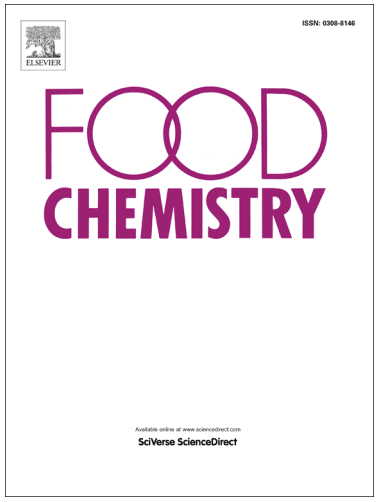

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Mass spectrometry-based phytochemical screening for hypoglycemic activity of Fagioli di Sarconi beans (*Phaseolus vulgaris L.*)

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Abstract

The present study deals with the evaluation of antidiabetic activities of Fagioli di Sarconi beans (*Phaseolus vulgaris*), including 21 ecotypes protected by the European Union with the mark PGI (i.e., Protected Geographical Indication), and cultivated in Basilicata (southern Italy). For this purpose, α-glucosidase and α-amylase assays were assessed; among all bean ecotypes, the tight green seed colour of Verdolino extracts exhibited the highest α-glucosidase and α-amylase inhibitory activity with IC_{50}=1.1 ± 0.1 μg/ml and IC_{50}=19.3 ± 1.1 μg/ml, respectively. Phytochemical compound screening of all Fagioli di Sarconi beans performed by flow injection-electrospray ionization-ultrahigh resolution mass spectrometry (uHRMS) and based on the calculation of elemental formulas from accurate *m/z* values, was helpful to annotate specific compounds, such as alkaloids, saponins, flavonoids, and terpenoids, which are most likely responsible for their biological activity.

Keywords: Fagioli di Sarconi beans; Phaseouls vulgaris L.; high-resolution mass spectrometry; phytochemical profile; anti-diabetic activity; α-glucosidase; α-amylase.

1. Introduction

The increasing prevalence of type 2 diabetes mellitus, and the negative clinical outcomes observed with the commercially available anti-diabetic drugs, have led to the investigation of new therapeutic and nutritional approaches, focused on controlling postprandial glucose levels (Howlett & Bailey, 1999). Among many enzymes, α-amylase is one which helps the human body to break down complex polysaccharides into oligosaccharides and disaccharides. α-Glucosidase then hydrolyzes these into simple absorbable monosaccharides, which are responsible for the increase in postprandial glucose level. The use of carbohydrate digestive enzyme inhibitors from natural resources was proposed as a possible strategy to block dietary sugar compound absorption with less adverse effects than synthetic drugs (Etxeberria, de la Garza, Campión, Martínez, & Milagro, 2012; Kumar, Prakash, Kumar, & Narwal, 2011). Currently, some of these drugs act mainly by inhibiting carbohydrate digestion and absorption. Acarbose (BAY g 5421) was the first-line drug used as α-glucosidase inhibitor available for diabetes treatment. Voglibose and miglitol are newer α-glucosidase inhibitors commercially available for therapy (Van de Laar et al., 2005). Although the efficiency of these drugs in maintaining postprandial blood glucose levels under control in many patients, their lack of specificity gives rise to several gastrointestinal side effects, such as abdominal cramping, flatulence and diarrhoea (Fujisawa, Ikegami, Inoue, Kawabata, & Ogihara, 2005). The prominent side effects of such drugs have driven the seeking of alternative therapies with less severe, or no, side effects. In this regard, natural α-glucosidase and α-amylase inhibitors from medicinal plants are being investigated as a new natural approach to treat diabetes: they seem to work without any major side effects, offering an economical alternative to the traditional hypoglycemic agents. The available literature shows more than 400 plant species with anti-diabetic activity (Ivorra, Paya, & Villar, 1989), but only a small number of these have received a scientific and medical evaluation to assess their efficiency. Among them, the genus *Phaseolus vulgaris*, including all species of legumes seeds, normally known as common beans, is gaining increasing attention as functional foods. Dry bean consumption has been reported to be associated with reduced risk for a number of chronic metabolic disorder, including diabetes mellitus (Jenkins et al., 2012; Singhal, Kaushik, & Mathur, 2014). Accordingly, the use of kidney bean extracts as α-amylase inhibitors for obesity and diabetes treatment has been discussed in different reviews (Helmstädter, 2010) and a great body of research has gone into the use of some extracts, specifically Phase 2®, which is a phaseolamin-based water extract of *P. vulgaris* that is commercialized as a dietary supplement with no side effects (Barrett & Udani, 2011). Several *in*

vitro studies have demonstrated the amylase inhibitory activity of different compounds that, as phaseolamin (specific for animal α-amylases), have been isolated from white kidney beans. However, these benefits are more probably associated with the whole phytochemical content (Savithramma, Rao, & Ankanna, 2011) and the synergistic or at least additive pharmacological effects of secondary metabolites occurring in legumes, thus evaluating for each of them the hypoglycemic activity (Chowdhury et al., 2016; Kumar et al., 2011). Therefore, the non-targeted metabolite profiling (simultaneous measurement of all metabolites in a given sample) is becoming an indispensable screening tool to better understand health-related food bioactivity. Several techniques, such as ultraviolet-visible (UV-Vis) spectrophotometry, Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) and mass spectrometry (MS), have been reported to obtain the metabolite profiling which is a critical point in natural product investigation. Furthermore, in the recent years, the metabolite investigation in non-cooked legumes is increasing because of significant reduction in phytochemical content due to preparation and cooking method (Fabbri & Crosby, 2016). The aim of this study was to evaluate the antidiabetic activity of 21 ecotypes of Fagioli di Sarconi beans (Basilicata, southern Italy) with the mark protected geographical indication, PGI (Kireeva, 2011), belonging to the species *P. vulgari*s, without any previous thermal processing, in order to promote their nutraceutical application rather than functional food proprieties. *In vitro* antihyperglycemic activity of these ecotypes was evaluated using α-amylase and α-glucosidase inhibition assays. Moreover, this work provides insight into the metabolite profile of bean extracts using magnetic resonance mass spectrometry (Fourier transform ion cyclotron resonance MS/FT-ICR–MS).

2. Materials and methods

2.1. Chemicals

Sodium phosphate (≥98%), sodium chloride (≥99.5%), 3,5-dinitrosalicylic acid (≥98%), α-amylase from porcine pancreas starch, α-glucosidase from *Saccharomyces cerevisiae*, potassium phosphate monobasic (≥99%), 4-nitrophenyl α-D-glucopyranoside (≥99%) and acarbose were acquired from Sigma–Aldrich (Milano, Italy). Ferric chloride, sulphuric acid, acetic anhydride, glacial acetic acid, chloroform, vanillin, sodium chloride, mercuric chloride, gelatin, bismuth nitrate, tartaric acid, iodine and potassium iodide to perform colorimetric assay-based phytochemical screening were acquired from Sigma–Aldrich (Milano, Italy). All the solvents used for sample pretreatment and MS analysis were of LC-MS grade and were purchased from Sigma-Aldrich (Milano, Italy). Ultrapure water was produced using a Milli-Q RG system from

Millipore (Bedford, MA, USA). Pure nitrogen (99.996%) was delivered to the MS system as the sheath gas.

2.2. Bean samples and metabolite extraction

The 21 ecotypes of Fagioli di Sarconi bean samples (*Cannellino, Cannellino nasello rosso, Cannellino rosso, Ciuoto o Regina, Marucchedda, Munachedda, Nasello nero, Nasello rosso, Nasello viola, Panzaredda, Riso bianco, Riso giallo, san Michele, san Michele rosso, Tabacchino, Tondino bianco, Tuvagliedda, Tuvagliedda marrone, Tuvagliedda nera, Tuvagliedda rossa, Verdolino*) were made available through the local agricultural farm of the consortium Fagioli di Sarconi PGI (Kireeva, 2011). The dried powder of Fagioli di Sarconi beans was extracted using a modified procedure based on the previously reported method (Marimuthu & Gurumoorthi, 2013). Briefly, 10 ml of 70:30 (v/v) water/ethanol solution was used to extract metabolites from 1g of finely ground beans in an ultrasonic bath for 6 h at room temperature (Sonorex Super RK 100/H sonicator; Bandelin electronic, Berlin, Germany) with a 35 kHz automatic frequency control and a high-frequency power of 80 W. After centrifugation at 5000 rpm (3000g) at 4 °C for 5 min (Kontron A8.24 rotor centrifuge), the supernatant was filtered through a 0.20 μm nylon syringe filter (Whatman, Maidstone, UK) and injected into the MS system without further pre-treatment. To carry out the enzymatic inhibition assays and phytochemical assays, the solvent was evaporated (Laborota 4000 efficient, Heidolph, Schwabach, Germany) and the sample was solubilized (see next sections) for further analysis.

2.3. In vitro antidiabetic activity: α-amylase and α-glucosidase enzymatic assays

The inhibition assays to evaluate *in vitro* antidiabetic activity were performed using a previously reported method (Milella et al., 2016). Acarbose, a widely used clinical antidiabetic drug, was used as a positive control.

Bean extracts were solubilized in 10% DMSO/MeOH solution and tested at different concentrations (0.005-0.00016 mg/ml). The α-amylase inhibitory activity was assayed using 10 μl of 20 mM sodium phosphate buffer (pH 6.9 with 6 mM NaCl) containing 0.5 mg/ml α-amylase (50 Units/mg) and then incubated at 25 °C for 10 min with 10 μl of bean extract. After this pre-incubation, 10 μl of 1% starch solution in 20 mM of sodium phosphate buffer, used as the substrate, was added to each sample and the reaction mixtures were incubated at 25° C for an additional time of 10 min. The reaction was stopped with 20 μl of dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 10 min, cooled to room temperature and after addition of 300 μl of distilled water, the absorbance was measured at 540

nm. The absorbance of blank samples (in which enzyme solution was added during the boiling process) and negative controls (10% DMSO/MeOH solution added in place of extract) were recorded. Acarbose solubilized in 10% DMSO/MeOH was tested at different concentrations (1.28-0.02 mg/ml). Analyses were performed in triplicate (independently repeated experiments) and the final value of sample absorbance (A540 nm) was obtained by subtracting its corresponding blank sample reading (Ranilla, Kwon, Apostolidis, & Shetty, 2010). The α-amylase inhibitory activities of bean extracts and acarbose were calculated, and its IC_{50} values were determined. The inhibitory activity (%) was calculated as follows (equation 1):

$$\%\ \text{Inhibition} = \frac{(\text{A540 Negative Control} - \text{A540 Sample})}{\text{A540 Negative Control}} * 100 \qquad \text{eq. 1}$$

The inhibitory activity of α-glucosidase enzyme was assessed in 96-well plates. In each well 10 μl of bean extract was solubilized in 10% DMSO/MeOH solution and tested at different concentrations (0.029-0.0018 mg/ml); 160 μl of 10 mM sodium phosphate buffer pH 7.0 and 60 μl of the substrate (2.5 mM 4-nitrophenyl α-D-glucopyranoside in 10 mM phosphate buffer) were added. The reaction started with the addition of 20 μl of enzyme (0.28 U/ml in 10 mM phosphate buffer) and the plates were incubated at 37° C for 10 min. The absorbance at 405 nm was measured before the addition of the enzyme ($T0$) and after 10 minutes of incubation ($T10$). Acarbose was solubilized in 10 μl 10% DMSO/MeOH and tested at different concentrations (0.057-0.0009 mg/ml). Negative control absorbance (10% DMSO/MeOH solution in place of extract) was also recorded. The inhibitory activity was calculated by using the formula (equation 2):

$$\%\ \text{Inhibition} = \frac{(\text{A405 Negative Control}_{T10-T0} - \text{A405 Sample}_{T10-T0})}{\text{A540 Negative Control}_{T10-T0}} * 100 \qquad \text{eq. 2}$$

The concentration of the extract required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by non-linear curve-fitting. The experiments were repeated thrice (independently repeated experiments).

2.4. Phytochemical assays

Screening of phytochemical constituents, i.e. alkaloids, anthraquinones, carbohydrates, coumarins, cardiac glycosides, gum and mucilage, lipids, protein and amino acids, tannins, phenols, quinones, saponins, steroids and terpenoids, occurring in the bean powder extracts was done using standard protocols, commonly used to investigate the presence of bioactive

compounds of medicinal plants (Atchibri et al., 2010; Marimuthu & Gurumoorthi, 2013; Savithramma et al., 2011; Senguttuvan et al., 2014; Yogeshwari & Kalaichelvi, 2017). In detail:

Test for alkaloids. 500 µl of bean extract was dissolved in 2 ml of hydrochloric acid 5%, after mixing and filtering, three aliquots were taken. Drops of Mayer, Wagner and Dragendorff reagents were added to each. A brown precipitate (Wagner), yellowish-white precipitate (Mayer), and red–orange precipitate (Dragendorff) indicated the presence of such metabolites.

Test for cardiac glycosides. Keller-Kiliani test: 500 µl of bean extract was solubilized in 1 ml of glacial acetic acid containing one drop of ferric chloride solution (5%). This solution was underlaid with 100 µl of 37% sulphuric acid and a brown ring obtained at the junction of two layers indicates the presence of glycosides.

Test for steroids. 500 µl of bean extract was suspended in 200 µl of chloroform, and concentrated sulphuric acid (300 µl) was carefully added to form a layer. A reddish brown coloration of the interface is indicative of the presence of steroids.

Test for terpenoids. Crude extract (500 µl) was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

Test for phenols. 500 µl of the extract was added to 500 µl of $FeCl_3$ (5%), a deep bluish green solution is formed when phenols are present.

Test for protein and amino acids. Biuret test: an aliquot of 500 µl of the filtrate was treated with a drop of 2% copper sulphate solution. To this, 250 µl of ethanol (95%) was added followed by an excess of potassium hydroxide pellets. The formation of pink colour in ethanol layer indicates the presence of proteins.

Test for tannins. To 500 µl of bean extract 1-2 drops a 1% solution of gelatin containing 10% sodium chloride, was added. White precipitate was observed for tannins.

Test for saponins. Makkar's test: 500 µl of the extract reacts with 1 ml of alcoholic vanillin (400 mg of vanillin in 5 ml of 99.5% ethanol) solution and adds few drops concentrated sulphuric acid. The formation of deep red colour indicates presence of saponins.

Test for coumarins. 10% of NaOH (500 µl) was added to 500 µl of the plant extract. The formation of yellow colour indicates the presence of coumarins.

Test for quinones. Concentrated sulphuric acid (500 µl) was added to 500 µl of plant extract. The formation of red colour indicates the presence of quinones.

Test for anthraquinones. Few drops of 2% HCl were added to 500 µl of extract. The appearance of the red colour indicates the presence of anthraquinones.

Test for gum and mucilage. The plant extract was diluted with 5 ml of distilled water and to this 25 ml of absolute alcohol was added with constant stirring. The formation of white or cloudy precipitate indicates the presence of gums and mucilage.

Test for lipids. 1g plant sample was dissolved in water: chloroform (50:50) and stirred for a hour. The mixture was centrifuged, organic supernatant dried and dissolved in ethanol. 2 ml of concentrated H_2SO_4 and 5 ml of phosphor-vanillin reagent (50 mg vanillin was dissolved in 800 µl of absolute ethanol before diluting to 8 ml with distilled water and mixing with 33 ml of concentrated H_3PO_4) was added to 100 µl of extract. Change of the colour indicates the presence of lipids.

Test for carbohydrates. Molisch's test: 500 µl crude extract was mixed with few drops of Molisch's reagent (10% alcoholic solution of α-naphtol) and the mixture was shaken properly. After that, 1 ml of concentrated H_2SO_4 was poured carefully along the side of the test tube. The appearance of a violet ring at the interphase indicated the presence of carbohydrates.

2.5. FT-ICR-MS analyses

High-resolution mass spectra were acquired on a Bruker (Bruker Daltonik GmbH, Bremen, Germany) solariX Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) equipped with a 12 Tesla superconducting magnet (Magnex Scientific Inc., Yarnton, GB) and a APOLLO II ESI source (Brucker Daltonik GmbH, Bremen, Germany) in the negative ionisation mode. 10 µl of each ethanol bean extract were diluted in 1 ml of methanol (Schmitt-Kopplin et al., 2012) prior to direct injection into the microelectrospay source at a flow rate of 120 µl h^{-1} with a nebulizer gas pressure of 32 psi, and drying gas flow rate of 4 l/min at 180°C. Spectra were acquired with a time domain of 4 mega-word and a mass range of m/z 100–1000. 300 scans were accumulated for each sample. Spectra were externally calibrated using a blank analysis of typical solvent impurities in methanol. The accuracy reached values of less than 0.1 ppm. Further internal calibrations were performed for each sample through the identification of ubiquitous fatty acids. Fourier transform ion cyclotron resonance (FT-ICR) mass spectra with *m/z* from 100 to 1000 were exported to peak lists at a signal-to-noise ratio (S/N) of 2 and higher (Schmitt-Kopplin et al., 2012). From these lists, possible elemental formulae were calculated for each peak using Data Analysis software (v4.1, Bruker Daltonik GmbH, Bremen, Germany); an elemental formulae assignment was obtained due to the ultra high resolution (R = 400.000 at *m/z* 500, thus differentiating two masses separated by the mass of an electron) and to the mass accuracy of 0.1 ppm (electron mass accuracy). Thousands of such compositions could be calculated, which contained C, H, O, N and S elements. The generated formulas were validated

by setting sensible chemical constraints: N rule; element counts: C ≤ 100, H ≤ 200, O ≤ 80, N ≤ 5, S ≤ 1 and only the masses in conjunction with their automated generated theoretical isotope pattern (existence of the ^{13}C isotope) were taken into consideration, according to available literature concerning elemental composition assignment to Fourier transform ion cyclotron resonance mass spectrometry data (Herzsprung et al., 2014). They were represented using van Krevelen diagrams, which sort them onto two axes according to H/C and O/C atomic ratios (Tziotis, Hertkorn, & Schmitt-Kopplin, 2011). Moreover, the *m/z* peak lists were used for further statistical analysis.

2.6. Data analyses

The IC_{50} values were estimated by non-linear curve-fitting and presented as their respective 95% confidence limits. All enzymatic assays were performed in triplicate, and results expressed as mean ± standard deviation (Mean ± SD). The Student's *t*-test (SPSS 19.0 for Windows; IBM SPSS Statistics, Armonk, NY, USA) was used to assess the presence of significant differences ($p<0.05$) among the extracts. All the statistical analyses were accomplished, using the computer software GraphPad Prism 3.02 for Windows (GraphPad Software, USA). High-resolution mass spectra were subjected to data processing and filtering by using DataAnalysis software (v4.1, Bruker Daltonik GmbH, Bremen, Germany). The reduced peak lists were submitted to orthogonal partial least square (OPLS) analysis for more detailed insight into the relations between the variables.

3. Results

3.1. Inibition of α-amylase and α-glucosidase assays

Potential anti-diabetic activities of 21 ecotypes of Fagioli di Sarconi beans were investigated using α-amylase and α-glucosidase inhibition assays alongside acarbose as a positive control. Results were expressed as either the content (mg/ml) of acarbose or that of bean extracts required to inhibit 50% of α-amylase and α-glucosidase activity (IC_{50}). Since low inhibition was observed for some bean extracts, both α-amylase and α-glucosidase enzymes were tested at the maximum concentration allowed (%I) (Nickavar & Abolhasani, 2013; Safamansouri et al., 2014; Wang, Du, & Song, 2010). %I is also used in inhibition analysis and thus was utilized as an alternative

parameter of IC_{50}. The α-amylase and α-glucosidase inhibitory activities of bean extracts along with acarbose, in term of IC_{50} and %I values are summarized in Table 1.

3.2. Preliminary phytochemical analysis

Colorimetric assay-based phytochemical screening is usually carried out as a preliminary investigation to discover active compounds of medicinal plants (Marimuthu & Gurumoorthi, 2013). Phytochemical screening of Fagioli di Sarconi bean extracts (i.e., 21 seeds), revealed the presence of alkaloids, carbohydrates, coumarins, cardiac glycosides, proteins, amino acids, phenols, saponins, steroids, tannins and terpenoids (Table 2). No presence of anthraquinones, quinones, lipids, gum, and mucilage was found in the sample extracts.

3.3. Metabolite Fingerprinting by magnetic resonance mass spectrometry

Although MS is particularly powerful when combined with LC separation of the analytes of interest (Bianco, Abate, Labella, & Cataldi, 2009; Cataldi, Bianco, & Abate, 2009), a shotgun approach, based on direct infusion negative-ion ESI ultrahigh resolution mass spectrometry (FT-ICR-MS), was employed for the rapid analysis of metabolites occurring in all extracts of Fagioli di Sarconi beans. Such non-targeted analysis generates a tremendous amount of data and requires visualization strategies to convert lists of accurate *m/z* values into metabolomic context, prior to the application of statistical tools (Kim, Kramer, & Hatcher, 2003). Since FT-ICR-MS offers the highest resolution performance, the interpretation of high-resolution mass spectra (Figure 1A) was made by converting accurate mass values into putative elemental compositions in order to better understand the chemical composition of this sample extract (Hertkorn et al., 2007). For each sample, up to 400 unambiguous elemental formulas were found (with 200 ppb tolerances), when considering only the composition based on C, H, N, O, and S (i.e., CHO, CHOS, CHON, CHONS). Due to the high complexity of metabolome, visualization strategy using van Krevelen diagram have been adopted. This diagram displays the hydrogen/carbon (H/C) *vs.* oxygen/carbon (O/C) ratios of these elemental formulas and provide a qualitative description of the molecular complexity of Fagioli di Sarconi data, never reported before (Figure 1B). This plot enables the localization of chemical species, particularly of the specific masses correlated to high α-glucosylase inhibition (Figure 1C) according to class metabolites, as carbohydrates and glycosylated compounds, peptides, polyphenols, fatty acids, saponins and condensed heterocycles (Figure 1D) (Schmitt-Kopplin et al., 2012).

In order to discriminate the Fagioli di Sarconi beans on the basis of their metabolites and biological activity (i.e., inhibition of α-glucosidase and α-amylase), reduced FT-ICR-MS data

were log-transformed and normalized. One sample was excluded from the model (*Marucchedda*) as detected as an outlier. In the model, the α-glucosidase assay was set as Y-variable and the data was modeled with an orthogonal partial least square (OPLS) analysis in order to find the *m/z* values much more related to the variable object of study. The sample trend is visualized in Figure 2. List of the most related *m/z* values with α-glucosidase were selected based on the highest regression coefficient values. The list was plotted in the Van Krevelen diagram (Figure 1C).

4. Discussion

Hypoglycemic activity of extracted samples of Fagioli di Sarconi beans was determined to be effective through α-glucosidase assays in comparison to acarbose a positive control. Data obtained showed that the inhibitory activities varied among the tested ecotypes. The most potent inhibition appeared to be present in extracts of *Verdolino, Tuvagliedda, Tuvagliedda nera, Tuvagliedda rossa, Cannellino, Cannellino rosso, Cannellino nasello rosso, Riso bianco, Riso giallo, san Michele, san Michele rosso* and *Tondino bianco* (%I>50%) at the concentration of 0.005 mg/ml (see Table 1). Therefore, the dose dependent α-glucosidase inhibitory activities of these ecotypes were further investigated and their IC_{50} values were estimated. All of them demonstrated a significant dose-dependent reduction in α-glucosidase activity, always higher than reference drug (acarbose) with an IC_{50}=135.6 ± 9.1 μg/ml. *Verdolino* extract exhibited the highest inhibitory effect ($p<0.05$) with an IC_{50}=1.1 ± 0.1 μg/ml (Figure 3 and Table 1). Note that very low inhibition ($p<0.05$) was observed for extracts of *Tabacchino* ecotype (%I<25%) at the concentration of 0.005 mg/ml (Table 1).

Additionally, α-amylase assays were performed, using acarbose as a positive control. Conversely to α-glucosidase assays, all tested beans do not exhibit favourable concentration dependent hypoglycemic activities: %I values for the 16 ecotypes extracts never exceeded 55% at the highest common tested concentration of 0.029 mg/ml; no dose dependent effect was observed on increasing the concentration for the remaining ecotypes (*Nasello nero, Nasello viola, Tabacchino, Tuvagliedda, Tuvagliedda rossa*) (Table 1). The highest inhibition was observed by *Verdolino* with an IC_{50}=19.3 ± 1.1 μg/ml, followed by *Cannellino rosso, Tuvagliedda nera, Riso giallo, Riso bianco* and *Cannellino nasello rosso* ecotypes which IC_{50} values ranging from 25.9 ± 0.7 μg/ml to 28.8 ± 1.1 μg/ml (Figure 3 and Table 1).

Interestingly, each of the 21 extracts of Fagioli di Sarconi beans inhibited α-glucosidase with different potencies, always better than positive control. Moreover, in the α-amylase inhibition

test, all ecotypes showed %I values lower or not significantly different ($p < 0.05$) compared to acarbose (Figure 4). Since α-amylase catalyzes the breakdown of starch into simple sugars, its inhibition increases the amount of unabsorbed polysaccharides in the intestine. Polysaccharides, remaining in the intestine, are broken down by enterobacteria, resulting in the production of gas and causing adverse effects as abdominal fullness and flatulence (Aoki, Muraoka, Ito, Togashi, & Terauchi, 2010). Therefore, the Fagioli di Sarconi beans can have advantages as α-glucosidase inhibitors for the postprandial hyperglycemia treatment in diabetic patients.

The reason for this antihyperglycemic activity of Fagioli di Sarconi seeds was investigated through metabolite screening. Preliminary colorimetric phytochemical analysis revealed the presence of alkaloids, carbohydrates, coumarins, cardiac glycosides, phenols, saponins, steroids, tannins, terpenoids, protein and amino acids (Table 2). These metabolites are reported to have many biological and therapeutic properties (Russo, Valentão, Andrade, Fernandez, & Milella, 2015; Senguttuvan, Paulsamy, & Karthika, 2014). The more detailed identification of the putative metabolite or class of metabolites occurring in beans under study was achieved by determining the elemental composition of experimental *m/z* values based upon accurate mass determinations (Figure 1) and visualization in van Krevelen diagrams. The frequency distribution of these elemental formulas in Figure 1B showed the most abundant chemical species in Fagioli di Sarconi matrix are the nitrogen containing compounds (CHON and CHONS) as compared to CHO and CHOS. Additionally, the van Krevelen diagram in Figure 1D localized the identified elemental compositions according to the main chemical families. The diagram shows that relatively abundant compounds were found in the peptides, polyphenols and condensed heterocycles regions. In detail, CHON species were found mainly in the peptide region according to the fact that *P. vulgaris* is a legume widely recognized as an excellent source of dietary and low-cost proteins. In a previous study (Sotelo, Sousa, & Sanchez, 1995), it is reported that cultivated beans showed a higher content of sulfur amino acids compared to the wild bean, thus explaining the presence of CHONS components in the region of peptides. Moreover, CHON components occurred also in the condensed nitrogen containing compound region (nitrogen in heterocycles): they could be associated with alkaloids, consistently with a seed composition. If we consider CHO formulas, the diagram shows that relatively abundant compounds were found in the condensed hydrocarbon, polyphenols and saponins regions, with also a few ones in the carbohydrate and glycosylated compounds section. The relatively low content of carbohydrates in Fagioli di Sarconi beans is apparently in contrast to Atchibri et al. (Atchibri, Kouakou, Brou, Kouadio, & Gnakri, 2010) who reported carbohydrates as major

phytoconstituents in *P. vulgaris* seeds. However, it must be underlined that carbohydrates are not easily ionized under ESI conditions (Boutegrabet et al., 2012).

The results of this study indicate that Fagioli di Sarconi bean extracts showed appreciable hypoglycemic effects thanks to their phytoconstituents, as reported in the literature. In detail, Heredia-Rodriguez et al. (Heredia-Rodríguez, de la Garza, Garza-Juarez, & Vazquez-Rodriguez, 2016), described anti-diabetic effects of bioactive peptides in common beans (*P. vulgaris*) due to inhibition of α-amylase and α-glucosidase and stimulation of glucose uptake. Flavonoids have been reported to stimulate peripheral glucose uptake and express the enzymes responsible for the metabolism of carbohydrates (Chowdhury et al., 2016). Alkaloids are also hypoglycemic in nature (Kumar et al., 2011) and tannins have α-amylase and α-glucosidase inhibition capability (Chowdhury et al., 2016). Moreover, lupeol type terpenoid is also reported for its α-amylase inhibition activity (Kumar et al., 2011). Among terpenoids, saponins can stimulate the beta cells and pancreatic islets with the consequent decrease of blood glucose (Zheng et al., 2012). Stimulation of 5-adenosine monophosphate activated protein kinase and insulin receptor/insulin receptor substrate 1/phosphatidylinositol 3-kinase/Akt signaling pathways leading to a decrease in blood glucose is also demonstrated for soyasaponins (X. Hu et al., 2014) that could be considered having potential hypoglycemic activity (Quan, Yin, Jin, & Shen, 2003). Isolation of the active phytoconstituents and further evaluation of their individual hypoglycemic activities are still needed to confirm the showing hypoglycemic property for Fagioli di Sarconi beans.

The use of OPLS (Figure 2) allowed a simple representation of ultrahigh resolution MS data showing the main correlations between α-glucosidase assay and the *m/z* values. The model gave the following values: $R^2(Y)=0.9$ and $Q^2(cum)=0.9$, indicating the goodness of the fit and prevision capability. This reasonable result could be explained because the 21 cultivars under study had the same geographic/environmental origin, harvest year and storage condition (C. Hu et al., 2014). Therefore, the different biological activity probably could be associated with different amounts of each active phytochemical (Wang et al., 2010), which need to be investigated.

5. Conclusion

This study improved the knowledge on metabolites occurring in traditional foodstuffs and it could be used for comparative evaluation of bioactive constituents occurring in Fagioli di Sarconi beans with other populations of P. vulgaris present in different parts of the word. The results of the present study also highlighted the health-promoting value of Fagioli di Sarconi

beans (*P. vulgaris*) correlated to their metabolites. In term of α-glucosidase inhibition, all 21 bean ecotypes possess hypoglycemic activity, thus suggesting a potential use to reduce dietary carbohydrate absorption with less adverse effects than traditional drugs; *Verdolino* bean extract exhibited the highest inhibitory effect. The preliminary MS-based phytochemical screening revealed that all 21 ecoptypes of Fagioli di Sarconi beans exhibit similar metabolite profiles consisting mainly of nitrogen bearing compounds, as well as possibly saponins and alkaloids; all of them have been reported as bioactive components responsible for the antidiabetic activity of medicinal plants, confirming thus a beneficial use of Fagioli di Sarconi beans in case of hyperglycemia. Further studies are needed to isolate, characterize and elucidate the structure of the bioactive compounds of this legume, thus developing promising antidiabetic formulations.

Acknowledgments

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FIGURE CAPTIONS

Figure 1. Visualization of the ESI(-)-FT-ICR-MS data of 21 extracts of Fagioli di Sarconi ecotypes. (A) ESI(-)-FT-ICR-MS spectrum of Fagioli di Sarconi beans extract in the mass ranges 150-1000 Da. (B) van Krevelen diagram (H/C vs O/C atomic ratios) of specific masses and (C) of specific masses correlated to high α-glucosylase inhibition. (D) van Krevelen diagram with the interpretation of molecular family (CHONS (red), CHO (blue), CHON (orange) and CHOS (green) elemental compositions).

Figure 2. Score scatter plot of the OPLS model ($R^2(Y)=0.9$ and $Q^2(cum)=0.9$, indices for the goodness of the fit and prevision capability). The data is modeled following the possible trend of the α-glucosidase assay.

Figure 3. Dose-dependent inhibitory effects of acarbose, chosen as a positive control, and the *Verdolino* ecotype, belonging to Fagioli di Sarconi beans under study, on α-glucosidase and α-amylase activities. Each point represents the mean of three experiments (n=3) and the vertical bars represent the SD.

Figure 4. Percentage inhibition of enzyme activity (%I) for the 21 ecotypes of Fagioli di Sarconi beans, in both α-glucosidase and α-amylase assays. Each value (mean ± SD) was normalized for maximum tested concentration: for all samples, it was 0.005 mg/ml and 0.029 mg/ml for α-glucosidase and α-amylase assays, respectively; for acarbose, it was 1.28 mg/ml in α-glucosidase assay and 0.057 mg/ml in α-amylase assay. Values marked by the same letter are not significantly different ($p < 0.05$).

Table 1. Morphological and growing traits of 21 ecotypes of Fagioli di Sarconi beans (*P. vulgaris*) under study (harvest year: 2014) and their enzyme inhibition parameters, in term of IC_{50} and %I values, compared to acarbose (positive control).

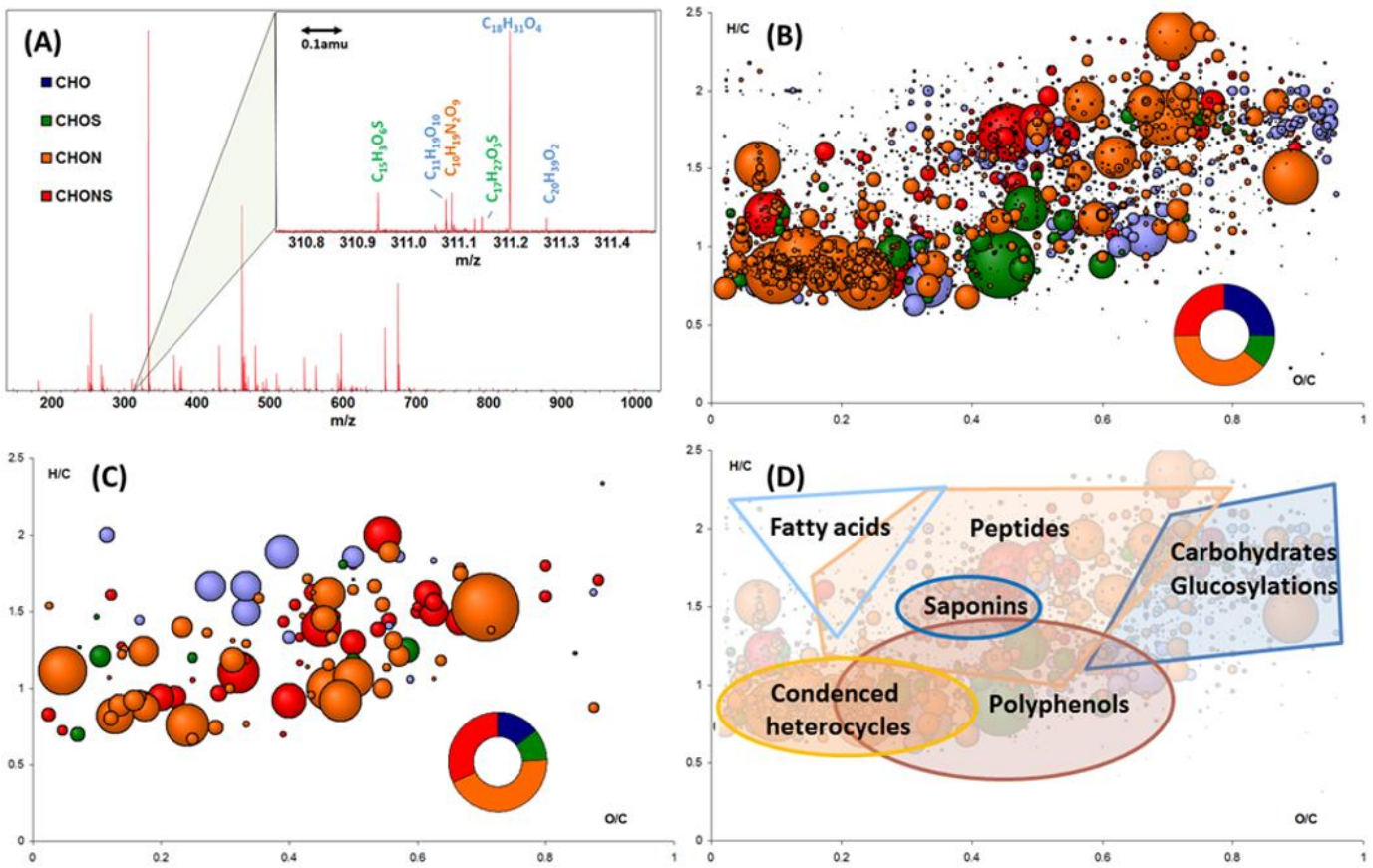
Inhibitor	**Morphological and grow traits**			**α-Glucosidase assay**		**α-Amylase assay**	
	Grown habit	**Seed coat pattern**	**Seed colour**	**%I[a] ± SD[b] (mg/ml)**	**IC_{50}[c] ± SD[b] (μg/ml)**	**%I[a] ± SD[b] (mg/ml)**	**IC_{50}[c] ± SD[b] (μg/ml)**
Cannellino	Dwarf	Striped	White (Red)	64.1 ± 1.9	2.5 ± 0.2	26.3 ± 2.2	-
Cannellino nasello rosso	Dwarf	Striped	White (Purplish Red)	62.0 ± 1.1	3.0 ± 0.2	50.7 ± 0.8	28.8 ± 1.1
Cannellino rosso	Dwarf	Absent	White	57.5 ± 2.7	4.0 ± 0.4	51.1 ± 2.8	25.9 ± 0.7
Ciuoto o Regina	Dwarf	Striped	Creamy White (Wine)	31.7 ± 1.1	-	44.5 ± 1.6	-
Marucchedda	Trailing	Striped	Crearti (Dark Green)	35.1 ± 0.4	-	35.7 ± 3.3	-
Munachedda	Trailing	Striped	Light Brown (White)	47.8 ± 2.2	-	37.9 ± 1.4	-
Nasello nero	Trailing	Striped	White (Black)	34.2 ± 2.0	-	-	-
Nasello rosso	Dwarf	Striped	White (Purplish Red)	48.2 ± 0.5	-	13.2 ± 1.0	-
Nasello viola	Trailing	Striped	White (Purple)	32.0 ± 0.4	-	-	-
Panzaredda	Trailing	Striped	White (Wine)	36.9 ± 1.7	-	19.6 ± 0.5	-
Riso bianco	Trailing	Absent	White	80.9 ± 0.7	1.2 ± 0.1	53.5 ± 0.2	26.4 ± 1.4
Riso giallo	Dwarf	Absent	Ocher	79.8 ± 0.5	1.5 ±0.1	53.0 ± 0.9	27.0 ± 1.2
san Michele	Trailing	Striped	Beige (Dark Red)	52.3 ± 0.9	4.6 ± 0.5	27.9 ± 2.0	-
san Michele rosso	Trailing	Absent	Ruby Red	58.9 ± 1.0	2.9 ± 0.3	47.8 ± 0.5	-
Tabacchino	Dwarf	Absent	Tobacco	23.2 ± 1.1	-	-	-
Tondino bianco	Dwarf	Absent	White	63.4 ± 0.9	3.2 ± 0.2	33.3 ± 2.0	-
Tuvagliedda	Trailing	Striped	White (Brown)	65.2 ± 1.8	2.0 ± 0.1	-	-
Tuvagliedda marrone	Trailing	Striped	White (Dark Brown)	44.3 ± 2.5	-	35.7 ± 0.6	-
Tuvagliedda nera	Trailing	Striped	White (Black)	74.7 ± 1.6	1.4 ± 0.1	54.4 ± 1.2	26.1 ± 0.9
Tuvagliedda rossa	Trailing	Striped	Ruby Red (White)	50.9 ± 1.1	4.4 ± 0.5	-	-
Verdolino	Dwarf	Absent	Tight Green	77.0 ± 1.2	1.1 ± 0.1	54.1 ± 1.2	19.3 ± 1.1
Acarbose (positive control)	-	-	-	96.3 ± 2.9	135.6 ± 9.1	92.2 ± 3.1	10.5 ± 1.2

[a]%I, percentage inhibition of enzyme activity at the maximum tested concentration: the concentration of all test samples was 0.005 mg/ml and 0.029 mg/ml for α-glucosidase and α-amylase assays, respectively. In the case of acarbose, the maximum concentration was 1.28 mg/ml in α-glucosidase assay and 0.057 mg/ml in α-amylase assay. [b]Values represent the means ± standard deviation (SD) of n = 3 triplicate assays (independently repeated experiments). [c]IC_{50}, concentration, expressed as μg/ml, resulting in 50% inhibition as compared to uninhibited activity.

Table 2. Detection of phytochemical constituents of 70% aqueous/ethanol extracts of Fagioli di Sarconi bean (*Phaseolus Vulgaris*).

Phytochemicals	**Present(+)/Absent(-)**
Alkaloids	+
Anthraquinones	-
Carbohydrates	+
Cardiac glycosides	+
Coumarins	+
Gum and mucilage	-
Lipids[a]	-
Proteins and amino acids	+
Phenols	+
Quinones	-
Saponins	+
Steroids	+
Tannins	+
Terpenoids	+

[a]Lipid extraction was performed by sulfo-phospo-vanillin reaction (Rasool, Ganai, Akbar, Kamili, & Masood, 2010).



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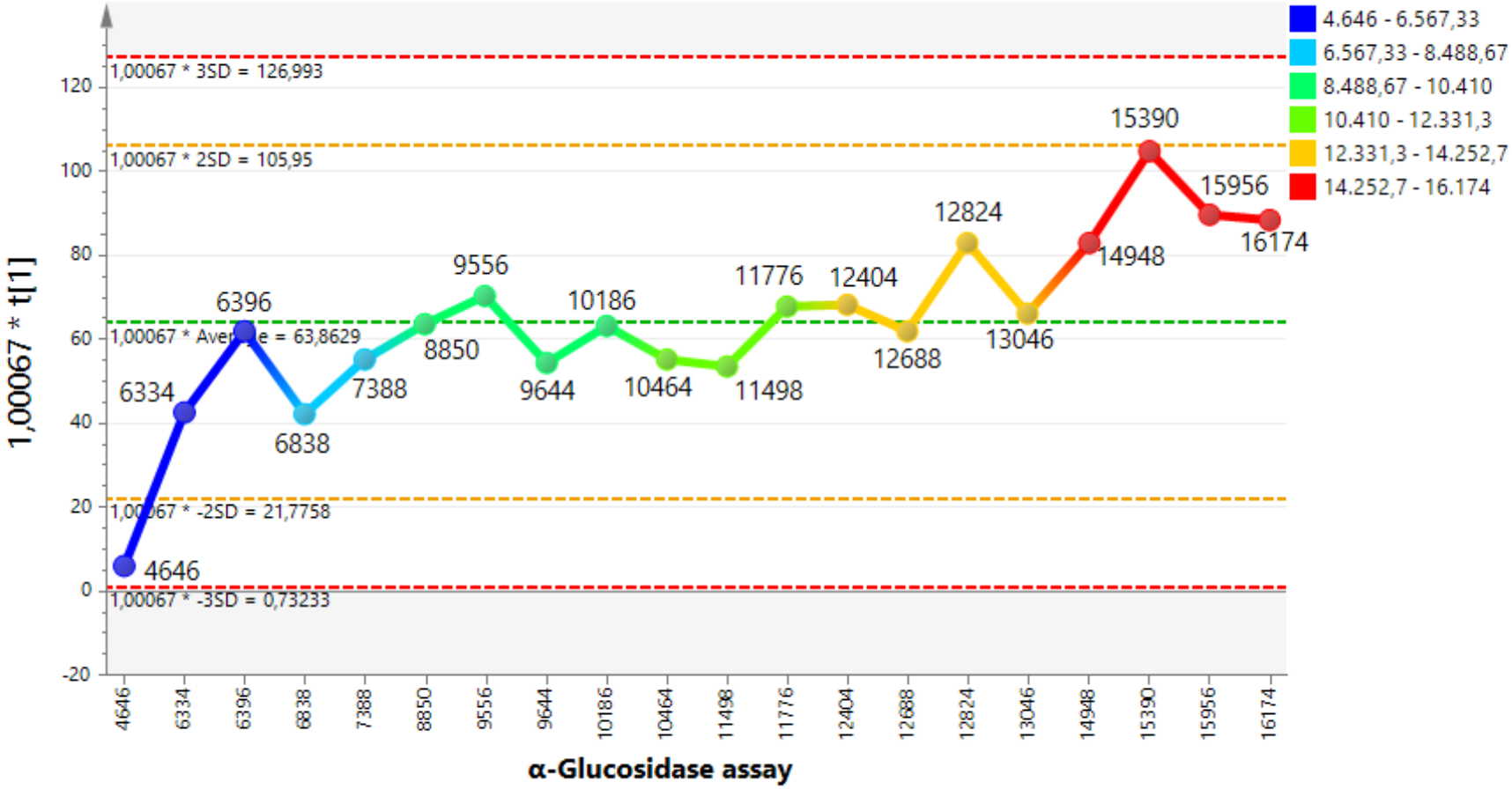

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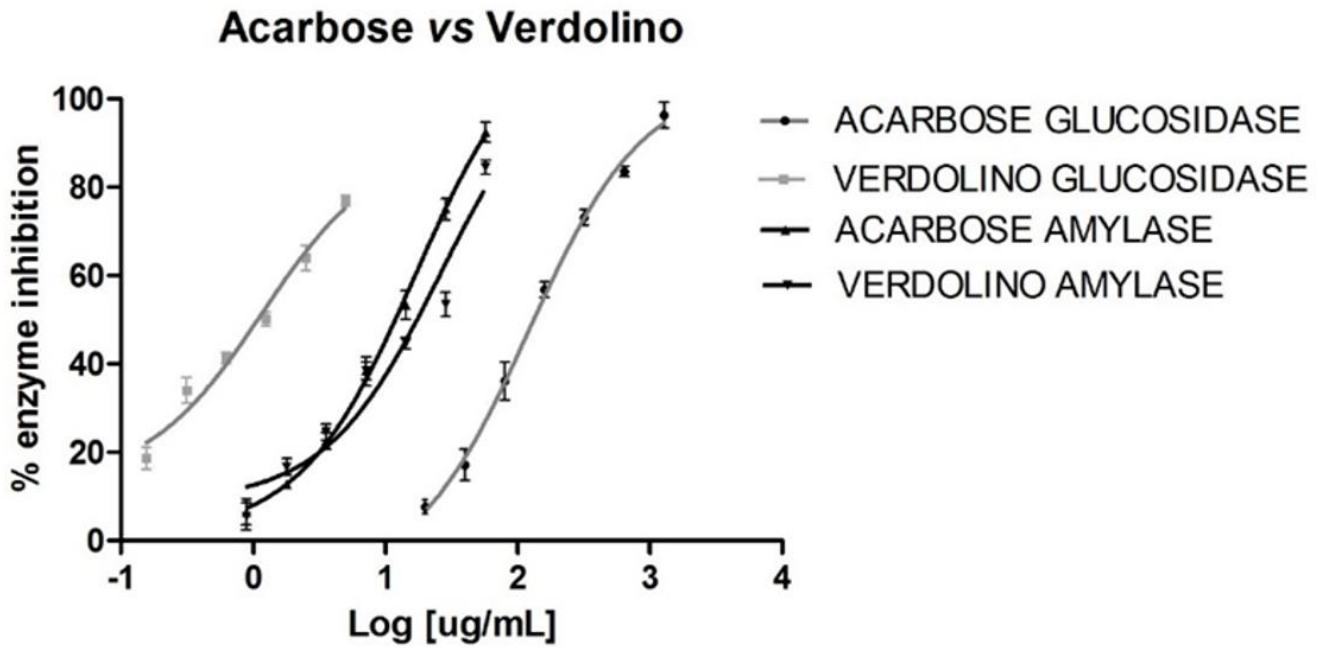

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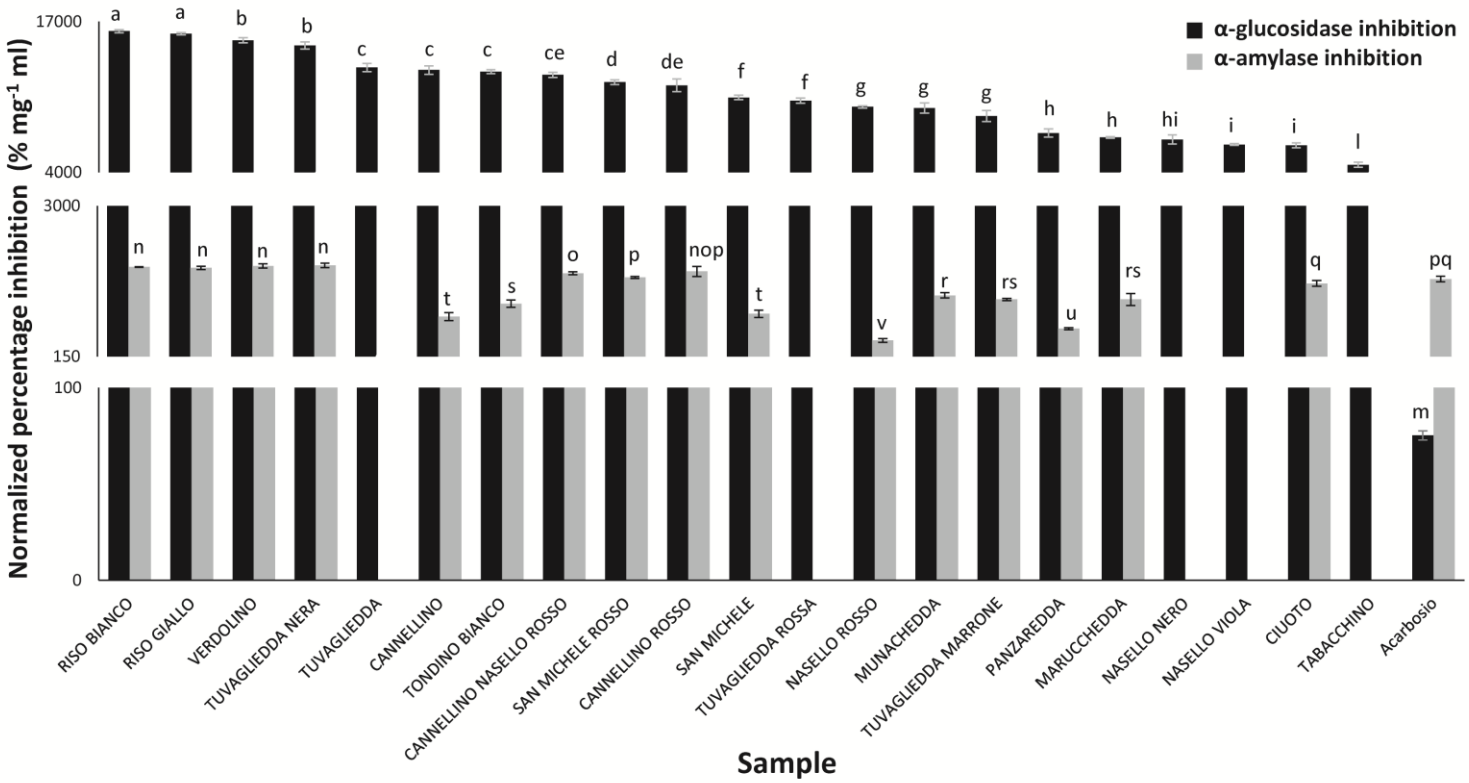

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Highlights

- All Fagioli di Sarconi beans showed in vitro antidiabetic activity.
- Flow injection-ESI-uHRMS was performed to investigate untarget metabolite profile.
- Elemental formulas were calculated from accurate *m/z* values.
- Alkaloids, flavonoids, and terpenoids were annotated.
- The secondary metabolites appear to be correlated to antidiabetic activity.