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High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry (HPLC-ESI-MSⁿ) Analysis and Bioactivity Useful for Prevention of “Diabetes” of *Allium commutatum* Guss

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Abstract

This study aimed at evaluating and comparing the chemical profile obtained by HPLC-ESI-MSⁿ analysis, the inhibitory activity of enzymes linked to obesity (α -amylase, α -glucosidase, and lipase) and the antioxidant properties (DPPH, ABTS, FRAP, and β -carotene bleaching tests) of ethanol extracts of bulbs (BE) and aerial parts (APE) from *Allium commutatum* Guss. (known in Italy as “aglio delle isole”). The chemical profile revealed alliin as the main abundant compound with values of 31.5 and 38.8 mg/g extract for BE and APE, respectively. APE is rich also in quercetin (38.5 mg/g extract) and luteolin (31.8 mg/g extract). Bulb extract exhibited the highest activity as inhibitor of enzymes linked to obesity. Except for DPPH test, APE showed the highest antioxidant potential with IC₅₀ of 7.6 and 56.6 μ g/mL in ABTS and β -carotene bleaching test after 60 min of incubation, respectively. In conclusion, the present investigation revealed *A. commutatum* bulbs and aerial parts as a promising source of inhibitors of enzyme linked to the obesity and of antioxidant compounds.

Keywords Garlic • Aerial parts • Bulbs • Carbohydrate-hydrolysing enzymes inhibition • Lipase inhibition • Antioxidant activity

Introduction

The genus *Allium* belongs to the Alliaceae family formerly considered part of Liliaceae and Amaryllidaceae. The first centre of evolution of the genus *Allium* extends along the Irano-Turanian region. Mediterranean basin and western North America represents the secondary centre of diversity. From these centres, *Allium* species have widely spread all over the northern hemisphere [1]. Several plants belonging to this genus such as *A. cepa*, *A. sativum*, *A. ampeloprasum*, *A. schoenoprasum*, and *A. ascalonicum* are of high economic value not only for their use as spice in traditional recipes but also for their taste and for their action as biopreservatives [1–4]. Several studies reported the beneficial effect of *Allium* species in metabolic syndrome and related diseases [3, 5]. *Allium commutatum* Guss. is known in Italy as “aglio delle isole”. This species is synonymous of *A.*

ampeloprasum var. *lussinense* and *A. bimetrale* in Italy, Greece, and Croatia [6]. According to World Health Organization (WHO), data report 2016 more than 1.9 billion adults were overweight and of these over 650 million were obese. Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health and is associated to a series of comorbidities in which diabetes type 2 (DM2) has a prevalent role [7]. The strict relationship between obesity and diabetes has led to coining the term “diabesity” [8]. Overweight or obese people are characterized by a progressive reduction in insulin secretion coupled with a progressive rise in insulin resistance. Moreover, a persistent blood hyperglycaemia as in DM2 determined, at cytosolic and mitochondrial level, an overproduction of Reactive Oxygen Species (ROS) [9]. The use of foods and spices able to induce body weight reduction by inhibition of digestive enzymes that interfere with the hydrolysis and absorption of lipids and carbohydrates is a largely investigated topic [10, 11]. The key enzymes involved in the hydrolysis of fat and dietary carbohydrates are pancreatic lipase, α -amylase, and α -glucosidase [7].

Herein, we investigated for the first time wild Sicilian (Italy) *A. commutatum* bulbs and aerial parts extracts were chemically characterized by HPLC-ESI-MSⁿ. and *in vitro* studied for their α -amylase, α -glucosidase and pancreatic lipase inhibitory activities. The antioxidant properties were also examined by different tests, namely ABTS, DPPH and β -carotene bleaching tests.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Co. Ltd. (Milan, Italy) and VWR International (Milan, Italy) and, unless specified otherwise, were analytical grade or higher.

Plant Materials and Extraction Procedure

Bulbs and aerial parts of *Allium commutatum* Guss. used in this work were collected in May 2018 near Capo Calavà, Gioiosa Marea located about 160 km east of Palermo (Italy)

(38°11'22.47" N, 14°55'17.75" E) from plants at the full flowering stage growing on an area of about 200 m². Samples were harvested from 60 plants in order to obtain a set of bulbs and aerial parts that are representative. Samples were identified by Prof. Vincenzo Ilardi, University of Palermo, Italy. A voucher specimen (PAL-109619) has been deposited in Herbarium Mediterraneum Panormitanum (PAL), Italy. Bulbs (100 g) and aerial parts (100 g) were dried at room temperature for seven days, manually separated, finely ground, and exhaustively extracted by maceration (3 × 24 h) by using ethanol as solvent. The extracts were filtered through Whatman No. 4 filter paper and the solvent completely evaporated by using rotary evaporator (Buchi model R-210, Cornaredo, Italy) under reduced pressure to obtain dry crude extracts. Yields of 14.3 and 5.9% were obtained for bulbs (BE) and aerial parts (APE), respectively.

Total Phenols Content

A. commutatum total phenols content was evaluated by using the Folin-Ciocalteu method as previously reported [12]. Briefly, the extract was mixed with Folin-Ciocalteu reagent (0.2 mL), sodium carbonate 15% (1 mL), and water (2 mL). After incubation at 25 °C for 2 h, the absorbance was read at 765 nm. Determinations were made in triplicate and the result was expressed as milligrams of chlorogenic acid equivalents/g of extract.

Total Flavonoids Content

The total flavonoids content was determined by using the aluminium chloride colorimetric assay as previously described [12]. In brief, *A. commutatum* extract was added to distilled water (4 mL) and sodium nitrite 5% (w/v) (0.3 mL). After 5 min, 0.6 mL of 10% (w/v) AlCl₃ was added. At 6 min 2 mL of 1 M NaOH and 2.1 mL of distilled water were added to the mixture. The absorbance was read at 510 nm. Determinations were made in triplicate and values were expressed as milligrams of quercetin equivalents/g of extract.

HPLC-ESI-MSⁿ Analysis

HPLC-ESI-MSⁿ was used for the analysis of *A. commutatum* constituents using a Chromatograph Agilent 1260 (Santa Clara, CA, USA) equipped with diode array detector (DAD) and Agilent/Varian MS-500 ion trap mass spectrometer. Agilent Eclipse XDB C-18 (3.0 × 150 mm, particle size

3.5 μ m) was used as stationary phase. The sample injection volume was 10 μ L. The mobile phase was 0.1% formic acid in water (A) and acetonitrile (B). The gradient elution start with 90% A and is changed to 0% of A in 33 min, flow rate was 0.4 mL/min. Rutin and S-allyl-L-cysteine sulfoxide -alliin were used as reference. Allicin content was estimated using alliin calibration curve while rutin for flavonoid derivatives. The chromatograms were monitored at different wavelength namely 240 nm for alliin and allicin while 350 nm for the flavonol derivatives acquiring the UV spectra in the range 200–400 nm. MS spectra were acquired using Electro Spray (ESI) as ion source the m/z 50–2000 range, operating in positive ion mode.

Pancreatic Lipase Inhibitory Activity

Pancreatic lipase inhibitory activity was determined by 96- well plate method based on the procedure proposed by El- shiekh et al. [13]. 4-Nitrophenyl octanoate (NPC), 5 mM in dimethylsulfoxide solution and an aqueous solution of porcine pancreatic lipase (1 mg/mL), and Tris-HCl buffer (pH 8.5) were prepared. *A. commutatum* extracts (2.5–40 mg/mL) were added in a well with 6 μ L of the enzyme, 6 μ L of NPC and 279 μ L of buffer. The mixture was incubated at 37 °C for 30 min. The absorbance was measured at 405 nm. Experiments were performed in triplicate. Orlistat was used as a positive control.

Carbohydrates-Hydrolysing Enzymes Inhibition

The α -amylase inhibitory assay was performed as previously described [14]. Absorbance was measured at 540 nm. The α -glucosidase inhibition test was performed following the procedure previously described [14]. Absorbance was measured at 500 nm. Acarbose used as positive control in both assays.

ABTS and DPPH Tests

The radical scavenging activity was investigated by using two different test namely ABTS and DPPH. ABTS test employed ABTS radical cation generated in aqueous phase by using potassium persulfate

as strong oxidizing agent. The reduction of blue-green ABTS radical cation by hydrogen-donating antioxidant was spectrophotometrically measured. To evaluate the ABTS radicals scavenging activity of *A. commutatum* extracts the procedure previously reported was applied [14]. ABTS scavenging activity (%) was calculated as follows: $[(A' - A)/A'] \times 100$, where A' is the absorbance of the control reaction and A is the absorbance in the presence of the extract. The DPPH test is based on the reduction of DPPH radical by substance that can donate a hydrogen atom. This reaction, measured at 517 nm, lead to a reduced form of DPPH with the loss of the violet color that characterize the radical. The DPPH radicals scavenging activity was assessed as previously reported [14]. Ascorbic acid was used as positive control in both assay.

143 β -Carotene Bleaching Test

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145 The protection of extract on lipid peroxidation was measured as previously described [14]. The
146 discoloration of aqueous emulsion of linoleic acid, β -carotene and 100% Tween 20, which is
147 generated by the spontaneous oxidation of the fatty acid at 50 °C was measured at 470 nm against a
148 blank at $t = 0$ and after 30 and 60 min of incubation. Propyl gallate was used as positive control.

149

150 Global Antioxidant Score (GAS)

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152 The T-score is calculated by the following equation: $T\text{-score} = (X - \min)/(\max - \min)$, where min
153 and max, respectively, represent the smallest and largest values of variable X among the
154 investigated extract [15].

155

156 Statistical Analysis

157

158 GraphPad Prism version 4.0 for Windows, GraphPad Software (San Diego, CA, USA) was used
159 to calculate the concentration-response curve and consequently the inhibitory concentration 50%
160 (IC_{50}). Differences within and between groups were evaluated by one-way ANOVA followed by
161 a multicomparison Dunnett's test ($\alpha = 0.05$): **** $p < 0.0001$,
162 *** $p < 0.001$ compared with the positive controls.

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165 Results and Discussion

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167 Chemical Profile

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169 HPLC-ESI-MSⁿ analysis of BE and APE lead to the identification of alliin, γ -glutamyl (S)-allin-
170 cysteine, allicin, γ -glutamyl (S)-methyl-cysteine, γ -glutamyl (S)-*trans*-propenyl-cysteine, and
171 cycloalliin (Table 1, Fig. 1). Allin represents the most abundant compound with values of 31.5 and
172 38.8 mg/g extract for BE and APE, respectively. *Allium* sulfur-containing compounds are hydrolysed
173 by means of alliinase into pyruvate, ammonia, and a mixture of both volatile and non-volatile sulfur
174 compounds, after the cutting tissue, mastication, and cooking [16]. Alliin and allicin are
175 characteristic garlic organo-sulphur compounds. Interestingly, our data demonstrated that *A.*
176 *commutatum* from Sicily presents a high content of both alliin (31.5 and 38.8 mg/g of extract for BE

and APE, respectively) and allicin (23.1 and 16.6 mg/g of extract for BE and APE, respectively). A significant difference in the content of alliin was detected in the extract of the garlic compared to the elephant garlic (23.63 vs 2.61 mg/g dw, respectively) [17]. A similar trend was observed for cycloalliin (2.21 vs 1.32 mg/g dw, for garlic and elephant garlic, respectively).

Table 1 HPLC-ESI-MS/MS profile of *A. commotatum* extracts (mg/g extract)

BE: bulbs extract, APE: aerial parts extract. Data are reported as mean $\pm$ standard deviation ($n = 3$). N.Q.: not quantified; tr: trace, Rt: retention time (average of $n = 3$ injections), MW: molecular weight

The spectrophotometric determinations of phenols and flavonoids evidenced the different content of these compounds in the analysed *A. commutatum* parts. To this regard, bulbs showed 41.4 and 14.7 mg/g extract for total phenols content and total flavonoids content, respectively, whereas values of 38.8 and 26.2 mg/g extract, respectively, were recorded in aerial parts. These values are in agreement with those reported by Ademoyeun et al. [18] but in disagreement with those reported for *A. cepa* cv. Bianca di Pompei (medium value of 5.32 mg GAE/g DW) [16]. In a previous study, Riggi et al. [19] analysed *A. cepa* ("cipolla di Giarratana") from Sicily (Italy). Some flavonoids were identified and quantified. In particular, quercetin, quercetin 4'-O-glucoside, and quercetin 3,4'-di-O-glucoside represent the main abundant compounds

199 not only in “cipolla di Giarratana” but also in red onion from Tropea and copper onion from Montoro
200 [20]. Phenols have raised huge attention in the last decade due to their role in the prevention of chronic
201 diseases. These compounds are involved in the “defensive characters” of the plants, and for this reason
202 their profile and content depend on genetic factors but it is also influenced by environmental and
203 agronomic practices, as extensively reported in recent literature [21]. Quercetin, apigenin and luteolin
204 have been identified by comparison with reference compounds, while other flavonol derivatives,
205 namely methoxy quercetin representing a glycosidic moiety formed by three hexoses as well as, three
206 different methoxy quercetin were identified [22, 23]. Among them APE showed a significant
207 flavonoid content with particular Inhibition of Enzymes Linked to Obesity

208
209 The search for natural and safe enzyme inhibitors to overcome overweight and obesity related to DM2
210 is amongst the most investigated matters especially if bioactive constituents are in commonly
211 consumed food or spice. *A. commutatum* extracts were investigated for their potential inhibitory
212 activity against pancreatic lipase and carbohydrate hydrolysing enzyme inhibitory activity. Data are
213 reported in Table 2. A concentration-dependent manner should be observed for all investigated
214 samples. Generally, BE showed a most promising “diabetes” linked enzyme inhibitory activities.

215 IC₅₀ values of 100.2 and 167.0 µg/mL were recorded against pancreatic lipase for BE and APE,
216 respectively. Regarding carbohydrates-hydrolysing enzymes, data evidenced that α-amylase was
217 more sensible than α-glucosidase to *A. commutatum* extracts. In fact, IC₅₀ values of 202.9 and
218

289.4 µg/mL were found against α-amylase for BE and APE, respectively. Bulbs extract was more active against α-glucosidase (IC₅₀ value of 302.5 µg/mL). Recently, Oboh et al. [24] compared the activity of aqueous extract of *A. sativum*, *A. cepa* var. *cepa*, and *A. cepa* var. *viviparum*. All these extracts evidenced a weak activity in comparison to our data. In particular, *A. cepa* var. *viviparum* inhibited α-amylase with IC₅₀ of 3.93 mg/mL, followed by *A. sativum* (IC₅₀ of 8.19 mg/mL) and *A. cepa* var. *cepa* (IC₅₀ of 8.27 mg/mL). No significant differences were reported for α-glucosidase inhibitory activity for all investigated *Allium* species. The positive impact of *Allium* species on metabolic disease was confirmed by *in vivo* study. *A. elburzense* bulbs hydroalcoholic extract orally administered (100–400 mg/kg die??? for seven days) in dexamethasone-induced dyslipidaemia, hyperglycaemia and oxidative-stress rats significantly reduced triglyceride, total cholesterol, low-density lipoprotein-cholesterol and serum blood glucose [25]. More recently, Zhai et al. [26] reported that alliin (0.1 mg/mL) orally administered in C57BL/6 J DIO mice for eight weeks alliin increased insulin sensitivity, enhanced glucose homeostasis, and improved the lipid profile.

235 Antioxidant Activity

The antioxidant activity of *A. commotatum* extracts was estimated by using two radical scavenging assays (ABTS and DPPH tests) and one test to evaluate the protection of lipid peroxidation (β-carotene bleaching test). Data are reported in Table 3. A different behaviour of extracts was observed in radical scavenging assays (ABTS and DPPH tests). In particular, APE showed the highest ABTS⁺ activity with IC₅₀ value of 7.6 µg/mL. A promising result was observed also with BE (IC₅₀ value of 10.5 µg/mL). Differently in DPPH assay BE was the most active with IC₅₀ value of 31 µg/mL. It will be possible to assume that *A. commutatum* extracts exert their antioxidant activity by hydrogen or electrons reduction of DPPH.

Pearson's correlation coefficient evidenced an *r* value of 1 for total phenols content and ABTS and β-carotene bleaching test and for total flavonoids content and DPPH assay. By the integrated approach GAS, APE showed the highest antioxidant potential. The comparison between *A. sativum* (garlic), *A. ampeloprasum* (elephant garlic) and *A. cepa* (onion) was done by Kim et al. [17]. Results evidenced the following trend of potency onion > elephant garlic > garlic. A positive correlation between organosulfur compounds content and bioactivity was found. Previously, Štajner et al. [27] compared the antioxidant effect of wild *Allium* species (*A. atroviolaceum*, *A. flavum*,

A. roseum, *A. scorodoprasum*, *A. subhirsutum*, *A. sphaerocephalum*, *A. ursinum*, and *A. vineale*) with the cultivated species *A. nutans*, *A. fistulosum*, *A. vineale*, *A. pskemense*, *A. schoenoprasum*, *A. cepa*, and *A. sativum*. Generally, both bulbs and leaves from cultivated species exerted a greater activity comparing to the wild ones. IC₅₀ values higher than that found in our study were reported by Suleria et al. [28] for *A. sativum* aqueous bulbs extract (1.03, 4.41, and 6.01 for immature garlic, air-dried garlic, and fresh garlic, respectively). A lower DPPH radical scavenging activity was found also with *A. cornutum* and *A. cepa* bulbs from Croatian coast and island with percentage of inhibition of 60.50 and 64.82% at 100 µg/mL, and *A. cepa* cv. Bianca di Pompei (EC₅₀ medium value of 19.94 mg/mL) [16]. A DPPH radical scavenging activity comparable to the positive control BHT was observed with *A. saralicum* leaves ethanol extract [29]. Kim et al. [17] evidenced that garlic exhibited a great radical scavenging activity in both DPPH and ABTS respect elephant garlic. Some studies reported organo-sulphur constituents as responsible for the antioxidant activities of *Allium* species whereas others have correlated the antioxidant activities mainly with phenols [17]. In our study, Pearson's correlation coefficient, calculated by using the sum of quantified compounds by HPLC-ESI-MSⁿ revealed positive correlations ($r = 1$) for both phytoconstituents classes and investigated bioactivity.

Conclusions

The present study assessed the chemical profile, and *in vitro* α -amylase, α -glucosidase, and lipase inhibitory activity, and the antioxidant activity of bulbs and aerial parts of wild Sicilian *A. commutatum* ("aglio delle isole"). Alliin was identified as the main abundant compound of both bulbs and aerial parts. Bulbs extract exhibited the highest enzymes linked to obesity inhibitory activity, while aerial parts extract showed the highest antioxidant potential. This extract was characterized by the presence of quercetin and luteolin as the most abundant identified flavonoids. Collectively, our results represent a starting point for further *in vivo* studies to promote the use of this species as nutraceutical or functional food product. Obtained data could help to support the identification of local

varieties to counter the globalization of agricultural production and promote the biodiversity.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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	Identified compound	Rt (min)	M W	M + H and fragments	UV-Vis data (nm)	BE	APE
1	γ -glutamyl-S-methyl cysteine	2.2	264	265, 136, 139	—	N.Q.	N.Q.
2	γ -glutammil S trans propenil cysteine	4.2	290	291, 145	—	N.Q.	N.Q.
3	Allicine	4.6	162	163,121 87	220	23.1 $\pm$ 0.1*	16.6 $\pm$ 0.3*
4	γ -glutammil (S)-allil- cysteine	4.8	290	291, 145	240	N.Q.	N.Q.
5	Methoxy quercetin trisaccharide	6.2	778	779, 493, 331, 313	350	12.3 $\pm$ 0.1	20.1 $\pm$ 0.1
6	Alliin	9.07	177	178, 88	220	31.5 $\pm$ 0.1	38.8 $\pm$ 0.1
7	Apigenin diglycoside	10.2	594	595, 271	350	6.5 $\pm$ 0.1	11.5 $\pm$ 0.1
8	Cycloalliin	11.6	177	178, 88	220	N.Q.	N.Q.
9	Quercetin	13.3	302	303, 285, 257, 229,166	350	12.1 $\pm$ 0.1	38.5 $\pm$ 0.1
10	Luteolin	15.1	286	287, 213, 166	350	14.3 $\pm$ 0.1	31.8 $\pm$ 0.1
11	Methoxy quercetin isomer 1	16.2	316	317, 302	350	1.5 $\pm$ 0.1	8.2 $\pm$ 0.1
12	Methoxy quercetin isomer 2	16.8	316	317, 302	350	tr	3.1 $\pm$ 0.2
13	Methoxy quercetin isomer 3	17.0	316	317, 302	350	4.1 $\pm$ 0.1	6.6 $\pm$ 0.2

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	α -Amylase	α - Glucosida se	Lipase
BE	202.9 $\pm$ 4.3****	302.5 $\pm$ 5.1****	100.2 $\pm$ 2.3****
APE	289.4 $\pm$ 4.6****	325.5 $\pm$ 5.3****	167.0 $\pm$ 2.9****
<i>Positive control</i>			
Acarbose	50.0 $\pm$ 0.9	35.5 $\pm$ 1.2	
Orlistat			37.4 $\pm$ 1.3

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			t 30 min	t 60 min
BE	31.9 $\pm$ 3.1****	10.5 $\pm$ 1.3****	72.8 $\pm$ 3.9****	75.3 $\pm$ 3.2****
APE	68.1 $\pm$ 2.3****	7.6 $\pm$ 1.1***	58.6 $\pm$ 2.0****	56.6 $\pm$ 2.3****
<i>Positive control</i>				
Ascorbic acid	5.0 $\pm$ 0.80	1.7 $\pm$ 0.06		
Propyl gallate			0.1 $\pm$ 0.004	0.1 $\pm$ 0.004
BHT				

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