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

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27

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 [Commission Regulation (EC) No 1263/96] 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; the ethanol/aqueous (30:70, v/v) solution extracts exhibited different potencies ranging from 23.2 ± 1.1% to 77.0 ± 1.2% and from 13.2 ± 1.0% to 54.1 ± 1.2% respectively, expressed as percentage inhibition of enzyme activity (%I). Among all bean ecotypes, the tight green seed color of *Verdolino* extracts exhibited the highest α-glucosidase inhibitory activity with IC_{50}=1.1 ± 0.1 μg/mL ($p<0.05$), which is at least 100-fold better than that of acarbose used as reference compound. Moreover, *Verdolino* beans showed the highest inhibition of α-amylase activity, IC_{50}=19.3 ± 1.1 μg/mL, followed by *Cannellino Rosso, Tuvagliedda Nera, Riso Giallo, Riso Bianco* and *Cannellino Nasello Rosso* ecotypes. Preliminary 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 nitrogen containing compounds, alkaloids, flavonoids, and terpenoids, which are most likely responsible of their biological activity. Results demonstrated that Fagioli di Sarconi bean extracts, especially *Verdolino*, *Tuvagliedda*, *Tuvagliedda nera*, *Tuvagliedda rossa*, *Cannellino*, *Cannellino rosso*, *Cannellino nasello rosso*, *Riso bianco*, *Riso giallo*, *san Michele*, *san Michele rosso* and *Tondino bianco* ecotypes, are important natural sources of hypoglycemic compounds, helpful to control the postprandial blood high glucose levels.

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

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 (Botero and Wolfsdorf, 2005; Howlett and Bailey, 1999). Among many enzymes, α-amylase is one which helps human body to breakdown 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 (El-Kaissi and Sherbeeni, 2011; Oh et al., 2015). 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 et al., 2012; Kumar et al., 2011; Tundis et al., 2010). Currently, some of these drugs act mainly by inhibiting carbohydrate digestion and absorption. Acarbose (BAY g 5421) was the first natural α-glucosidase inhibitor available for diabetes treatment. Voglibose and miglitol are newer α-glucosidase inhibitors commercially available for therapy (Van de Laar, 2008; Van de Laar et al., 2005). Although 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 like abdominal cramping, flatulence and diarrhea (Fujisawa et al., 2005; Hsieh et al., 2011; Iwamoto et al., 2010; Li et al., 2011). The prominent side effects of such drugs have driven for seeking alternative therapies with less severe or no side effects. In this regard, natural α-glucosidase and α-amylase inhibitors from plant medicines 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 (Bailey and Day, 1989; Ivorra et al., 1989; Konkon et al., 2008), but only a small number of these have received 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; Longo-Mbenza and Muaka, 2013; Singhal et al., 2014; Szkudelski, 2001). Accordingly, the use of kidney bean extracts as α-amylase inhibitors for obesity and diabetes treatment has been discussed in different reviews (Helmstädter, 2010; Obiro et al.,

87 2008) and a great body of research has gone into the use of some extracts, specifically Phase 2®,
88 which is a water extract of *P. vulgaris* that is commercialized as a dietary supplement with no
89 side effects (Barrett and Udani, 2011). Several *in vitro* studies have demonstrated the amylase
90 inhibitory activity of different compounds that, as phaseolamin (specific for animal α-amylases),
91 have been isolated from white kidney beans (Payan, 2004). However, these benefits are more
92 probably associated with the whole phytochemical content (Savithramma et al., 2011) and their
93 synergistic or at least additive pharmacological effects of secondary metabolites occurring in
94 legumes, thus evaluating for each of them the hypoglycemic activity (Chowdhury et al., 2016;
95 Kumar et al., 2011). Therefore, the non-targeted metabolite profiling (simultaneous
96 measurement of all metabolites in a given sample) is becoming an indispensable screening tool
97 to better understand health-related food bioactivity. Several techniques, such as ultraviolet-
98 visible (UV-Vis) spectrophotometry, Fourier transform infrared (FT-IR) spectroscopy, nuclear
99 magnetic resonance (NMR) and mass spectrometry (MS), have been reported to obtain the
100 metabolite profiling which is a critical point in natural product investigation. Furthermore, in the
101 recent years, the metabolite investigation in not-cooked legumes is increasing because of
102 significant reduction in phytochemical content due to preparation and cooking method (Fabbri
103 and Crosby, 2016). The aim of this study was to evaluate the antidiabetic activity of 21 ecotypes
104 of Fagioli di Sarconi Beans (Basilicata, southern Italy) with the mark protected geographical
105 indication, PGI (Kireeva, 2011), belonging to the species *P. vulgari*s, without any previous thermal
106 processing, in order to promote their nutraceutical application rather than functional food
107 proprieties. *In vitro* antihyperglicemic activity of these ecotypes was evaluated by using α-
108 amylase and α-glucosidase inhibition assays. Moreover this work provides insight into the
109 metabolite profile of bean extracts using magnetic resonance mass spectrometry (Fourier
110 transform ion cyclotron resonance MS / FT-ICR–MS).
111

112 # 2. Results

113 **2.1 Inibition of α-amylase and α-glucosidase assays**

114 Potential anti-diabetic activities of 21 ecotypes of Fagioli di Sarconi beans were investigated by
115 using α-amylase and α-glucosidase inhibition assays alongside acarbose as a positive control.
116 Results were expressed as either the content (mg/mL) of acarbose or that of bean extracts
117 required to inhibit 50% of α-amylase and α-glucosidase activity (IC_{50}). Since low inhibition was

118 observed for some bean extracts, both α-amylase and α-glucosidase enzymes were tested at the
119 maximum concentration allowed (%I) (Nickavar and Abolhasani, 2013; Nickavar and Mosazadeh,
120 2009; Nickavar and Yousefian, 2011, 2009; Safamansouri et al., 2014; Sudha et al., 2011; Wang
121 et al., 2010). %I is also used in inhibition analysis and thus was utilized as an alternative parameter
122 of IC_{50}. The α-amylase and α-glucosidase inhibitory activities of bean extracts along with acarbose
123 in term of IC_{50} and %I values are summarized in **Table 1**.

124 **Table 1**. Morphological and growing traits of 21 ecotype of Fagioli di Sarconi beans (*P. vulgaris*) under study (harvest year: 2014) and their enzyme
125 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] (mg/mL)**	**%I[a] ± SD[b] (mg/mL)**	**IC_{50}[c] ± SD[b] (mg/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

126 [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
127 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
128 in α-amylase assay. [b]Values represent the means ± standard deviation (SD) of n = 3 triplicate assays. [c]IC_{50}, concentration, expressed as μg/mL, resulting in 50%
129 inhibition as compared to uninhibited activity.

130 **2.2 Preliminary phytochemical analysis**

131 Colorimetric assay-based phytochemical screening are usually carried out as preliminary
132 investigation to discover active compounds of medicinal plants (Yadav et al., 2011). Phytochemical
133 screening of Fagioli di Sarconi bean extracts (i.e., 21 seeds), revealed the presence of alkaloids,
134 carbohydrates, coumarins, glycosides, proteins, aminoacids, phenols, saponins, steroids, tannins
135 and terpenoids (**Table 2**). No presence of anthraquinones, quinone, lipids, gum and mucilage was
136 found in the sample extracts.

137

138 **Table 2.** Detection of phytochemical constituents of
139 70% aqueous/ethanol extracts of Fagioli di Sarconi
140 bean (*Phaseolus Vulgaris*).

Phytochemicals	Present(+)/Absent(-)
Alkaloids	+
Anthraquinones	-
Carbohydrates	+
Coumarins	+
Glycosides	+
Gum and mucilage	-
Lipids[a]	-
Protein and aminoacid	+
Phenols	+
Quinones	-
Saponins	+
Steroids	+
Tannins	+
Terpenoids	+

141 [a]Lipid extraction was performed by sulfo-phospo-
142 vanillin reaction (Rasool et al., 2010).

143

144 **2.3 Metabolite Fingerprinting by magnetic resonance mass spectrometry**

145 Although MS is particulary powerful when combined with LC separation of the analyte of
146 interest(Cataldi et al., 2009; Bianco et al., 2009)., a shotgun approach, based on direct infusion
147 negative-ion ESI ultrahigh resolution mass spectrometry (FT-ICR-MS), was employed for the rapid
148 analysis of metabolites occurring in all extracts of Fagioli di Sarconi beans Such non-targeted
149 analysis generates a tremendous amount of data and requires visualisation strategies to convert
150 lists of accurate *m/z* values into metabolomic context, prior to the application of statistical tools

151 (Kim et al., 2003). An initial exploratory metabolite fingerprinting was performed classifying
152 arbitrarily detected accurate *m/z* values into 100 Da lists (assuming single charge) (Mensack et al.,
153 2010). The frequency of detected *m/z* values in each group reveals that a distinguishing
154 characteristic of all ecotypes of Fagioli di Sarconi beans is the presence of a large number of
155 compounds in the mass ranges from 100 to 500 Da with the most numerous group of species among
156 200 and 300 Da (see supplementary material **Figure S1**). Since FT-ICR-MS offers the highest
157 resolution performance, an additional interpretation of high-resolution mass spectra (**Figure 1A**)
158 was made by converting accurate mass values into putative elemental compositions in order to
159 better understand chemical composition of this sample extract (Hertkorn et al., 2007). For each
160 sample, up to 400 unambiguous elemental formulas were found (with 200 ppb tolerances), when
161 considering only the composition based on C, H, N, O and S (i.e., CHO, CHOS, CHON, CHONS). Due
162 to the high complexity of metabolome, visualization strategy using van Krevelen diagram have been
163 adopted. This diagram displays the hydrogen/carbon (H/C) *vs.* oxygen/carbon (O/C) ratios of these
164 elemental formulas and provide a qualitative description of the molecular complexity of Fagioli di
165 Sarconi data, never reported before (**Figure 1B**). This plot enables the localization of chemical
166 species, particulary of the specific masses correlated to high glucosylase inhibition (**Figure 1C**)
167 according to class metabolites, as carbohydrates and glycosylated compounds, peptides,
168 polyphenols, fatty acids and condensed heterocycles (**Figure 1D**) (Minor et al., 2014)

169

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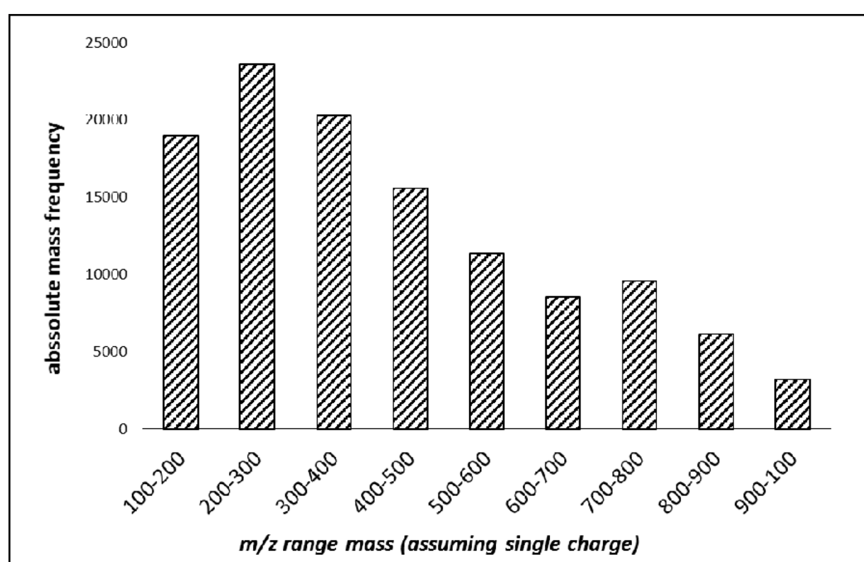
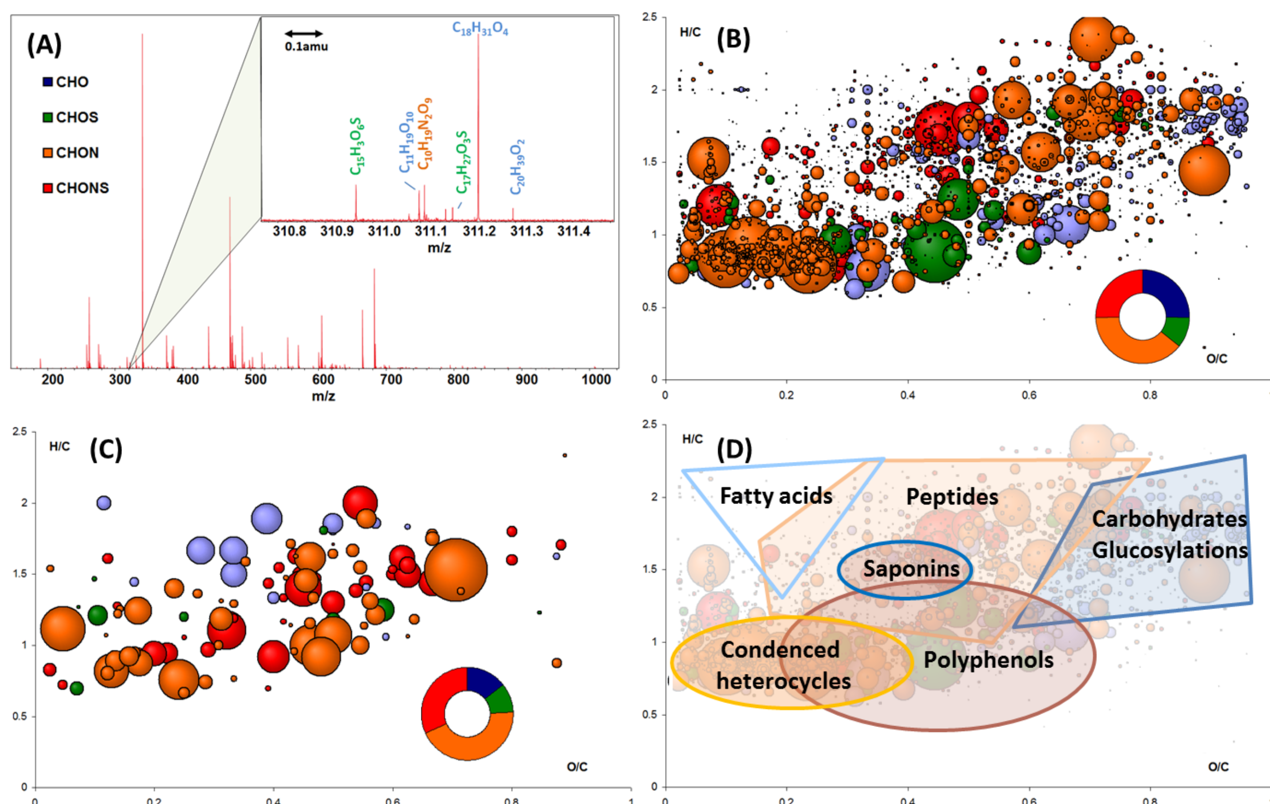


171 **Figure S1**. Visualisation of the ESI(-)-FT-ICR-MS data of 21 extracts of Fagioli di Sarconi ecotypes as
172 the relative frequency histograms classified by mass into 100 Da lists (assuming single charged
173 species) for the sum of all ecotypes.

174



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

181

182 In order to discriminate the Fagioli di Sarconi beans on the basis of their metabolites and biological
183 activity (i.e., inhibition of α-glucosidase and α-amylase), reduced FT-ICR-MS data were log-
184 transformed and normalized. One sample was excluded from the model (Marucchedda) as detected
185 as outlier. In the model the α-Glucosidase assay was set as Y-variable and the data was modelled
186 with an orthogonal partial least square (OPLS) analysis in order to find the *m/z* values much more
187 related with the variable object of study. The sample trend is visualized in the **Figure 2.** List of the
188 most related *m/z* values with α-Glucosidase were selected based on the highest regression
189 coefficient values. The list was plotted in the Van Krevelen diagram (**Figure 1C**).

190

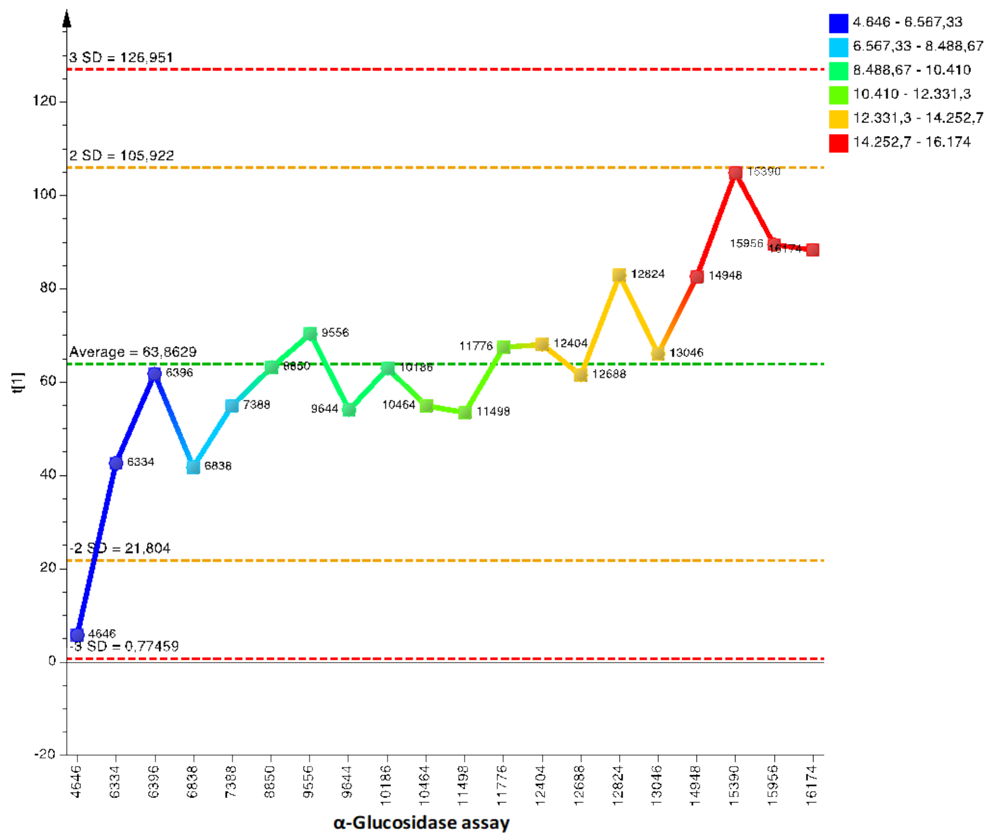
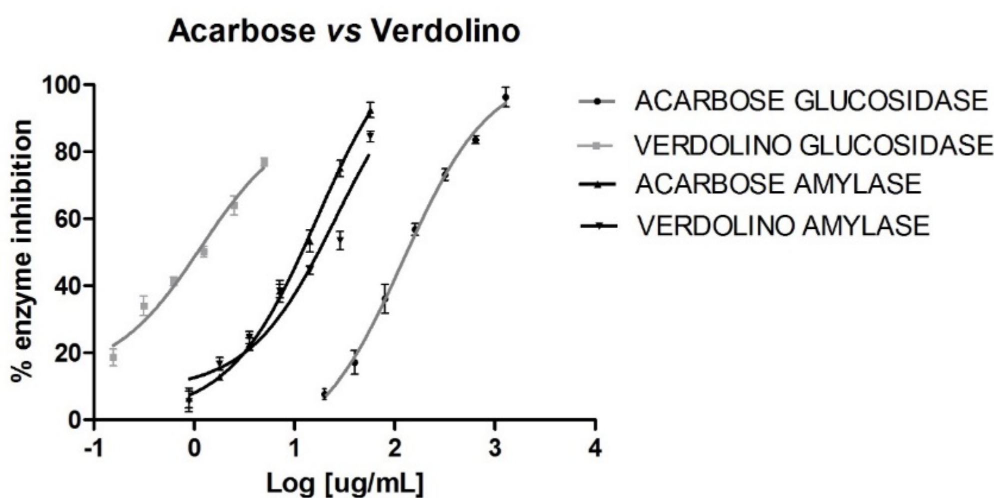


Figure 2. Score scatter plot of the OPLS model ($R^2(Y)=0.9$ and $Q^2(cum)=0.9$, indices for the goodness of the fit and prevision capability). The data is modelled following the possible trend of the α-glucosidase assay.

3. Discussion

Hypoglycemic activity of extracted samples of Fagioli di Sarconi beans was determined to be effective through α-glucosidase assays in comparison to acarbose as 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 significant dose-dependent reduction in α-glucosidase activity, always higher than reference drug with an $IC_{50}=135.6 \pm 9.1$ μg/mL. *Verdolino* extract exhibited the highest inhibitory effect ($p<0.05$) with an $IC_{50}=1.1 \pm 0.1$ 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**).

208 Additionally, α-amylase assays were performed, using acarbose as positive control. Conversely to α-
209 glucosidase assays, all tested beans do not exhibit favourable concentration dependent
210 hypoglycemic activities: %I values for the 16 ecotypes extracts never exceeded 55% at the highest
211 common tested concentration of 0.029 mg/mL; no dose dependent effect was observed on
212 increasing the concentration for the remaining ecotypes (*Nasello Nero*, *Nasello Viola*, *Tabacchino*,
213 *Tuvagliedda*, *Tuvagliedda Rossa*) (**Table 1**). The highest inhibition was observed by *Verdolino* with
214 an IC_{50}=19.3 ± 1.1 μg/mL, followed by *Cannellino Rosso, Tuvagliedda Nera, Riso Giallo, Riso Bianco*
215 and *Cannellino Nasello Rosso* ecotypes which IC_{50} values ranging from 25.9 ± 0.7 μg/mL to 28.8 ± 1.1
216 μg/mL (**Figure 3** and **Table 1**).



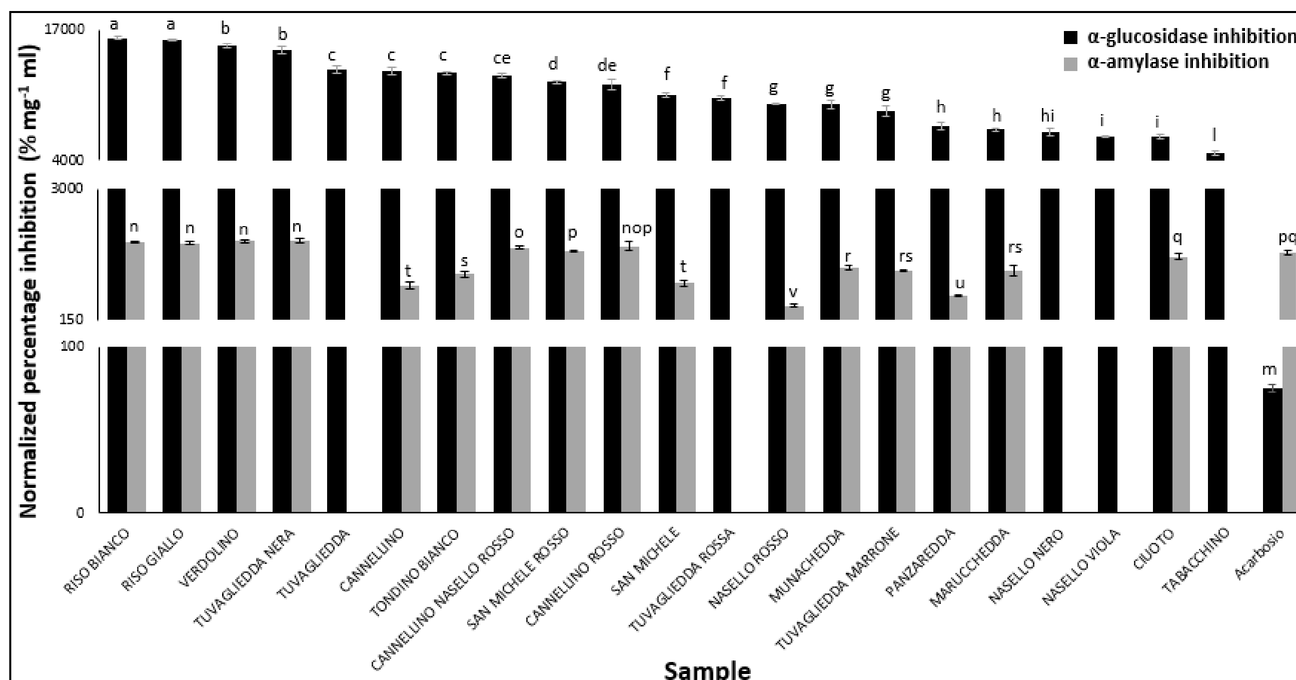
217

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

222

223 Interestingly, each of the 21 extracts of Fagioli di Sarconi beans inhibited α-glucosidase with
224 different potencies, always better than positive control. Moreover, in the α-amylase inhibition test,
225 all ecotypes showed %I values lower or not significantly different ($p < 0.05$) compared to acarbose
226 (**Figure 4**). Since α-amylase catalyses the breakdown of starch into simple sugars, its inhibition
227 increase the amount of unabsorbed polysaccharides in the intestine. Polysaccharides remaining in
228 the intestine are broken down by enterobacteria, resulting in the production of gas and causing
229 adverse effect as abdominal fullness and flatulence (Aoki et al., 2010; Kageyama et al., 1997).
230 Therefore, the Fagioli di Sarconi beans can have advantages as α-glucosidase inhibitors for the

231 postprandial hyperglycemia treatment in diabetic patients who are constipated, have firm stools
232 and/or flatus.



233

234 **Figure 4**. Percentage inhibition of enzyme activity (%I) for the 21 ecotypes of Fagioli di Sarconi beans,
235 in both α-glucosidase and α-amylase assays. Each value (mean ± SD) was normalized for maximum
236 tested concentration: for all samples, it was 0.005 mg/mL and 0.029 mg/mL for α-glucosidase and
237 α-amylase assays, respectively; for acarbose, it was 1.28 mg/mL in α-glucosidase assay and 0.057
238 mg/mL in α-amylase assay. Values marked by the same letter are not significantly different ($p <$
239 0.05).
240

241 The reason for this antihyperglycemic activity of Fagioli di Sarconi seeds was investigated through
242 metabolite screening. Preliminary colorimetric phytochemical analysis revealed the presence of
243 alkaloids, glycosides, phenols, saponins, steroids, tannins, terpenoids (**Table 2**). These secondary
244 metabolites are reported to have many biological and therapeutic properties (Dekdouk et al., 2015;
245 Russo et al., 2015; Senguttuvan et al., 2014). Remarkably, when these compounds were stratified
246 by m/z values , the number of compounds was heavily weighted toward polar secondary
247 metabolites exhibiting <500 Da (assuming singly charged species). More detailed identification of
248 the putative metabolite or class of metabolites occurring in beans under study was achieved by
249 determining elemental composition of experimental *m/z* values based upon accurate mass
250 determinations (**Figure 1**) and visualization in van Krevelen diagrams. The frequency distribution of
251 these elemental formulas in **Figure 1B** showed the most abundant chemical species in Fagioli di
252 Sarconi matrix are nitrogen containing compounds (CHON and CHONS) as compared to CHO and

253 CHOS. Only few CHO compounds were found in the region of saponins. Additionally, the van
254 Krevelen diagram in **Figure 1D** localized the identified elemental compositions according to the main
255 chemical families. The diagram shows that relatively abundant compounds were found in the
256 peptides, polyphenols and condensed heterocycles regions. In detail, CHON species were found
257 mainly in the peptide region according to the fact that *P. vulgaris* is a legume widely recognized as
258 an excellent source of dietary and low-cost proteins. In a previous study (Sotelo et al., 1995) it is
259 reported that cultivated beans showed a higher content of sulfur amino acids compared to wild
260 bean, thus explaining the presence of CHONS components in the region of peptides. Moreover,
261 CHON components occurred also in the condensed nitrogen containing compound region (nitrogen
262 in heterocycles): they could be associated with alkaloids, consistently with a seed composition. If
263 we consider CHO formulas, the diagram shows that relatively abundant compounds were found in
264 the condensed hydrocarbon and saponins regions, with also a few ones in the carbohydrate and
265 glycosylated compounds section. The relatively low content of carbohydrates in Fagioli di Sarconi
266 beans is apparently in contrast to Atchibri et al. (A. L. O. A. Atchibri et al., 2010) who reported
267 carbohydrates as major phytocostituents in *P. vulgaris* seeds. However, it must be underlined that
268 carbohydrates are not easily ionised (protonated) under ESI conditions (Boutegrabet et al., 2012).
269 The results of this study indicate that Fagioli di Sarconi bean extracts showed appreciable
270 hypoglycemic effects thanks to their phytoconstituents, as reported in literature. In detail, Heredia-
271 Rodriguez et al., described anti-diabetic effects of bioactive peptides in common beans (*P. vulgaris*)
272 due to inhibition of α-amylase and and α-glucosidase and stimulation of glucose uptake (Heredia-
273 Rodriguez et al., 2016) Flavonoids have been reported to stimulate peripheral glucose uptake and
274 express the enzymes responsible for metabolism of carbohydrates (Brahmachari, 2011). Alkaloids
275 are also hypoglycemic in nature (Kumar et al., 2011) and tannins have α-amylase and α-glucosidase
276 inhibition capability (Kunyanga et al., 2011). Moreover, lupeol type terpenoid is also reported for its
277 α-amylase inhibition activity (Kumar et al., 2011). Among terpenoid, saponins can stimulate the beta
278 cells and pancreatic islets with the consequent decrease of blood glucose (Samaddar et al., 2016;
279 Zheng et al., 2012). Stimulation of 5-adenosine monophosphate activated protein kinase and insulin
280 receptor/insulin receptor substrate 1/phosphatidylinositol 3-kinase/Akt signaling pathways leading
281 to a decrease in blood glucose is also demonstrated for soysaponin (X. Hu et al., 2014) that could be
282 considered having potential hypoglycemic activity (Quan et al., 2003). Isolation of the active
283 phytoconstituents and further evaluation of their individual hypoglycemic activities are still needed
284 to confirm the showing hypoglycemic property for Fagioli di Sarconi beans.

285 The use of OPLS allowed a simple representation of ultrahigh resolution MS data showing the main
286 correlations between α-glucosidase assay and the m/z values. The model gave the following values:
287 $R^2(Y)=0.9$ and $Q^2(cum)=0.9$, indicating the goodness of the fit and prevision capability. This
288 reasonable result could be explained because the 21 cultivars under study had same genotype,
289 geographic/environmental origin, harvest year and storage condition (C. Hu et al., 2014; Masi et al.,
290 2009). Therefore, the different biological activity probably could be associated to different amounts
291 of each active phytochemicals (Wang et al., 2010), which need to be investigated.

292

293 # 4. Conclusion

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

305

306 # 5. Experimentals

307 **5.1 Chemicals**

308 Sodium phosphate (≥98%), sodium chloride (≥99.5%), 3,5-dinitrosalicylic acid (≥98%), α-amylase
309 from hog pancreas starch, α-glucosidase from *Saccharomyces cerevisiae*, potassium phosphate
310 monobasic (≥99%), 4-nitrophenyl α-D-glucopyranoside (≥99%) and acarbose were acquired from
311 Sigma–Aldrich (Milano, Italy). Ferric chloride, sulphuric acid, acetic anhydride, glacial acetic acid,
312 chloroform, vanillin, mercuric chloride and potassium iodide to perform colorimetric assay-based
313 phytochemical screening were acquired from Sigma–Aldrich (Milano, Italy). All the solvents used for

314 sample pretreatment and MS analysis were of analytical grade, and were purchased from Sigma-
315 Aldrich (Milano, Italy). Ultrapure water was produced using a Milli-Q RG system from Millipore
316 (Bedford, MA, USA). Pure nitrogen (99.996%) was delivered to the MS system as the sheath gas.

317

318 **5.2 Bean samples and metabolite extraction**

319 The 21 ecotypes of Fagioli di Sarconi bean samples (*Cannellino*, *Cannellino Nasello Rosso*, *Cannellino*
320 *Rosso*, *Ciuoto o Regina*, *Marucchedda*, *Munachedda*, *Nasello Nero*, *Nasello Rosso*, *Nasello Viola*,
321 *Panzaredda*, *Riso Bianco*, *Riso Giallo*, *San Michele*, *San Michele Rosso*, *Tabacchino*, *Tondino Bianco*,
322 *Tuvagliedda*, *Tuvagliedda Marrone*, *Tuvagliedda Nera*, *Tuvagliedda Rossa*, *Verdolino*) were made
323 available through the local agricultural farm of the consortium Fagioli di Sarconi PGI (Kireeva, 2011).
324 The dried powder of Fagioli di Sarconi beans was extracted by using a modified procedure based on
325 previously reported method (Awoyinka et al., 2007; Marimuthu and Gurumoorthi, 2013). Briefly, 10
326 mL of 70:30 (v/v) water/ethanol solution was used to extract metabolites from 1 g of finely ground
327 beans in an ultrasonic bath for 6 h at room temperature (Sonorex Super RK 100/H sonicator;
328 Bandelin electronic, Berlin, Germany) with a 35 kHz automatic frequency control and a high-
329 frequency power of 80 W). After centrifugation at 5000 rpm (3000g) at 4 °C for 5 min (Kontron A8.24
330 rotor centrifuge), the supernatant was filtered through a 0.20 µm nylon syringe filter (Whatman,
331 Maidstone, UK) and injected into the MS system without further pre-treatment. To carry out the
332 enzymatic inhibition assays and phytochemical assays, the solvent was evaporated (Laborota 4000
333 efficient, Heidolph, Schwabach, Germany) and the sample was solubilized (see next sections) for
334 further analysis.

335

336 **5.3 *In vitro* antidiabetic activity: α-amylase and α-glucosidase enzymatic assays**

337 The inhibition assays to evaluate *in vitro* antidiabetic activity were performed using previous
338 methods (Milella et al., 2016; Saltos et al., 2015). Acarbose, a widely used clinical antidiabetic drug,
339 was used as a positive control.
340 The α-amylase inhibitory activity was assayed using 10 μL of 20 mM sodium phosphate buffer (pH
341 6.9 with 6 mM NaCl) containing 0.5 mg/mL α-amylase (50 Units/mg) and then incubated at 25 °C
342 for 10 min with 10 μL of bean extract. Extracts were solubilized in 10% DMSO/MeOH solution and
343 tested at different concentrations. After this pre-incubation, 10 μL of 1% starch solution in 20 mM

344 of sodium phosphate buffer, used as substrate, was added to each sample and the reaction mixtures
345 were incubated at 25° C for an additional time of 10 min. The reaction was stopped with 20 μL of
346 dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 10
347 min, cooled at room temperature and after addition of 300 μL of distilled water the absorbance was
348 measured at 540 nm. The absorbance of blank samples (in which enzyme solution was added during
349 the boiling process) and negative controls (10% DMSO/MeOH solution added in place of extract)
350 were recorded. Acarbose solubilized in 10% DMSO/MeOH was tested at different concentrations.
351 Analyses were performed in triplicate and the final value of sample absorbance (A540 nm) was
352 obtained by subtracting its corresponding blank sample reading (Ranilla et al., 2010). The
353 concentration of acarbose and bean extracts required to inhibit 50% of α-amylase activity under the
354 conditions was defined as the IC_{50} value. The α-amylase inhibitory activities of bean extracts and
355 acarbose were calculated, and its IC_{50} values were determined. The inhibitory activity (%) was
356 calculated as follows (equation 1):

357 $$\% \text{ Inhibition } = \frac{(\text{A540 Negative Control} - \text{A540 Sample})}{\text{A540 Negative Control}} * 100 \qquad \text{eq. 1}$$

358

359 The inhibitory activity of α-glucosidase enzyme was assessed in 96-well plates. In each well 10 μL of
360 bean extract was solubilized in 10% DMSO/MeOH solution and tested at different concentrations;
361 160 μL of 10 mM sodium phosphate buffer pH 7.0 and 60 μL of substrate (2.5 mM 4-nitrophenyl α-
362 D-glucopyranoside in 10 mM phosphate buffer) were added. The reaction started with the addition
363 of 20 μL of enzyme (0.28 U/mL in 10 mM phosphate buffer) and the plates were incubated at 37° C
364 for 10 min. The absorbance at 405 nm was measured before the addition of the enzyme (T0) and
365 after 10 minutes of incubation (T10). Acarbose was solubilized in 10 μL 10% DMSO/MeOH and
366 tested at different concentrations. Negative control absorbance (10% DMSO/MeOH solution in
367 place of extract) was also recorded. The inhibitory activity was calculated by using the formula
368 (equation 2):

369 $$\% \text{ Inhibition } = \frac{(\text{A405 Negative Control}_{\text{T10}-\text{T0}} - \text{A405 Sample}_{\text{T10}-\text{T0}})}{\text{A540 Negative Control}_{\text{T10}-\text{T0}}} * 100 \qquad \text{eq. 2}$$

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

372

373 **5.4 Phytochemical assays**

374 Screening of phytochemical constituents, i.e. glycosides, tannins, phenols, flavonoids, alkaloids,
375 saponins, steroids and terpenoids, occurring in the bean powder extracts was done using standard
376 protocols, commonly used to investigate the presence of bioactive compunds of medicinal plants
377 (A. L. O. Atchibri et al., 2010; A. L. O. A. Atchibri et al., 2010; Marimuthu and Gurumoorthi, 2013;
378 Mbagwu et al., 2011; Savithramma et al., 2011; Senguttuvan et al., 2014; Yogeshwari and
379 Kalaichelvi, 2017). In detail:

380 *Test for alkaloids.* Meyer's test: to the 500 µL of bean extract, add 500 µL of Mayer's reagent
381 (potassium mercuric iodide solution). Whitish yellow or cream coloured precipitate indicates the
382 presence of alkaloids.

383 *Test for glycosides.* Keller-Kiliani test: 500 µL of bean extract was solubilized in 1 mL of glacial acetic
384 acid containing one drop of ferric chloride solution (5%). This solution was underlayed with 100 µL
385 of 37% sulphuric acid and a brown ring obtained at the junction of two layers indicates the presence
386 of glycosides.

387 *Test for terpenoids and steroids.* Salkowski test: 500 µL of bean extract was suspended in 200 µL of
388 chloroform, and concentrated sulfuric acid (300 µL) was carefully added to form a layer. A reddish
389 brown coloration of the interface is indicative of the presence of terpenoids.

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

392 *Test for tannins.* To 500 µL of bean extract solution 1 mL of water and 1-2 drops of ferric chloride
393 solution (5%) was added. Green-Black color was observed for tannins.

394 *Test for saponins.* Makkar's test: 500 µL of the extract reacts with 1 mL of alcholic vanillin (400 mg
395 of vanillin in 5 mL of 99.5% ethanol) solution and adds few drops concentrated sulfuric acid. The
396 formation of deep red colour indicates presence of saponins.

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

399 *Test for quinones.* Concentrated sulphuric acid (500 µL) was added to 500 µL of plant extract. The
400 formation of red colour indicates the presence of quinone.

401 *Test for anthraquinones.* Few drops of 2% HCl were added to 500 µL of extract. Appearance of the
402 red colour indicates the presence of anthraquinones.

403 *Test for gum and mucilage.* The plant extract was diluted with 5 ml of distilled water and to this 25
404 mL of absolute alcohol was added with constant stirring. The formation of white or cloudy
405 precipitate indicates the presence of gums and mucilage.
406 *Test for lipids.* 1g plant sample was dissolved in water:chloroform (50:50) and stirred for a hour.
407 Mixture was centrifuged, organic supernatant dried and dissolved in ethanol. 2 mL of concentrated
408 H_2SO_4 and 5 mL of phosphovanillin reagent (50 mg vanillin was dissolved in 800 μL of absolute
409 ethanol before diluting to 8 mL with distilled water and mixing with 33 mL of concentrated H_3PO_4)
410 was added to 100 μL of extract. Change of the colour indicates the presence of lipids.
411 *Test for carbohydrates.* Molisch's test: 500 μL crude extract was mixed with few drops of Molisch's
412 reagent (10% alchoolic solution of α-naphtol) and the mixture was shaken properly. After that, 1 mL
413 of concentrated H_2SO_4 was poured carefully along the side of the test tube. Appearance of a violet
414 ring at the interphase indicated the presence of carbohydrate.
415

416 **5.5 FT-ICR-MS analyses**

417 High-resolution mass spectra were acquired on a Bruker (Bruker Daltonik GmbH, Bremen, Germany)
418 solariX Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) equipped with a
419 12 Tesla superconducting magnet (Magnex Scientific Inc., Yarnton, GB) and a APOLLO II ESI source
420 (Brucker Daltonik GmbH, Bremen, Germany) in the negative ionisation mode. 10 µL of each ethanol
421 bean extract were diluted in 1 mL of methanol (Schmitt-Kopplin et al., 2012) prior to direct injection
422 into the microeletrospay source at a flow rate of 120 µL h^{-1} with a nebulizer gas pressure of 32 psi,
423 and drying gas flow rate of 4 L/min at 180°C. Spectra were acquired with a time domain of 4 mega-
424 word and a mass range of m/z 100–1000. 300 scans were accumulated for each sample .Spectra
425 were externally calibrated using a blank analysis of typical solvent impurities in methanol. The
426 accuracy reached values of less than 0.1 ppm. Further internal calibrations were performed for each
427 sample through the identification of ubiquitous fatty acids. Fourier transform ion cyclotron
428 resonance (FT-ICR) mass spectra with *m/z* from 150 to 1000 were exported to peak lists at a signal-
429 to-noise ratio (S/N) of 2 and higher (Schmitt-Kopplin et al., 2010). From these lists, possible
430 elemental formulae were calculated for each peak using Data Analysis software (v4.1, Bruker
431 Daltonik GmbH, Bremen, Germany); an elemental formulae assignment was obtained due to the
432 ultra high resolution (R = 400.000 at *m/z* 500, thus differentiating two masses separated by the mass
433 of an electron) and to the mass accuracy of 0.1 ppm (electron mass accuracy). Thousands of such
434 compositions could be calculated, which contained C, H, O, N and S elements. The generated

435 formulas were validated by setting sensible chemical constraints: N rule; element counts: C ≤ 100,
436 H ≤ 200, O ≤ 80, N ≤ 5, S ≤ 1 and only the masses in conjunction with their automated generated
437 theoretical isotope pattern (existence of the ^{13}C isotope) were taken into consideration, according
438 to available literature concerning elemental composition assignment to Fourier transform ion
439 cyclotron resonance mass spectrometry data (Herzsprung et al., 2014). They were represented using
440 van Krevelen diagrams, which sort them onto two axes according to H/C and O/C atomic ratios
441 (Hertkorn et al., 2007; Tziotis et al., 2011). Moreover, the *m/z* peak lists were used for further
442 statistical analysis.

443

444 **5.6 Data analyses**

445 The IC_{50} values were estimated by non-linear curve-fitting and presented as their respective 95%
446 confidence limits. All enzymatic assays were performed in triplicate, and results expressed as mean
447 ± standard deviation (Mean ± SD). The Student's *t*-test (SPSS 19.0 for Windows; IBM SPSS Statistics,
448 Armonk, NY, USA) was used to assess the presence of significant differences ($p<0.05$) among the
449 extracts. All the statistical analyses were accomplished, using the computer software GraphPad
450 Prism 3.02 for Windows (GraphPad Software, USA). High-resolution mass spectra were subjected to
451 data processing and filtering by using DataAnalysis software (v4.1, Bruker Daltonik GmbH, Bremen,
452 Germany). The reduced peak lists were submitted to multivariate principal component analysis
453 (PCA) using XLSTAT Version 2015.1 (Addinsoft Inc., New York, NY, USA) for more detailed insight in
454 the relations between the variables.

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460

461 **This article contains supporting information.**

462

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