents. Raw CHT extract has been recognized as a plant biostimulant for its capacity to enhance plant rooting, and improve early P uptake (starter effect), as a seed-transplant seedling local treatment. It has been investigated, in particular on tobacco, also for its ability to enhance plant resistance to nematodes. More recently, some CHT fractions obtained by process stream fractioning and enriched in selected polyphenolic subclasses with antimicrobial and antioxidant effects were identified, chemically characterized and tested for their potential in promoting selected aspects of plant yield, quality and protection, and maintaining and improving feed and food quality during processing. A method of application of a CHT fraction with a biostimulant effect on plant root system was developed, to protect tobacco plant in *Meloidogyne* spp. infested fields. Results of this application consistently did not differ from better treatments.

Keywords: *Castanea sativa* Mill.; sweet chestnut; hydrolysable tannins; HPLC-DAD-ESI-MS; plant biostimulant; antimicrobial activity; antioxidant activity; nematodes; *Meloidogyne* spp

1. Introduction

Hydrolysable tannins, water-extracted from sweet chestnut (*Castanea sativa* Mill) biomass (CHT) and membrane concentrated, have several remarkable effects as antioxidant, superoxide radical scavenging, antimicrobial, and metal complexing agents. As antioxidants, they were used to reduce lipid oxidation in food, e.g., dry-cured sausage and liver pâté [1,2], but also as antioxidant supplement in animal nutrition [3,4]. Extracts of leaves of chestnut Italian and Lovran's marrone cv were found to be efficient radical scavengers, while catkin, leaves, chestnut bark, and spiny burs extracts had the highest antimicrobial activity on several bacteria strains [5]. CHT exhibited also anti-yeast and antiviral activities [6,7]. Most of the antibacterial activity of sweet chestnut extracts applies to prevention and cure of clostridial and other diseases in animals [8], and therefore they were proposed as a co-adjuvant in the therapy of diarrhea, due to their antispasmodic or spasmolytic effects and delaying gastrointestinal transit [9]. For their metal complexing activity, and correlated antioxidant capacity, CHT found practical applications in agriculture, as an iron fertilizer [10], and in industry, as an iron and steel metal protection agent [11,12]. Recent studies revealed that its antioxidant activity seems to be correlated with its copper chelating capacity [13].

Raw CHT extract has been recognized as a plant biostimulant for its capacity to enhance plant rooting, and improve early P uptake (starter effect), as a seed-transplant seedling local treatment [14]. It has been investigated also for its ability to enhance plant resistance to nematodes, an increasing threat to agricultural productions world-wide. In vitro tests, and some pot experiments, indicated nematostatic, nematocidal, and disorienting activity of CHT on gall-nematode *Meloidogyne javanica* (Treub) Chitwood, and cyst-nematodes *Globodera rostochiensis* (Woll), and *Heterodera carotae* Jones [15-17]. However, recent pot tests on *Meloidogyne incognita* (Kofoid et White) Chitwood indicated that a higher concentration of free gallic acid in CHT extract is apparently related to an increased biocidal activity, in agreement with previous results described for *Tagetes* spp. extracts [18], while a biostimulant effect on plant root system predominates when its free gallic acid content is relatively low [14]. More recently, by process stream fractioning, some CHT fractions with antimicrobial, antioxidant, and biostimulant effects were identified and tested for their potential in promoting selected aspects of plant yield, quality, and protection, and for maintaining and improving feed and food quality during processing. This paper describes this activity of fraction characterization, and the results of two-year field tests on Virginia Bright tobacco, aiming at investigating the consistency of the biostimulant effect of a CHT formulation relatively low in gallic acid, in comparison with other products of natural origin or inspiration (botanicals and biological control) and ordinary agrochemicals, used to prevent and counteract nematode attacks.

2. Materials and Methods

2.1. CHT fraction characterization

2.1.1. General

The products under examination (courtesy: Gruppo Saviola srl, Viadana, MN, Italy) consist of ten process streams of the tannin extraction unit operating in Radicofani (SI), Tuscany (Figure 1): (1) filtered tannin broths; (2) permeate from nanofiltration step-1; (3) concentrate from nanofiltration step-

1; (4) concentrate from nanofiltration step-1 (after cooling); (5) permeate from nanofiltration step-2; (6) concentrate from nanofiltration step-2; (7) osmosis permeate; (8) osmosis concentrate; (9) settled fraction from clarification step; and (10) spray-dried material obtained from fraction 6. Gallic acid and ellagic acid were of analytical grade, purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France). The DPPH•(1,1-diphenyl-2-picrylhydrazyl) radical (analytical grade) and the Folin-Ciocalteu reagent were purchased from Sigma (St Louis, MO, USA). All solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Company Inc. (Milwaukee, Wisconsin, USA).

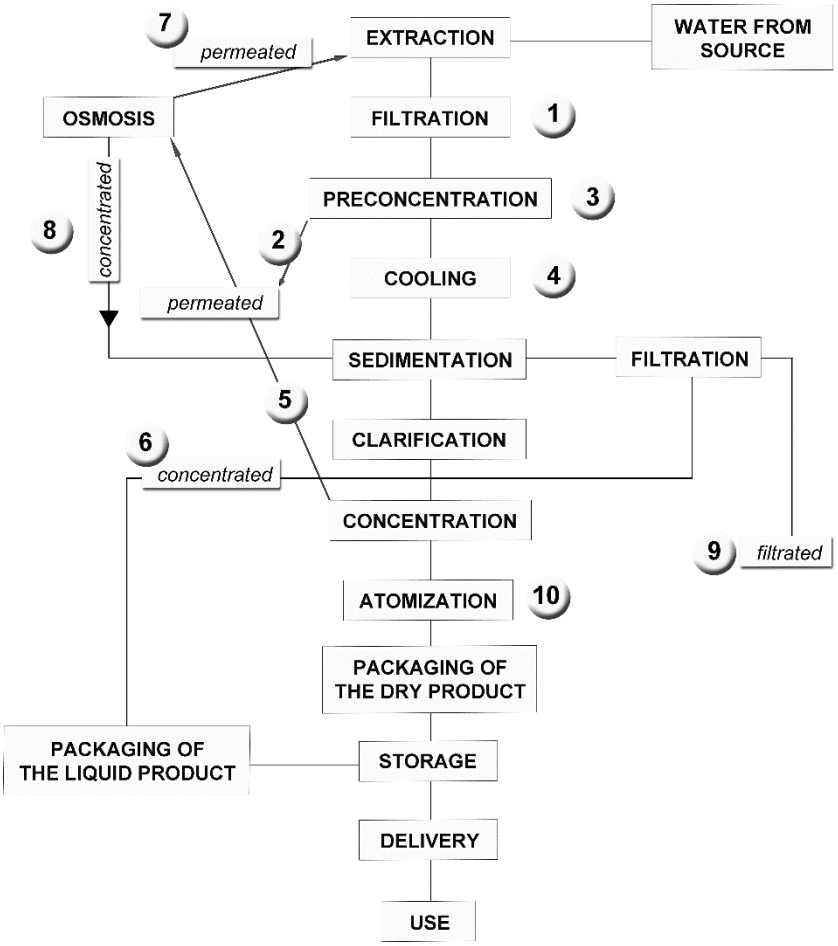

Figure 1. Operating diagram of the Gruppo Saviola's extraction and fractionation plant.

2.1.2. HPLC-DAD-ESI-MS Analysis

The HPLC-DAD-ESI-MS analyses were conducted using an HP-1100 liquid chromatograph equipped with a DAD detector and an HP 1100 MSD API-electrospray (Agilent Technologies) operating in negative ionization mode. A Luna, C18 250 × 4.60 mm, 5 μm column (Phenomenex), operating at 26 ºC was used. The eluents were H_2O (adjusted to pH 3.2 with HCOOH) and CH_3CN. A four-step linear solvent gradient starting from 100% H_2O (A) up to 100% CH_3CN (B) was performed with a flow rate of 0.8 mL/min over a 55-min period, as previously described [19]. Mass spectrometer operating conditions were: gas temperature 350 ºC at a flow rate of 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 ºC and capillary voltage 3500 V. The fragmentor was 120 eV. Identification

of individual tannins was carried out using their retention times, and both spectroscopic and spectrometric data. Quantification of the single compounds was directly performed by HPLC-DAD using a five-point regression curve built with the available standards. Curves with an $r^2 > 0.9998$ were considered. Calibration was performed at the wavelength of the maximum UV-Vis absorbance, by applying the correction of molecular weight. In particular, galloyl-glucosides and gallic acid amounts were calculated at 280 nm using gallic acid as a reference. Ellagic tannins and ellagic acid were calibrated at 254 nm using ellagic acid as a reference. The determinations of the polyphenol contents were carried out in triplicate; the results are given as means and the standard error was < 5%.

2.2. Tobacco field tests

- General

CHT extract of the fraction 10 was used to prepare formulations both of a microgranule fertilizer for local application during transplanting (in cogranulation with gypsum), and as a wettable powder, to be applied in microirrigation. This is a standardized extract of almost the whole phytocomplex, which is soon stabilized by spray-drying. This process determines consistent characteristics in the product for a long time, thence consistent performance over the time. In 2013 and 2014 two field experiments on 3-replicated 500 m^2 plots, arranged in a fully randomized block design, were carried out on Virginia Bright tobacco cv ITB678 (2013) and K326 PVY (2014) grown at Fattoria Autonoma Tabacchi (Città di Castello, Perugia, Italy), in the area localized around the Gauss-Boaga point X:2290792; Y: 48007721. Crops were grown according to the indications of the Agro-Ecological Measures and Production Technical Specifications of Regione Umbria [20]. Microirrigation tapes (1 L m^{-1} h^{-1}) were set soon after

Table 1. Nematode field experiments. 2013 and 2014 treatments on Virginia Bright tobacco

Treatm. No.	Year		AI & Formulation[a]	Commercial Product (CP)	Manufacturer	CP Rate (kg ha^{-1})
	2013	2014				
0	X	X	Control	-	-	-
1	X	X	Etoprofos 10% MG	Mocap	Certis Europe	60
2	X	X	Oxamyl 5% MG	Vydate	DuPont CropProt	60
3	X		Paecilomyces lilacinus strain 251, 1.25%	BioAct WG	Biogard-CBC Eur.	10
4	X		CHT extract 40% MG + CHT 75% WP	Saviostart 40 + Saviotan WP	Gruppo Saviola	20 + 45
5		X	CHT extract 18% MG + CHT 75% WP	Saviostart 18 + Saviotan WP	Gruppo Saviola	30 + 45
6		X	Bacillus firmus I-1582 5% WG	FlocteR	Bayer CropScience	80
7		X	Azadirachtine 2.4% WG	Oikos	Sipcam Italy	10

[a] MG = microgranules; WG = wettable granules; WP = wettable powder

transplanting, along each plant row; any microirrigation treatment considered the application of 5 m^3 ha^{-1} of water; products to be applied by microirrigation were first diluted in 10 L water, and then injected by a Venturi type injector at mid-irrigation. Treatments are indicated in Table 1 and 2. In 2013 a sandy-loam soil (sand 548, clay 155, Organic Carbon 13 g kg^{-1}; pH_w 7.9), with a pre-plant *Meloidogyne arenaria* Chitwood mean count of 244/200 cm^3 of soil (April 15, 2013), was selected for the experiment. Tobacco, after a previous tobacco crop, was transplanted (110 × 35 cm) on June 14, topped on Aug 19, and harvested between Aug 23 and Oct 19, 2013. In 2014 a sandy-loam soil (sand 630, clay 73, Organic Carbon 10 g kg^{-1}; pH_w 7.23), with a pre-plant *Meloidogyne arenaria* Chitwood mean count of 555/200 cm^3 of soil (April 30, 2014), was transplanted (same distances), after a previous tobacco crop, on May 24, topped on Aug 12, and harvested between Aug 21 and Sept 29, 2014. Each replicated plot consisted of 4 rows, and determinations were carried on the plants of the two central rows. Crop growth, tobacco yield, and quality, Barker's crop root infestation grade [21], and nematode count in soil post-harvest were determined, and statistically analyzed (ANOVA).

Table 2. Nematode field experiments 2013 and 2014. Product application dates and rates (kg ha^{-1})

Treatm.No.	2013								
	06-14	06-21	06-26	07-03	07-12	07-19	07-25	-	-
1	30[a]	30[a]	-	-	-	-	-	-	-
2	30[a]	30[a]	-	-	-	-	-	-	-
3	6[b]	-	1[c]	1[c]	1[c]	1[c]	-	-	-
4	20[a]	-	7.5[c]	7.5[c]	7.5[c]	7.5[c]	7.5[c]	-	-
	2014								
	05-23	05-24	06-10	06-12	06-18	06-26	07-01	07-08	07-31
1	-	30[a]	30[a]	-	-	-	-	-	-
2	-	30[a]	30[a]	-	-	-	-	-	-
5	-	30[a]	-	7.5[c]	7.5[c]	7.5[c]	7.5[c]	7.5[c]	7.5[c]
6	80[d]	-	-	-	-	-	-	-	-
7	-	1[b]	-	1.5[c]	1.5[c]	1.5[c]	1.5[c]	1.5[c]	1.5[c]

[a] Microgranule box; [b] Pre-transplant tray immersion; [c] microirrigation; [d] Pre-transplant broadcasted and soil incorporated

3. Results and Discussion

3.1. Chemical fractioning

Each fraction from the productive process was characterized both by subclasses and single compounds through HPLC-DAD-ESI-MS analysis. Figure 2 shows the chromatographic profile of the commercial liquid fraction of sweet chestnut (fraction 6), registered at 254 and 280 nm, with the list of the detected compounds. The profile at 254 nm emphasizes the presence of ellagic acid and its derivatives; at 280 nm, gallic and its derivatives are more evident. The single compounds were identified using both retention times and their spectroscopic and spectrometric data, according to previously reported data when available [22]. Table 3 shows the identification of the single compounds, numbered as in Figure 2. In the analyzed samples, only HTs were present, and, on the basis of their different chemical structures, UV-Vis spectra, and mass fragmentation patterns, it was possible their

attribution to various molecular subclasses (Figure 3). The compounds based on glucose and HHDP (hexahydroxydiphenoyl-) and/or NHTP (nonahydroxytriphenoyl-) units have UV-Vis absorbances with maxima at wavelengths lower than 240 nm and decreasing to 0 mAU between 300 and 400 nm [23]. This kind of UV-Vis spectra has a shoulder around 260–290 nm, more easily detected by observing them in combination with their first and second derivatives, the profiles of which deviate significantly as a result of slight variations in the spectrum [19]. The ESI-MS fragmentation patterns confirm the structure described above, showing the presence of the ion at *m/z* 301 (HHDP-unit or deprotonated ellagic acid). These identified compounds are: vescalin (1), castalin (2), pedunculagin I (3), roburin D (7), vescalagin (8), castalagin (10) and O-galloyl-castalagin isomer (12).

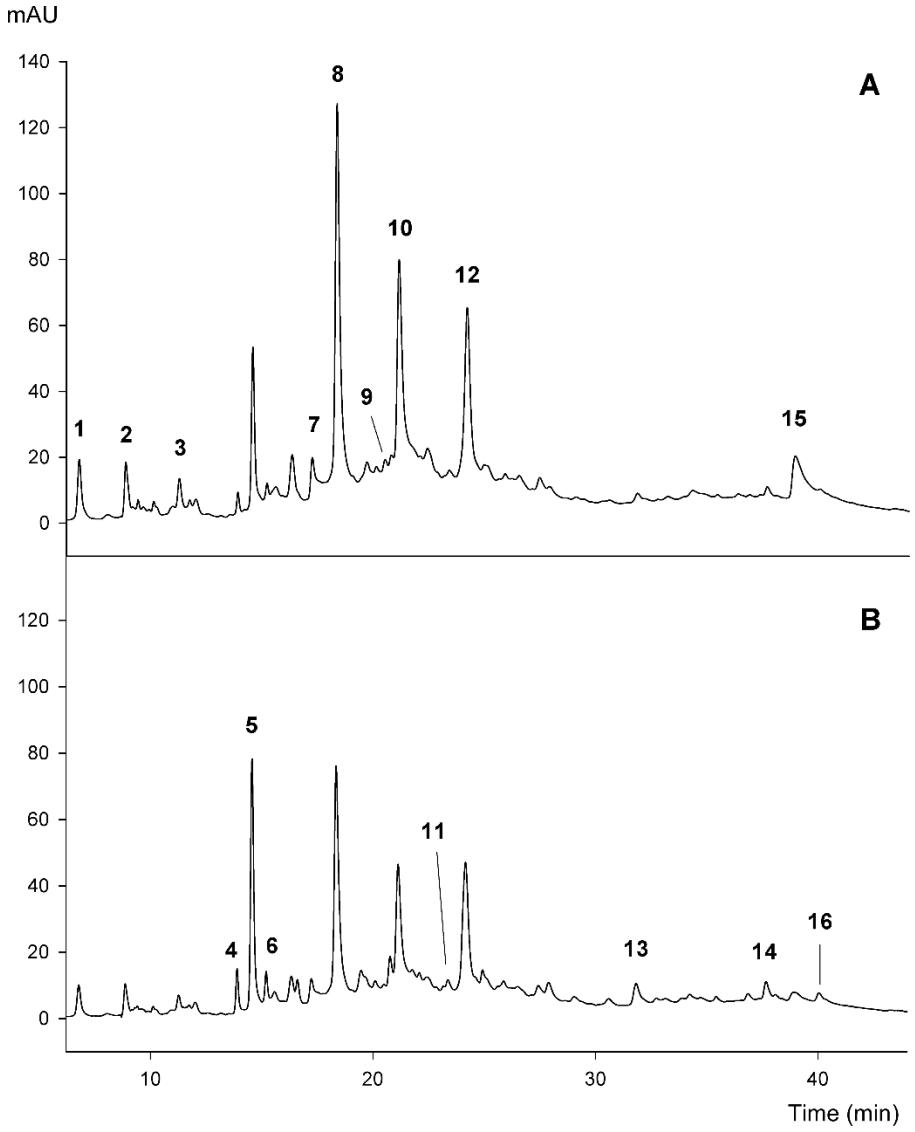

Figure 2. Chromatographic profile of the commercial liquid fraction of sweet chestnut (fraction 6), registered at 254 nm (A) and 280 nm (B). Peaks: 1. Vescalin; 2. Castalin; 3. Pedunculagin I; 4. Monogalloyl glucose I; 5. Gallic acid; 6. Monogalloyl glucose II; 7. Roburin D; 8. Vescalagin; 9. Dehydrated tergallagic-C-glucoside; 10. Castalagin; 11. Digalloyl glucose; 12. O-galloyl-castalagin isomer; 13. Trigalloyl glucose; 14. Tetragalloyl glucose; 15. Ellagic acid; 16. Pentagalloyl glucose.

The identification of pedunculagin I was performed by comparing the spectral data with those in literature: this compound has already been detected in other vegetable matrices such as fruits and leaves of strawberry (*Fragaria x ananassa*) and walnut (seeds of *Juglans regia* L.), but to the authors' knowledge, not yet in sweet chestnut wood [24-26]. Concerning the compounds 8 and 10, the spectrophotometric and spectrometric data, and previously reported data, make possible an identification as vescalagin and castalagin (Figure 3A). These two epimers, having identical UV-Vis profiles and ESI-MS spectra, are distinguishable only based on their chromatographic retention times (Rt), allowing for identifying compound 8 (Rt = 18.3 min) as vescalagin, and compound 10 (Rt = 21.1 min) as castalagin [23]. The same holds for compounds 1 and 2, castalin and vescalin, which are hydrolysis products of 8 and 10 respectively, lacking the HHDP residue. They are not always present in chestnut extracts, but were detected in all the fractions analyzed in this study. The compound 12 shows an ESI-MS intense signal at *m/z* 1085 (the most abundant fragment), consistent with a structure based on HHDP- or NHTP- glucose, possibly an isomer of *O*-galloyl-castalagin or *O*-galloyl-vescalagin. 1-*O*-galloyl-castalagin was recently tentatively identified in chestnut extracts, and the reported spectrometric and spectrophotometric data make it plausible that the identified compound and our compound 12 are two isomers [27]. Therefore, the compound 12 was tentatively identified as *O*-galloyl-castalagin isomer. The compounds 9 and 15 have UV-Vis spectra with absolute absorbance maxima at $\lambda \leq 254$ nm and a maximum relative absorbance around 370 nm, similar to that of ellagic acid as shown in Figure 3 B. These compounds are based on a glucose core esterified with at least one unit of ellagic acid. Compound 15 was identified as ellagic acid, while the compound 9 is tentatively identified as dehydrated tergallagic C1-glucopyranoside according to its spectrophotometric and spectrometric data, and previous data reported for Acorns (*Quercus spp*.) and cork from *Quercus suber* L. Such a compound could derive from tergallagic acid C1-glucoside (MW 631) via intramolecular esterification of the carboxyle group of one gallic acid unit and the oxydryl group linked to the C2 of cyclic glucose, with the loss of one molecule of water [28,29]. Finally, the UV-Vis spectra with maximum absorbance between 270 and 280 nm, the profile of which is similar to that of gallic acid as shown in Figure 3 C, characterize the esters between glucose and gallic acid.

Among these, the compound 5 was identified as gallic acid (MW 170). The ESI-MS fragmentation patterns of the other similar compounds 4, 6, 11, 13, 14 and 16, show quasi-molecular ions respectively at *m/z* 331, 483, 635, 787 and 939; the intense signal at *m/z* 169 confirms the presence of gallic acid and the loss of an increasing number of deprotonated gallic acid units from a central glucose core, allowing the identification of the compounds 4 and 6 as two different monogalloyl D-glucopyranose isomers, and the compounds 11, 13, 14 and 16 as digalloyl-, trigalloyl-, tetragalloyl- and pentagalloyl-glucopyranose respectively. The intense ESI-MS peak at *m/z* 271, [monogalloyl glucopyranose-H-60]$^-$, due to the cross-ring fragmentation of the quasi-molecular ion [30,31], was well evident in particular for the compounds 4, 6 and 11. Compound 16 also shows the doubly-charged quasi-molecular ion (*m/z* 469). Moreover, we can note the absence of the typical spectral characteristics of the polygalloyl derivatives with depsidic links: a slight batochromic shift due to the increased conjugation inside the molecule, and the formation of a shoulder at about 300 nm, increasing with the number of the depside links, and clearly evident when observing the UV-Vis spectra in first and second derivatives [19,32].

Each fraction of the production process has been quantitatively characterized by single compounds; by way of example, the results for the two commercial fractions are shown in Table 4. The total tannins are 11.1 and 22.5% weight in the liquid fraction and in the dry one respectively. The

most abundant tannins are vescalagin and castalagin, with a predominance of the first one. The weight percentage of vescalagin + castalagin on total tannins is 43.0 (liquid fraction) and 37.8% (spray-dried fraction). Vescalin and castalin are present in smaller amounts, and it can be can easily hypothesized that their higher percentage, where the corresponding percentage of vescalagin and castalagin is lower, is due to the partial hydrolysis of the latter. Gallic acid and ellagic acid amounts in the dry fraction are higher than in the liquid one, and this difference reinforces the hypothesis of low stability of the high molecular weight GTs and ETs, and their partial hydrolysis during the spray-drying process. In actual fact, only pentagalloyl glucose seems to be definitely unstable, since its content, 3.3% in the liquid fraction, drops to 0.0% in the dry fraction. Nevertheless, it is difficult to make assumptions about the stability of the single HTs, all present in the same sample, especially when we consider that the percentages of high molecular weight ETs, such as roburin D and dehydrated tergallagic C1 glucoside, are higher in the spray-dried than in the liquid fraction.

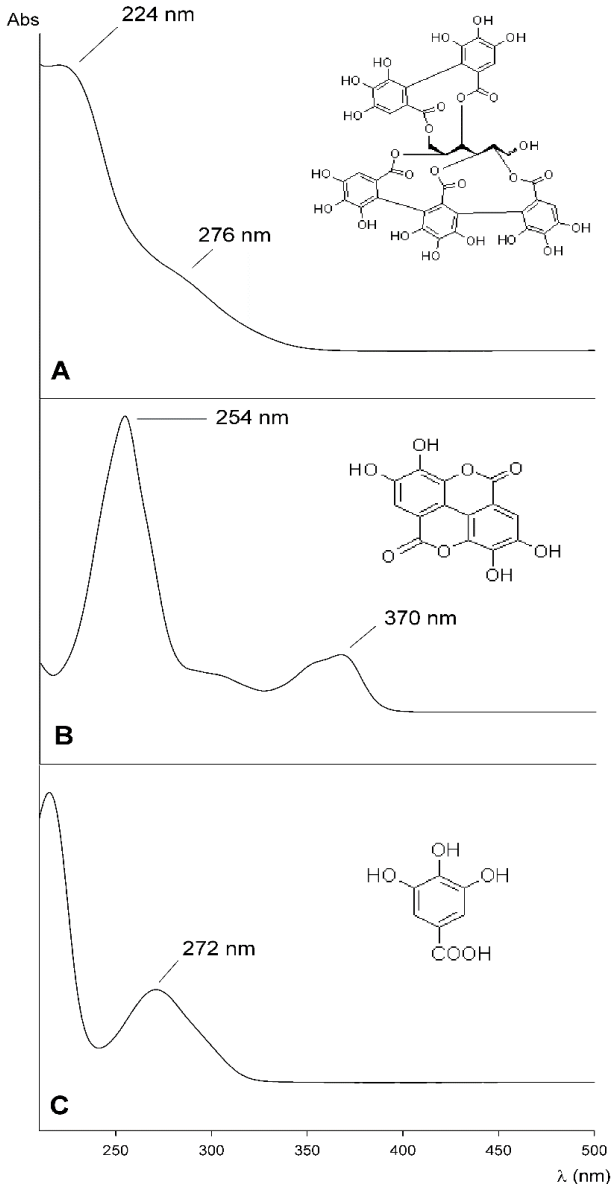

Figure 3: Chemical structures and UV-Vis spectra of vescalagin and castalagin (A), ellagic acid (B) and gallic acid (C). λ (nm) of absorbance maxima and shoulders are indicated.

Table 3: Single compounds qualitative analysis of the Sweet Chestnut liquid commercial fraction (fraction 6). R_t = retention time (min); λ_{max} = maximum absorbance wavelength (nm); sh = shoulder. *m/z* of the most abundant ESI-MS ion is underlined

peak	Rt	compound	λ_{max}	MW	negative ions *m/z*
1	6.8	vescalin	230, 280 sh	632	631 $[M-H]^-$
2	8.9	castalin	230, 280 sh	632	631 $[M-H]^-$
3	11.3	pedunculagin I	222, 282 sh	784	783 $[M-H]^-$, 481
4	13.9	monogalloyl glucose I	274	332	331 $[M-H]^-$, 271, 169
5	14.6	gallic acid	272	170	169 $[M-H]^-$, 125
6	15.2	monogalloyl glucose II	274	332	331 $[M-H]^-$, 271, 169
7	17.2	roburin D	228, 286 sh	1850	933, 924, 915, 683, 301
8	18.3	vescalagin	224, 276 sh	934	933 $[M-H]^-$, 466, 301
9	20.5	dehydrated tergallagic-*C*-glucoside	250, 374	614	613 $[M-H]^-$, 301
10	21.1	castalagin	224, 276 sh	934	933 $[M-H]^-$, 466, 301
11	23.4	digalloyl glucose	274	484	483 $[M-H]^-$, 377, 271, 169
12	24.2	*O*-galloyl-castalagin isomer	220, 280 sh	1086	1085, 520, 542
13	31.8	trigalloyl glucose	276	636	635 $[M-H]^-$, 465, 241, 169
14	37.7	tetragalloyl glucose	276	788	787 $[M-H]^-$, 356, 169
15	38.9	ellagic acid	254, 370	302	301 $[M-H]^-$
16	40.1	pentagalloyl glucose	274	940	939 $[M-H]^-$, 469, 169

The identification and quantification of the tannin subclasses present in each fraction from the productive process is showed in Table 5. The compounds identified are almost the same for each fraction, while significant differences were found with regard to the quantitative aspects. The total tannin content varies between 2.68 and 138 mM for fractions 7 and 8 respectively (liquid fractions), and is 372 μmol/g for the spray-dried (fraction 10). The ET percentages are higher than those of the GTs in the liquid fractions concentrated from nanofiltration (3, 4, 6), whereas in the permeated ones (2 and 5), the GTs are predominant. Hence, the process of nanofiltration is quite effective in separating the GTs from the ETs. Fraction 5, already highly enriched with GTs (74.3%), gets filtrated via a reverse osmosis process to give a concentrate (fraction 8) with about the same qualitative composition and percentages as the tannic derivatives, but more highly concentrated (43.2 mM total tannins for fraction 5 and 138 mM for fraction 8). The osmosis permeate (fraction 7), that is now reintroduced in the production process as extraction water, only contains 2.68 mM of tannins, and 100% of gallic acid. This suggests an optimization of the filtration/concentration steps sequence to obtain a final fraction containing all the gallic acid present in the raw extract, purified and ready to be dried and marketed as a chemical. The vescalagin and castalagin content varies between 0.0 in fraction 7 and 40.5% in fraction 3, confirming that large amounts of high molecular weight compounds are removed during the nanofiltration process [22].

The dry commercial fraction was stable at the 6-month follow-up analytical control, while diluted solutions had some stability problems, in particular vescalagin and castalagin were partially hydrolyzed yielding vescalin and castalin.

Table 4. Single compounds quantitative analysis of Sweet Chestnut commercial fractions: liquid (fraction 6) and dry (fraction 10). Peak numbers correspond to those in the previous Table 1; results are expressed in mg/g and μmol/g of sample. RSD% = Relative Standard Deviation (%).

Compound	Content in commercial liquid fraction			Content in commercial dry fraction		
	mg/g	μmol/g	RSD %	mg/g	μmol/g	RSD %
vescalin	3.57	5.65	1.9	8.68	13.7	4.6
castalin	3.21	5.08	0.6	8.83	14.0	4.9
pedunculagin I	3.31	4.22	1.2	10.1	12.9	4.4
monogalloyl glucose I	2.51	7.57	1.3	4.69	14.1	2.4
gallic acid	1.53	9.02	1.9	13.6	79.8	2.1
monogalloyl glucose II	2.68	8.08	4.7	5.03	15.2	4.3
roburin D	4.03	4.36	1.2	9.61	10.4	3.4
vescalagin	26.7	28.6	2.4	45.2	48.4	3.6
dehydrated tergallic-*C*-glucoside	1.77	2.88	1.6	6.03	9.82	4.5
castalagin	21.0	22.5	1.8	39.7	42.5	3.6
digalloyl glucose	3.79	7.84	1.2	12.2	25.3	2.0
O-galloyl-castalagin isomer	20.8	19.2	1.9	32.0	29.5	2.2
trigalloyl glucose	5.03	7.91	1.4	12.1	19.0	3.1
tetragalloyl glucose	4.72	5.99	3.2	9.18	11.6	2.4
ellagic acid	2.50	8.26	0.8	7.80	25.8	8.7
pentagalloyl glucose	3.67	3.90	2.0	0.00	0.00	-
total tannins	111	151	1.0	225	372	2.5

3.2. Agronomic tests

Climate in 2013 cropping season was drier (11–47% less monthly rainfall) and in 2014 wetter vs. the same period of the previous two decades: in 2014 monthly rainfall in the July–September was 1.9–3.5 times greater than the historical reference (data not presented).

In 2013, among the ordinary agrochemicals, Oxamyl had a significantly better nematode control than Ethoprophos, with a strong impact on nematode infestation and Barker grading (less root attacks). *Paecilomyces lilacinus* strain gave results not different from the control, both for nematode count at the end of the experiment, and Barker grading. CHT apparently did not affect nematode count in the soil, however the treated plants exhibited a number of nematode attacks on the roots (Barker grading) not different from Oxamyl. This parameter was strongly and positively correlated to tobacco yield and quality (Table 6).

In 2014 nematode control in the soil was better in soils treated with *Bacillus firmus* I-1582 and Azadirachtine, while nematode count at the end of the experiment was unaffected by the application of the two agrochemicals, and CHT. However, less root attacks (lower Barker grading) was observed with *Bacillus firmus* I-1582 and CHT, and this aspect was directly related to tobacco quality index. The two botanicals, CHT and Azadirachtine, did not significantly differ from the biological agent for leaf yield, achieving yield levels greater than the control and the ordinary agrochemicals.

Table 5. Quantitative analysis by subclasses (% calculated from their contents expressed in mM) for all the fractions from the productive process. A. % gallic acid on total tannins; B. % gallic acid on total GTs; C. % castalagin + vescalagin on total tannins; D. % castalagin + vescalagin on total ETs; E. % GTs on total tannins; F. % ETs on total tannins.

	A	B	C	D	E	F	TOT[a]
1	42.6	74.3	27.3	63.9	57.3	42.7	27.2
2	74.4	86.4	10.6	75.8	86.1	13.9	15.7
3	24.3	69.4	40.5	62.3	35.0	65.0	82.0
4	38.3	79.0	33.1	64.1	48.4	51.6	75.3
5	62.2	83.6	17.1	66.4	74.3	25.7	43.2
6	6.00	17.9	33.8	50.7	33.3	66.7	121
7	100	100	0.00	-	100	0.0	2.68
8	63.0	84.0	15.1	60.1	74.9	25.1	138
9	31.8	59.6	31.1	66.7	53.3	46.7	63.6
10	21.4	48.4	24.4	43.9	44.4	55.6	372

[a] Total tannins content expressed as mM for the liquid fractions and as μmol/g for the fraction 10 (spray-dried).

The different behaviour, in particular of Oxamyl, in the two years is probably related to the heavy rainfall in August and September, which negatively affected the residual activity of this agrochemical. Nematicides on tobacco and most vegetable crops are labelled for applications carried out at least 60 days before harvest, to avoid a.i. residues, and this requirement may represent a major limit when the final part of the season is wet, and therefore prone to nematode late infestation. Under these conditions, botanicals applied in repeated treatments exhibited a better response.

Under the standpoint of the mechanism of action, field observations of plant root system across each plot and nematode counting at the end of the experiment are likely to confirm previous findings on the biostimulant effect of CHT [14]. In fact, the product did not determine a nematode control as in the case of the agrochemical Oxamyl, the biological agent *Bacillus firmus* I-1582, and the botanical Azadirachtine; however, a low evidence of nematode feeding on tobacco roots in CHT plots was found according to Barker grading. This effect is apparently related to the development of a larger root system (a kind of "diluting" effect), which is generally associated with higher plant yield and quality. Therefore, treating plant with CHT apparently activates a mechanism of root development which counterbalances the nematode attack.

4. Conclusion

The present study addresses for the first time the possibility of fractionating the raw aqueous extract to obtain new fractions with differentiated chemical compositions, for new, sustainable and innovative uses in a variety of fields. Further investigations are in progress on the possibility of stabilizing the liquid diluted fractions in quali-quantitative point terms, and the process repeatability, compatibly with the fact that the composition of the vegetable matrix, in any case, maintains a natural variability over the time. A further added value is given by the ecological and economical sustainability of the process that uses a wood co-product, is solvent-free, and the water used as a solvent is partially recycled from the process itself as an osmosis permeate.

Table 6. Nematode field experiments 2013 and 2014. Results of the determinations. Soil for nematode count and plant root samplings were made the same day of last harvest.

Treatm.No.	2013							
	Nematodes count No. in 200 cm^3 soil		Barker grading[a]		Yield Index		Quality Index	
Control	405	d	4.7	b	91	b	86.1	b
1	109	b	4.2	b	99	b	88	b
2	29	a	2.8	a	123	a	104.8	a
3	488	d	4.9	b	81	c	90	b
4	233	c	3	a	120	a	103.4	a
PP nematode count	244							
Leaf yield, field mean t/ha	2.65							
Treatm.No.	2014							
	Nematodes count No. in 200 cm^3 soil		Barker grading[a]		Yield Index		Quality Index	
Control	1102	c	4.6	c	91	b	87.9	d
1	633	b	4.3	bc	90	b	88	cd
2	552	b	3.9	b	92	b	93	c
5	181	a	3	a	110	a	110.4	a
6	225	a	3.7	b	102	a	101.8	b
7	556	b	3.1	a	106	a	106.9	a
PP nematode count	555							
Leaf yield, field mean t/ha	2.69							

Means within a column (belonging to the same year) followed by the same letters are not significantly different (P = 0.05).

For the first time, one of the CHT fractions was formulated as a microgranulated fertilizer and a fully dispersible powder for application at low rates in efficient, localized repeated placements along each transplant row. The experiments here reported are among the very first field applications on tobacco, in comparison with nematicides, and botanicals, and biological controlling agents for nematodes. These experiments confirm that CHT acts as a biostimulant on plant root systems, enhancing indirectly plant resistance to nematodes. This mechanism could positively affect CHT efficacy in the medium-long term, because it determines a lower selective pressure on nematode population as compared to more aggressive a.i. It should also be noticed, that this sustainable approach was demonstrated to be coupled with a null toxicity profile on model organisms and microorganisms by researchers of the same project EVERGREEN [34].

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Conflict of interest

The authors declare no conflict of interest in publishing this paper.

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