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A comparative study on phytochemical fingerprint of two diverse *Phaseolus vulgaris var.* Tondino del Tavo and Cannellino Bio extracts

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Abstract: Common bean (*Phaseolus vulgaris*) represents one of the most famous food with anti-obesity activity showing a significant efficacy against fat accumulation, insulin resistance and dyslipidaemia. In this work two Italian varieties of common bean *e.g.* Tondino del Tavo and Cannellino Bio from the centre of Italy were studied to characterise their phenolic profile by HPLC-PDA in relation to different fractions after a straightforward extraction procedure. Antioxidant property and enzymatic inhibition power were also evaluated in order to delineate a possible biological profile. Results show a considerable phenolic content (0.79 and 1.1 μg/mg of 3-hydroxy benzoic acid for hexane extract of Tondino del Tavo and Cannellino Bio respectively; 0.30 μg/mg *p*-coumaric acid for *n*-hexane extract of Tondino del Tavo) for both varieties, and a strong antioxidant activity according to the major phenolic concentration of the extracts. Anti-inflammatory activity of the decoction extracts was also investigated through zymosan-induced edema formation assay, revealing a moderate ability for both of them to reduce the edema formation. These preliminary data prompt us to further explore the nutrient components of these two varieties in the next future.

Keywords: extraction; fractions; phenols; enzymes; HPLC profile; protein; flavonoids

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1. Introduction

Metabolic syndrome is a pathological condition of obese people characterised by hypercholesterolemia, hepatic steatosis, abdominal obesity and many concomitant biochemical alterations such as the accumulation of cytoplasmatic triglycerides, insulin resistance and changes in glucose metabolism [1,2]. A new strategy for the management of metabolic syndrome consists in the use of nutraceuticals [3]; in particular common bean extracts (*Phaseolus vulgaris*) exhibit some beneficial effects against metabolic syndrome [1,2]. Common bean is the most diffuse legume in the world and represents the half of total legume consumption in human diet [4]. It is ~~reach~~ rich in proteins, fibres, unsaturated fatty acids, vitamins, minerals, and flavonoids [4,5]. Its extracts exhibit the capacity to inhibit α-amylase thus reducing body absorption of carbohydrates leading to a decrease of body

weight, glycemia, triglycerides and cholesterol blood level [1,2,4,5]. The anti-obesity activity of common bean extracts is confirmed by many different preclinical studies in rat model. Tormo and co-workers have isolated and purified an inhibitor of human α-amylase from common bean extracts, demonstrating the hypoglycaemic and anorexigenic power of this inhibitor *in vivo* [6]. Neil and co-workers reported that consumption of common bean extracts plays a role in body weight control by reducing the abdominal fat accumulation in mice model with an increase of intestinal mass, without modification of crypts height and mucin content [7]. ~~The same group has highlighted that there are~~ No differences in weight loss between the group treated with common bean extract in slimming regimen (dietary modification, exercise) and the control group (slimming regimen without common bean extracts) during a short period of time have been registered in this study [8]. ~~This study has been further implemented by~~ A meta-analysis on anti-obesity activity of common bean extracts confirmed the statistically significant effect of weight loss in humans treated with ~~subjected to~~ common bean extracts ~~diet has been confirmed~~. In fact ~~according to this meta-analysis,~~ *Phaseolus vulgaris* extracts reduced the body weight by 1.08 Kg and the body fat by 3.26 Kg (both results have a 95% CI) [9]. The anti-obesity activity of common bean extract is due to the presence of an α-amylase inhibitor isoform I called *phaseolamin* (α-AI), able to block the α-amylase digestive enzyme activity, preventing the carbohydrates metabolism and its absorption [2,4,8,10]. ~~Inhibition activity of *phaseolamin* on human salivary α-amylase has been evaluated;~~ De Gouveia and co-workers have tested commercial *phaseolamin in vitro* and *in vivo*, demonstrating ~~their~~ its capacity to inhibit α-amylase activity *in vitro*, thus reducing blood glucose level [11]. ~~In the same study the polypeptide profile of these commercial samples has been also characterised:~~ The SDS-PAGE analysis showed the presence of many type of proteins such as α-AI (16-11 kDa), *phaseolin* (50-35 kDa) the most representative glycoprotein in common bean and ~~the~~ *phytohemagglutinin* (35-25 kDa); ~~however~~ no toxicity was observed in rats, ~~instead~~ while a low hemagglutination activity was ~~observed~~ detected on all types of human erythrocytes [11]. Also Barret and Udani confirmed the role of ~~the~~ *phaseolamin* to inhibit α-amylase and to reduce body weight and fat accumulation in humans [12,13]. ~~A particular product called *Phase 2* consists in a common bean's water extract able to induce an effective weight loss at a dose of 500–300 mg per day, both as single and fractionated doses. *Phase 2* is a dietary supplement used as powders, tablets, capsules chewable and it is present on the market in 200 brands as weight loss products. *Phase 2* assumption provokes the reduction of blood post prandial spikes of glucose and insulin, thus decreasing the risk to develop insulin resistance in type 2 diabetic people [12]. Subjects treated with carbohydrate rich diet associated with *Phase 2* consumption have significantly reduced body weight, mass Index and fat mass, still maintaining the lean body mass compared to the placebo [13].~~ Other examples of beans with antidiabetic activity are mung and adzuki bean. Mung beans contain~~s~~ a series of polyphenols exhibiting ~~that explain~~ α-amylase and α-glucosidase inhibition activity, suggesting a potential control of postprandial glucose level [14]. Adzuki bean extracts contain~~s~~ polyphenols ~~showing interesting ability~~ able to reduce total hepatic lipid, and triglycerides accumulation ~~and anti-inflammatory response~~ [15]. Polyphenols can reduce hydrolysis of *phaseolin* thus interfering with its digestion [16]. In this work we have focused our attention on two varieties of *Phaseolus vulgaris, e.g.* Tondino del Tavo and Cannellino Bio from the center of Italy. The Tavo is an Abruzzo river that originates from the Apennine peak of the Gran Sasso, it flows in the hilly area among lush valleys. ~~The soils of these areas have proved to be excellent for the cultivation of a specific variety of legume, which takes its name from the river and its spherical shape.~~ The round bean from Tavo is small and round like a pea, with a candid pearly color ranging from milky white to ivory, it has excellent nutritional properties and a very thin skin, which allows for faster cooking and easy digestion [17]. Irrigation is essential in times of low rainfall, which coincide with the period of pod enlargement. ~~Weeds are kept under control thanks to the practice of weeding, carried out during the preparation of the soil. Manual and gradual harvesting begins in the second half of October: the dried pods are on the plant and left~~

~~to dry in the sun to eliminate residual moisture.~~ ~~The shelling of the pods, which historically was carried out by hand, nowadays also takes place with mechanical procedures, which facilitate the work.~~ The beans are kept in jute bags, away from sources of light and heat. Cannellino Bio from Colfiorito (Perugia, Umbria, Italy) is very smooth, it is characterized by an ovoid shape and uniform snow white color. It is considered one of the best varieties for production and organoleptic qualities~~, quick cooking with very tender and non-existent skin after cooking~~. It is part of the culinary and cultural heritage of the Umbria region. This legume can be considered an excellent substitute for meat and pasta, although it contains very little fat, it is highly caloric representing an important source of carbohydrates and proteins; due to the presence of fiber, it also helps to maintain normal cholesterol levels [18]. In this preliminary work we performed a separation of diverse fractions through a well-established extraction procedure~~, rich in protein, free phenols, conjugate phenols, and bound phenolic compounds~~ for each variety (Figure 1).

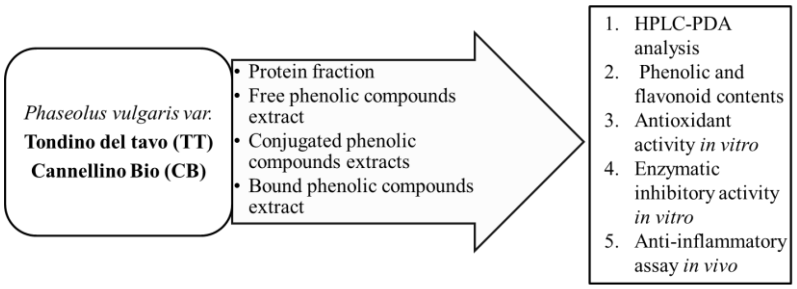

Figure 1. Work flow of comparative analysis for diverse extracts of *Phaseolus vulgaris var*. Tondino del Tavo (TT) and Cannellino Bio (CB).

~~Quantitative analysis via HPLC PDA was performed to delineate their content in gallic acid, catechin, chlorogenic acid, *p*-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, *p*-coumaric acid, rutin, ferulic acid, sinapinic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzoc acid, naringin, so as to determine a fingerprint profile. Total phenolic and flavonoid contents were also assessed via colorimetric assay for each fraction as well as antioxidant and enzymatic inhibition activity *in vitro*. Lastly anti-inflammatory activity of the decoction extracts for both species was also tested *in vivo* in zymosan induced edema formation.~~ Each fraction has been analysed through HPLC-PDA showing a significative phenolic content (0.79 and 1.1 μg/mg of 3-hydroxy benzoic acid for *n*-hexane extract of Tondino del Tavo and Cannellino Bio respectively; 0.30 μg/mg *p*-coumaric acid for *n*-hexane extract of Tondino del Tavo) in both varieties. Antioxidant assays revealed a strong activity according to their major phenolic concentration. Then decoction extracts of were tested for their anti-inflammatory activity through zymosan assay, indicating a moderate ability to reduce the edema formation.

2. Materials and Methods

2.1 Materials

Tondino del Tavo (Loreto Aprutino, Pescara, Abruzzo, Italy) and Cannellino Bio (Colfiorito, Perugia, Umbria, Italy) were selected as common bean variety (*Phaseolus vulgaris*) belonging to the genus *Phaseolus*. They were collected and stored according to their use for human consumption. Tondino del Tavo was purchased by Passeri Carlo Company (Loreto Aprutino, Pescara) and Cannellino Bio (Colfiorito, Perugia) by a local market. Methanol, ethanol, *n*-hexane, ethyl acetate, acetonitrile for HPLC ~~use~~, NaOH, HCl and

NaCl were purchased by Sigma Aldrich (Milan, Italy). Gallic acid, catechin, chlorogenic acid, *p*-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, *p*-coumaric acid, rutin, ferulic acid, sinapinic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzoc acid, naringin, 2,3-dimethoxybenzoic acid, *o*-coumaric acid, quercetin, cinnamic acid, carvacrol and naringenin were purchased by Sigma Aldrich (Milan, Italy). The centrifuge is an Eppendorf 5702 (Hamburg, Germany). The lyophilizator is a Buchi L-100 (BUCHI Italia s.r.l.Cornaredo, Italy).

2.2 Samples preparation

One hundred gram ~~100 g~~ of common beans for each variety were lyophilized, the so obtained material was weighted, shredded into a mortar and transferred into a plastic vial far from sun light and stored at 20°C. Around one gram of Cannellino Bio and Tondino del Tavo were powdered with a blender and freeze-dried. Five hundred milligram of Cannellino Bio and Tondino del Tavo were suspended in 70% ethanol/water solution (20 mL) and boiled for 5 min. The powder was filtered and freeze-dried again. The prepared extracts were obtained with 18% and 20% yields respectively.

2.3 Extraction method

2.3.1. Protein fraction

Five gram ~~5g~~ of lyophilized material was put into a reaction flask with 40 mL of NaCl 1.5% m/v solution (8 mL/g of sample) and stirred for 4 hours at room temperature [17]. The supernatant was transferred into 5 vials of 6 mL each and then centrifugated for 90 minutes at 4.400 rpm. After centrifugation, supernatant was transferred into a reaction flask, acidified at pH 6 and heated at 70°C for 20 minutes under reflux. Then supernatant was put into 8 vials of 6 mL each and centrifuged for 40 minutes at 4.400 rpm. Supernatant was separated by the precipitate and it was diluted with ethanol in a ratio of 3:7 then it was put into fridge at 4°C for 60 minutes. Finally, supernatant was centrifuged again for 15 minutes at 4.400 rpm. The supernatant was wasted and the solid precipitated was dried under high vacuum overnight [17]. The yield of the white bean protein extract was 2.61g/100g and 2.57g/100g of dried bean for Tondino del Tavo (TT) and Cannellino Bio (CB) respectively.

2.3.2. Free phenolic compounds fraction

Free phenols extraction was performed following the method developed by Telles *et al*., five gram of lyophilized material was put into a reaction flask with 17 mL of methanol and stirred for 1 hour [18]. The mixture was rested for 15 minutes, and the solvent removed. 17 mL of methanol was added to the powder and the mixture was stirred again for 90 minutes. This procedure was repeated twice. The tree solutions of methanol were gathered, put into 4 vials of 6 mL each and centrifuged for 5 minutes at 4.400 rpm. The supernatant was put into 4 vials of 6 mL each, then a solution of $ZnSO_4$ 0.1M (2.1 mL) and $Ba(OH)_2$ solution 0.1M (2.1 mL) were added for clarification. The mixtures obtained were rested for 30 minutes, centrifuged for 5 minutes at 4.400 rpm. This procedure was repeated twice, after clarification the supernatants were put together into a reaction flask and the solvent was removed by rotavapor. The yield was 1.36 g/100 g and 1.12 g/ 100g of dried bean for Tondino del Tavo (TT) and Cannellino Bio (CB) respectively.

2.3.3. Conjugated phenolic compounds fraction

Ten gram ~~10 g~~ of lyophilized material was weighted and put into a reaction flask with 33.3 mL of ethanol (10 mL/3g lyophilized material) and stirred for 10 minutes. The solid was filtered on Buckner funnel and extracted with 33.3 mL of ethanol and stirred for 10 minutes. The supernatant was centrifuged for 10 minutes at 4.400 rpm in 7 vials of 6 mL each. Finally, the supernatant was put into a reaction flask and the solvent was evaporated with rotavapor. The yield was 2.14 g/100 g and 2.08 g/ 100g of dried bean for Tondino del Tavo (TT) and Cannellino Bio (CB) respectively.

2.3.4. Bound phenolic compounds fraction

Half of the conjugated phenolic compounds fraction (paragraph 2.3.3.) was added to 16.6 mL of *n*-hexane (10 mL/3g of product) and stirred in a reaction flask for 10 minutes. The obtained supernatant was put into 4 vials and centrifuged for 35 minutes at 4.400 rpm. The supernatant was wasted and the precipitate was dried into high vacuum. The solid was put into a reaction flask with 70 mL of NaOH 4M and stirred for 3 hours. Then the solution was acidified at pH 1.0 with HCl 6M, centrifuged and put into separation funnel. The solution was extract two times with ethyl acetate, put into a flask and dried with dry sodium sulphate for 15 minutes. The solvent was removed in rotavapor until to obtain a white solid. The yield of the white bean extract was 1.16 g/100 g and 1.05 g/ 100g of dried bean for Tondino del Tavo (TT) and Cannellino Bio (CB) respectively.

~~*2.4 Decoction extracts preparation*~~

~~Around 1 gram of Cannellino Bio and Tondino del Tavo species were powdered with a blender and freeze-dried. 500 mg of Cannellino Bio and Tondino del Tavo were suspended in 70% ethanol/water solution (20 mL) and boiled for 5 min. The powder was filtered and freeze-dried again. The prepared extracts were obtained with 18% and 20% yields respectively.~~

2.4 HPLC-PDA analysis

The HPLC-PDA procedure ~~as reported in literature,~~ was performed on a model 600 solvent pump coupled with 2996 PDA detector (Waters Spa, Milford, MA, USA) [19]. A C18 reversed-phase column (Prodigy ODS-3, 4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA, USA) thermostated at 30 ± 1°C using a Jetstream2 Plus column oven, was used for the separation. The UV/Vis acquisition wavelength was set in the range of 200–500 nm. The quantitative analyses were achieved at maximum wavelength for each compound, in particular 271 nm, 278 nm, 324 nm, 256 nm, 260 nm, 278 nm, 274 nm, 275 nm, 278 nm, 310 nm, 256 nm, 324 nm, 315 nm, 285 nm, 299 nm, 275 nm, 276 nm, 367 nm, 280 nm, 276 nm, 290 nm, 275 nm for gallic acid, catechin, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic acid, naringenin, and carvacrol, respectively. The injection volume was 20 µL. The mobile phases were directly *on-line* degassed by using Biotech DEGASi, mod. Compact (LabService, Anzola dell'Emilia, Italy). The mobile phases consist of solution A (3% solution of acetic acid in water) and solution B (3% solution of acetic acid in acetonitrile) in a ratio 93:7 (*v:v*) and the gradient mode was applied (93-2 % eluent A in 45 min). The flow rate was set at 1 mL/min throughout the analysis. Empower v.2 Software (Waters Spa, Milford, MA, USA) was used to collect and analyse the raw data obtained after the sample suspension (accurately weighted) in the mobile phase, sonicated, centrifuged at 12000 rpm, and directly injected (20 µL) into HPLC-PDA system.

2.5 Total phenolic and flavonoid content

The total concentrations of phenols and flavonoids were determined using previously described methods [20]. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/g dry extract, while total flavonoids were expressed as mg rutin equivalents (RE)/g dry extract. The assays were performed in triplicate, and ANOVA (Tukey's test) was used to determine the differences in the extracts.

2.6 Antioxidant and enzyme inhibitory assays

The antioxidant activity of the extracts was determined using a variety of assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical scavenging capacity (CUPRAC), ferric ion reducing antioxidant power (FRAP), metal chelating ability (MCA), and phosphomolybdenum assay (PBD ~~PDA~~) [21]. The data for the DPPH, ABTS, CUPRAC, and FRAP assays were expressed in mg Trolox equivalents (TE)/g extract, whereas the data for MCA and PDA were expressed

in mg EDTA equivalents (EDTAE)/g extract and mmol TE/g extract, respectively. In cholinesterase assays, galanthamine was used as a positive control, and data were expressed as mg galanthamine equivalents (GALAE)/g extract. In the tyrosinase inhibitory assay, kojic acid was used as a standard inhibitor, and the results were expressed as mg kojic acid equivalents (KAE)/g extract [22]. In the anti-diabetic assays, acarbose was chosen as an inhibitor of both amylase and glucosidase, and the results are expressed as mmol acarbose equivalents (ACAE)/g extract. AChE and BChE are acetylcholinesterase and butyrylcholinesterase enzymes respectively. ~~The assays were performed in triplicate, ANOVA (Tukey's test) was used to determine the differences in the extracts.~~

2.7 Data analysis

The assays were performed in triplicate, ANOVA (Tukey's test) was used to determine the differences in the extracts. The relationship between biological activity assays and total bioactive components was demonstrated using Pearson correlation analysis. The analysis was carried out utilizing Graph Pad Prism (version 9.2). For *in vivo* assay Statistical analysis was performed by using two-way ANOVA followed by Dunnett's multiple comparisons test.

2.8 Zymosan induced edema formation

Male CD-1 mice (Harlan, Italy) of 3-4 weeks (25 g) were used for all the experiments. Mice were housed in colony cages, under standard conditions of light, temperature, and relative humidity for at least 1 week before starting experimental sessions. All experiments were performed according to Legislative Decree 27/92 and approved by the local ethics committee (Approval number 198/2013-B). Mice received a subcutaneous (s.c.) administration (20 µL/paw) of zymosan A (2.5% w/v in saline) into the dorsal surface of the right hind paw. Paw volume was measured 3 times before the injections and at 1, 2, 3, 4, and 24 h thereafter using a hydroplethysmometer apparatus (UgoBasile, Italy). The increase in paw volume was then evaluated as the percentage difference between the paw volume at each time point and the basal paw volume [23]. Cannellino Bio and Tondino del Tavo decoction extracts were dissolved in DMSO:saline (ratio 1:3 v/v) and were administered s.c. into the dorsal surface of the right hind paw at the dose of 100 µg/20 µL paw 15 min before or 150 min after zymosan. ~~Statistical analysis was performed by using two-way ANOVA followed by Dunnett's multiple comparisons test.~~

3. Results and discussion

To evaluate the phenolic content of the different *Phaseolus vulgaris* extracts HPLC-PDA analysis was used, and small content of phenolic substances were found in each type of extract as reported in Table 1. The major phenolic content of these secondary metabolites was found in *n*-hexane extract of Tondino del Tavo (#4TT), in which 3-hydroxybenzoic acid (0.79 µg/mL) and *p*-coumaric acid (0.30 µg/mg) were detected ~~found~~. The other metabolites were found in low concentration (below limit of quantification). Similar results were obtained for Cannellino Bio, in which the major phenolic content was also measured ~~found~~ in #4CB, in which ~~and~~ 3-hydroxybenzoic (1.1 µg/mg) was the most abundant (#4CB). Quantities below the quantification limit of *p*-OH benzoic acid, sinapinic acid and *t*-ferulic acid, were found in #4TT. The quantities of *p*-coumaric acid and sinapinic acid in #2CB/TT and #3CB/TT were also found to be below the quantification limits. The low concentration of *t*-ferulic acid and sinapinic acid was also reported in other studies based on a comparative analysis among diverse Phaseolus species [24].

Table 1. Quantitative analysis (µg/mg) of the *Phaseolus Vulgaris* extracts.

Sample ID	*p*-OH benzoic acid	3-hydroxybenzoic acid	*p*-coumaric acid	Sinapinic acid	*t*-ferulic acid
#1TT					
#2TT			BLQ	BLQ	
#3TT			BLQ	BLQ	

#4TT	BLQ	0.79±0.08	0.30±0.02	BLQ	BLQ
#1CB					
#2CB				BLQ	
#3CB					
#4CB	BLQ	1.1±0.1	BLQ	BLQ	BLQ

Values are reported as media ± standard deviation of three parallel experiment; **BLQ**: below limit of quantification; **TT:** Tondino del Tavo; **CB:** Cannellino.

Diverse factors influence the TPC, TFC and composition of common beans, for example the environmental conditions, genotype, storage, and processing methods. Several works stressed the major influence of genotype over location in the determination of the TPC of common beans [25]. Compared to TPC, there are fewer reports about the effect of germination on the TFC of common beans; Xue et al. reported that the TFC of black beans increased after a prolonged germination time, reaching the highest value on the 5th day [26]. Bioactive compounds such as gallic aldehyde, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, *p*-hydroxybenzoic aldehyde, *trans*-feruloyl aldaric acid and sinapoyl aldaric acid were found in the insoluble fractions of *Phaseolus vulgaris* L., while no hydroxy-benzoic compounds were identified in the insoluble fraction of the germinated beans [27]. Furthermore extraction techniques have a key role in the recovery of polyphenols. In this context, several matrices have been used such as raw beans, seed coats, cotyledons, germinated and fermented common beans. Actually there is a lack of data about the optimal extraction condition of common bean polyphenols. On the other hand, extraction solvent is crucial to reach a high efficiency polyphenol extraction; Methanol/water/acid systems are most commonly used for phenolic acids, however these are not suitable to gain soluble phenolic fraction [28]. In order to obtain polyphenol-rich extracts, can be purified to remove impurities by diverse types of adsorbents [29].

The Folin-Ciocalteu test did not show a significative total phenolic content in both varieties of *Phaseolus vulgaris* according to HPLC analysis. The major total phenolic content was found in #4CB (25.42 mg of GAE/g) and #4TT (11.59 mg GAE/g) (Table 2).

Table 2. Phenolic content of the tested samples.

SAMPLES	TPC (mg GAE/g)	TFC (mg RE/g)	DPPH (mg TE/g)	ABTS (mg TE/g)
#1TT	6.63±0.11^{c}	0.02±0.01^{e}	na	0.76±0.03^{e}
#2TT	3.46±0.06^{d}	1.68±0.01^{a}	na	na
#3TT	10.86±0.11^{b}	0.06±0.02^{cde}	na	0.71±0.01^{e}
#4TT	11.59±0.31^{b}	0.07±0.02^{cd}	1.04±0.02^{b}	16.11±0.07^{b}
#1CB	7.22±0.02^{c}	0.61±0.02^{b}	na	2.90±0.22^{c}
#2CB	3.32±0.02^{d}	0.08±0.01^{c}	na	na
#3CB	11.84±0.03^{b}	0.02±0.01^{de}	na	1.30±0.02^{d}
#4CB	25.42±1.23^{a}	0.61±0.03^{b}	6.65±0.11^{a}	55.96±0.01^{a}

Values are reported as media ±DS of three parallel experiment. TPC: Total Phenolic Content; TFC: Total Flavonoid content; GAE: Gallic Acid Equivalent; RE: Rutin Equivalent; TE: Troxol Equivalent. na: not active. Different superscripts (a-e) in same column indicate significant differences in the samples (by ANOVA (Tukey's test), $p<0.05$)

In vitro antioxidant activity was evaluated with DPPH and ABTS assays, while reducing power with CUPRAC and FRAP tests. The best antioxidant activity was found in #4CB (55.96 mg TE/g in ABTS), which is active on DPPH (6.65 mg TE/g) as well as #4TT (ABTS: 16.11 mg TE/g). These extracts reported the best reductive activity in CUPRAC (64.69 mg TE/g for CB and 37.05 mg TE/g for TT). Similar results were obtained in FRAP (#4CB: 42.94 mg TE/g,#4TT: 21.01 mg TE/g) (Table 3).

Table 3. Antioxidant activity of the tested samples.

SAMPLES	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Chelating activity (mg EDTAE/g)	PBD (mg TE/g)
#1TT	19.97±0.57^{f}	6.17±0.07^{e}	16.24±0.07^{a}	0.26±0.02^{d}
#2TT	15.14±0.13^{g}	7.40±0.28^{d}	3.34±0.87^{cd}	0.56±0.09^{c}
#3TT	30.43±0.13^{c}	6.96±0.03^{de}	7.64±0.21^{b}	1.13±0.05^{b}
#4TT	37.05±0.92^{b}	21.01±0.71^{b}	15.68±2.31^{a}	1.18±0.05^{b}
#1CB	24.14±1.53^{e}	6.78±0.02^{de}	16.51±0.06^{a}	0.12±0.01^{e}
#2CB	16.95±0.67^{g}	9.48±0.69^{c}	2.65±0.52^{d}	0.59±0.03^{c}
#3CB	27.48±0.35^{d}	6.83±0.12^{de}	1.81±0.10^{d}	1.10±0.02^{b}
#4CB	64.69±0.55^{a}	42.94±0.03^{a}	5.63±0.10^{bc}	1.55±0.01^{a}

Values are reported as media ±DS of three parallel experiment. TE: Troxol Equivalent; EDTAE: EDTA Equivalent. Different superscripts (a-f) in same column indicate significant differences in the samples (by ANOVA (Tukey's test), $p<0.05$)

These results are directly linked to the major phenolic concentration obtained through the extraction procedure applied (paragraph 2.1.). In the present work, the total phenolic and flavonoid contents in the tested extracts were examined by colorimetric methods. The highest level of total phenolic was determined in the bound phenolic extract of Cannellino Bio (#4CB: 25.42 mg GAE/g), followed by conjugated phenolic extract of Cannellino Bio (#3CB: 11.84 mg GAE/g) and bound phenolic extract of Tondino del Tavo (#4TT: 11.59 mg GAE/g). However, #2TT contained the highest level of total flavonoids (1.68 mg RE/g). Generally, the tested extracts exhibit low levels of flavonoids (<1 mg RE/g), actually different levels of total phenolic and flavonoids in *Phaseolus vulgaris* samples were observed in literature [30-33]. In a recent paper by Rossi *et al.*, the effects of germinating time and gastrointestinal digestion on chemical composition and biological activities of two common *Phaseolus vulgaris* cultivars were analysed, revealing a strong correlation between their phenolic contents and the soils composition [34]. Looking at the radical scavenging and reducing power data, the bound extracts of both varieties were the most active; this could be explained by the higher amounts of total phenolic content in respect to the other fractions.

In the phosphomolybdenum assay, the activity of analysed samples can be ranked as follow: #4CB (1.55 mmol TE/g) > #4TT (1.18 mmol TE/g) > #3TT (1.13 mmol TE/g) > #3CB (1.10 mmol TE/g) > #2CB (0.59 mmol TE/g) > #2TT (0.56 mmol TE/g) > #1TT (0.26 mmol TE/g) > #1CB (0.12 mmol TE/g). This behaviour was almost the same for CUPRAC and FRAP assays. Otherwise other antioxidant assays, the best metal chelating abilities were recorded for #1CB (16.51 mg EDTAE/g) and #1TT (16.24 mg EDTAE/g). Several researchers reported metal chelating abilities for some peptides and the antioxidant properties of *Phaseolus vulgaris* extracts [35,36]; ~~in a recent paper by Rossi *et al.*, the effects of germinating time and gastrointestinal digestion on chemical composition and biological activities of two common *Phaseolus vulgaris* cultivars were analysed [28].~~ Furthermore Hernandez-Guerrero *et al.* studied the correlation between secondary metabolites identified through NMR technique and the biological activities of *Phaseolus vulgaris* cultivars [37]. ~~Bento *et al.* also reported the effects of cooking method on metabolomics and biological properties of some *Phaseolus vulgaris* cultivars, further supporting the importance of this cultivar in human diet, deserving functional applications in nutraceutical field [30].~~ Generally colored bean cultivars exhibit superior antioxidant activity compared to white common beans [38]. The extracts were also tested for their inhibitory activity against cholinesterases, amylase, glucosidase and tyrosinase (Table 4).

Table 4. Enzymatic inhibition of the tested samples.

SAMPLES	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mg TE/g)	Glucosidase (mmol ACAE/g)
#1TT	0.02±0.01^{d}	4.30±0.01^{a}	15.07±0.57^{d}	0.05±0.01^{e}	na
#2TT	2.57±0.03^{a}	3.96±0.18^{bc}	57.94±0.23^{c}	0.30±0.02^{d}	1.11±0.04^{a}
#3TT	na	3.85±0.01^{c}	62.02±0.20^{b}	0.39±0.02^{bc}	1.15±0.01^{a}
#4TT	2.18±0.03^{b}	na	66.44±0.14^{a}	0.43±0.01^{b}	1.15±0.01^{a}
#1CB	na	0.61±0.07^{e}	15.73±1.15^{d}	0.05±0.00^{e}	na
#2CB	2.51±0.07^{a}	4.07±0.08^{b}	60.93±0.40^{b}	0.30±0.01^{d}	1.14±0.02^{a}
#3CB	2.57±0.04^{a}	3.76±0.04^{cd}	65.45±0.49^{a}	0.38±0.02^{c}	1.11±0.01^{a}
#4CB	1.90±0.02^{c}	3.57±0.01^{d}	61.36±0.67^{b}	0.57±0.03^{a}	na

Values are reported as media ±DS of three parallel experiment. GALAE: Galatamin equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent. AChE: acetylcholinesterase; BChE: butyrylcholinesterase; na: not active. Different superscripts (a-e) in same column indicate significant differences in the samples (by ANOVA (Tukey's test), $p<0.05$)

In AChE inhibition, the most active extracts were #3CB and #2TT, while #3TT and #1TT were not active on AChE. Regarding BChE inhibitory assay, the best action was observed in #1TT (4.30 mg GALAE/g). Tyrosinase is a key enzyme in the synthesis of melanin; it plays a vital role in the progression of hyperpigmentation problems [39]. All extracts exhibited inhibitory effects on tyrosinase and the most active one was #4TT (66.44 mg KAE/g). Amylase and glucosidase are considered as antidiabetic enzymes and their inhibition is an important mechanism to control blood glucose in the diabetic patients [40]. In amylase inhibition, the bound phenolic extracts displayed the strongest inhibitory effects (#4CB: 0.57 mmol ACAE/g; #4TT: 0.43 mmol ACAE/g), #1CB and #4CB were not active on glucosidase. These data are partially in line with recent literature devoted to the study of *Phaseolus vulgaris* extracts. ~~For example~~ Schisano *et al.* reported the amylase inhibitory effects of Controne ecotype of *Phaseolus vulgaris,* for which the inhibition was found to be 70% [41]. Furthermore Micheli *et al.* investigated the amylase inhibition ability of *Phaseolus vulgaris* in mice, highlighting its ~~their and the samples exhibited~~ significant activity to alleviate diabetic symptoms [2]. Fonseca-Hernández *et al.* investigated tyrosinase inhibitory effects of black bean polyphenol extracts. The purified extracts (IC50: 0.143-0.147 mg/mL) displayed stronger inhibitory activity ~~effects~~ than crude extracts (IC50: 2.59-9.92 mg/mL) [42]. In order to complete our panel of biological activity screening, *in vivo* zymosan induced edema formation was applied for decoction extracts of Cannellino Bio and Tondino del Tavo, with the aim to highlight a possible anti-inflammatory activity (Figure 2). Cannellino Bio and Tondino del Tavo reduced edema formation when administered 15 min before or 150 min after zymosan, but these effects did not reach statistical significance.

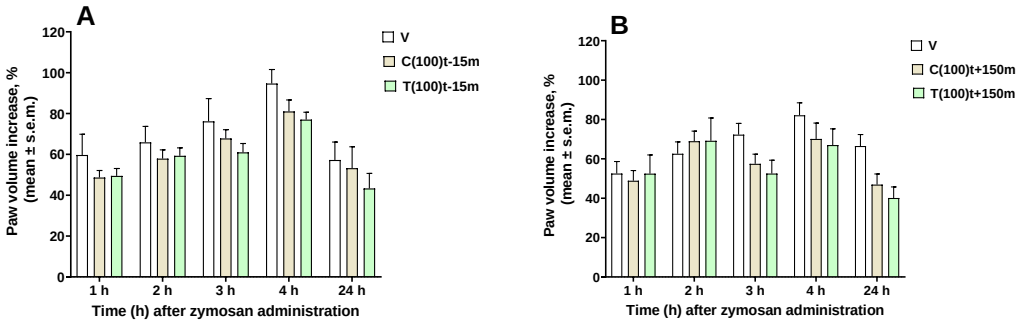

Figure 3. Effects induced by Cannellino Bio (C) and Tondino del Tavo (T) decoction extracts administered s.c. into the mice hind paw at the dose of 100 µg/20 µL paw, 15 min before (A) or 150 min after (B) zymosan (2.5% *w/v* in saline, 20 µL/paw) administration in the same paw. Statistical analysis was performed by using two-way ANOVA followed by Dunnett's multiple comparisons test. C and T induced a reduction in zymosan-induced edema, but this effect did not reach statistical significance, both in A and B. N=7.

In order to obtain new insights between the total bioactive components and the antioxidant and enzyme-inhibiting effects of the tested extracts, we performed a correlation analysis. The results are shown in Figure 3. In fact, the antioxidant properties correlate strongly with the total phenolic levels of the tested extracts, with the exception of metal chelating ability. In particular, the correlation values for free radical scavenger and reducing power assays were higher than 0.8. The results clearly showed that the phenolic compounds were the main contributors to the antioxidant properties of the tested extracts. Consistent with our findings, many researchers have reported that phenolics in the plant extracts are the key players in antioxidant properties [43,44]. The conflicting results in terms of metal chelating ability can be explained by the presence of non-phenolic chelating agents such as polysaccharides or peptides [35]. However, the correlation values of enzyme inhibition assays differed from antioxidant assays. Among the assays, only the amylase inhibition assay showed moderate correlation with total phenol content ($R > 0.6$). Other enzymes were almost weakly linked with total phenolic content. At this point, the observed ability to inhibit enzymes could be related to the non-phenolic enzyme inhibitors such as alkaloids or terpenoids [44,45]. However, we suggested that the individual components could be isolated through further analysis techniques and then their enzyme inhibitory effects could be better evaluated in future studies.

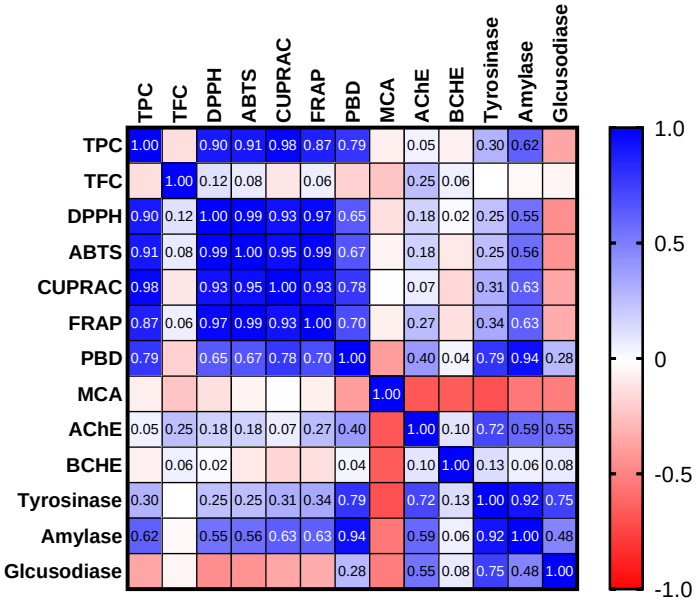

Figure 2. Pearson correlation values between biological activity assays (p<0.05). TPC: Total phenolic content; TFC: Total flavonoid content; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid; DPPH: 1,1-diphenyl-2-picrylhydrazyl; CUPRAC: Cupric reducing antioxidant capacity; FRAP: Ferric reducing antioxidant power; AChE: acetylcholinesterase; BChE: butyrylcholinesterase; MCA: Metal chelating ability; PBD: Phosphomolybdenum.

Findings by Cardador-Martinez *et al.* demonstrated the potent antimutagenic activity and antioxidant properties of diverse fractions of *Phaseolus vulgaris* obtained using different extraction's solvents, further supporting our hypothesis [46]. Nonetheless our study demonstrates the feasibility of producing fractions of bean with considerable level of phenolic antioxidants by means of this methodology, which could be potentially applied to the extraction of protein-rich legume such as soybeans, lentils, split peas and Graminacee derived natural products such as barley and grain. Overall these results could be partially explained by the extraction process applied; changing the solvents used to treat the lyophilized samples could represent a useful strategy to improve the extraction yield and at the same time, to obtain fractions rich in bioactive compounds such as flavonoids.

3. Conclusions

In this short communication an easy and straightforward extraction procedure has been assessed and applied to the separation of different fractions of two Italian varieties of *Phaseolus vulgaris*, *e.g.* Tondino del Tavo and Cannellino, which were analysed by quantitative HPLC-PDA to determine their phenolic fingerprint. Results show a considerable total phenolic content for both varieties and a growing antioxidant activity related to the major phenolic content of each extract. On the other hand a low enzymatic inhibition profile against AChE, BChE and glucosidase enzyme was also detected for all of them, with the exception of the activity found against tyrosinase enzyme. Despite these are preliminary data, we are confident in further development of this work starting from the enrolled extraction procedure, which can be employed to determine the protein content and complete phenolic profile for each of them in the next future.

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