Antioxidant, enzyme-inhibitory and antitumor activity of the wild dietary plant *Muscari comosum* (L.) Mill.

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Abstract

Conventional medicines used to treat obesity and cancer frequently exhibit high side effects, so that researchers are focusing on new therapies and drugs based on natural products. Total extracts from bulbs of *Muscari comosum* were tested for i) free radical scavenging activity, ii) *in vitro* enzymatic inhibition of pancreatic α-amylase and lipase, and iii) inhibition of the growth of breast adenocarcinoma cells. Three treatments were considered: bulbs boiled in water for 15 min (traditional cooking method; BB); bulbs steam-cooked for 15 min (alternative cooking method; SB); raw bulbs (RB). The polyphenol content and antioxidant capacity of bulb extracts were related to the inhibition of pancreatic lipase and α-amylase, whose activities have been found to have a half maximal inhibitory concentration (IC_{50}) of 0.28, 2.14 and 3.22 mg/mL for lipase, and 0.16, 0.73 and 0.69 mg/mL for α-amylase in RB, SB and BB, respectively. The analysis on breast adenocarcinoma MCF-7 cells revealed that RB extracts, and in a lesser extent BB, exerted a dose-dependent inhibition on cell proliferation. Considering that the potential of natural products for the treatment of obesity are under exploration, *M. comosum* could be an excellent plant for the development of future anti-obesity drugs, also able to prevent cancer.

Introduction

According to the World Health Organization,[1] overweight and obesity are defined as abnormal or excessive fat accumulation that increases the risk of chronic metabolic diseases. Chronic inflammation, characterized by the hypersecretion of pro-inflammatory adipokines, that aggravates the production of reactive oxygen species (ROS), is a central characteristic of obesity.[2] ROS are inevitably produced in biological systems due to oxidative metabolism, and are known to cause various degenerative disorders.[3,4]

Plant phenols are defense secondary metabolites produced by plants. They exert a ROS-scavenger activity but also have interesting biological activities related to enzymes inhibition, such as lipases and amylases.[5] In this context, the dietary polyphenols have a potential as nutritional strategies for the prevention and treatment of obesity and cancer, and their associated inflammations. Indeed, these two diseases have often been associated to a reduced consumption of fruit and vegetables, containing phenols.[6] Several studies have explored the relationship between overweight/obesity with an increased risk for cancer, mainly due to over-production of hormones, such as insulin or estrogen.[7,8] Due to their ROS-scavenging and anti-oxidative action, plant phenols have a dose-dependent inhibition on the growth and proliferation of cancer cells.[9,10] One of the most important strategies in the treatment of obesity includes inhibitors of nutrient digestion in an attempt to reduce energy intake through pancreatic lipase and amylase inhibition.[11,12] The lipase hydrolyzes a molecule of triglyceride in position 1 and 3, releasing fatty acid and 2-monoglyceride. The inhibition of lipase prevents the release of fatty acids, and then promote the non-absorption of fats. This inhibitory effect is has already been found for the anti-obesity drug *Orlistat*, containing tetrahydrolipstatin as active ingredient. On the other hand, pancreatic *α*-amylase catalyzes the hydrolysis of 1,4-*α*-D-glucosidic oligosaccharides and polysaccharides containing three or more residues, so yielding glucose and maltose, that enter glycolysis and, if in excess, are then converted in fats.

Interestingly, the effects of lipid-lowering, anti-obesity and anti-cancer plant extracts are becoming a sound topic in science research of food and nutrition.[13,14] A variety of natural products, including crude extracts and pure isolated natural compounds containing phenolss can promote the reduction of adipose tissue mass and they have been widely used in the treatment of diet-induced obesity.[15,16] Several authors studied phytochemical aspect related to phenols content in wild species traditionally consumed in the Mediterranean diet.[17,18]

In this study, we investigated a product of the culinary culture of the Mediterranean diet: cooked and raw bulbs of a wild onion species (*Muscari comosum*), commonly called *lampascioni*. The genus *Muscari* belongs to the family of *Liliaceae* and includes about 50 species manly distributed in Southern Italy, Greece, Turkey and Iran.[19,20] Particularly, *M. comosum* has been studied for its peculiar organoleptic,[21,22] economic,[23] and antioxidant characteristics,[24] but its pharmaceutical properties are mostly unknown. For this purpose, bulbs of lampascioni were screened for their content in total polyphenols and flavonoids, and tested for antioxidant properties, inhibiting activity of pancreatic lipase and *α*-amylase and antiproliferative activity against human cancer cells.

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Contributions: TC conducted the experiments about extraction, inhibition of amylase and antioxidant activity; AS conducted statistical analysis and interpretation of statistical data; IC conducted the experiments about cell proliferation; MM conducted the experiments about inhibition of lipase; FC collected test data and drafted the manuscript; GAS designed the study and interpreted the results.

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Materials and Methods

Plant material and preparation of samples

Bulbs of *Muscari comosum* were collected in field in Paludi (39°31'12.91"N, 16°41'41.95"E - 39°31'9.23"N; 16°41'47.13"E) (Cosenza district, Calabria Region, Italy) during July 2015. The bulbs were stored in a cool and dry environment and subsequently were deprived of the roots and soil residues. Three treatments were

considered: bulbs boiled in water for 15 min (traditional cooking method; BB); bulbs steam-cooked for 15 min (alternative cooking method; SB); raw bulbs (RB). Each sample was extracted through Naviglio® extractor (Atlas Filtri S.r.L., Limena, PD, Italy) for 36 h at 20°C and high pressure (8 bar), using two cycles with 2 L ethanol each. The average extraction yield (fresh sample/ethanol) was 21%. Solvent was evaporated using a Rotavapor® R-220 SE (BÜCHI Labortechnik AG, Flawil, Switzerland).

Determination of total phenol compounds

Total phenol content of the total extracts was determined using Folin-Ciocalteu reagent and chlorogenic acid as standard.[25] A 20-mg aliquot of the extracts was vortexed with 25 mL of the extraction solvent (95:5, water:37% HCl). during the tests, we compared absorbance of the extract with different methods. We verified that the absorbance values changed in the various tests. We thus tested strong acid at different concentrations (1%, 2%, 3% up to 5%). In fact, in this last concentration of HCl the result on the determination of the polyphenols has stabilized. We decided to use H2O: HCl the 5:95 ratio. Then, the samples were heated at 60°C (water bath) for 1 h, allowed to cool to room temperature, and finally homogenized. An amount of 200 µL (three replicates) was introduced into screw cap test tubes. Then, 1.0 mL of Folin-Ciocalteu reagent and, after 3 min, 1.0 mL of Na_2CO_3 (7.5%) were added. The tubes were vortexed and heated at 40°C (water bath) for 30 min. The calibration curve was determined with seven standards with concentrations ranging from 50 to 1200 µg/mL. The absorption at 726 nm was measured (Perkin-Elmer Lambda 40 UV/VIS spectrophotometer) and the total phenol content expressed as mg of chlorogenic acid equivalents (CAE) per g of fresh material (FW).

Determination of total flavonoids

The total flavonoid content of crude extract was determined on the same extracts used for total phenols determination by the $AlCl_3$ colorimetric method.[26] In brief, 1 mL of EtOH was added to 2 mg of crude extract. After 5 min of incubation, 1 mL di 2% $AlCl_3$ aqueos solution was added and the mixture was allowed to stand for 15 min. The calibration curve was determined with eight standard concentrations, ranging from 25 to 900 µg/mL. The absorbance was measured at 430 nm. Total flavonoid content was expressed as mg quercetin equivalent (QE) per g of fresh material (FW).

Antioxidant properties

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was adapted from Marrelli and others.[25] In an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (final concentration = 1.0×10^{-4} M), extracts at different concentrations were added. The reaction mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance of the resulting solutions was measured at 517 nm, against a blank with DPPH. Decreasing absorbance values of the DPPH solutions indicated an increase of DPPH radical scavenging activity. The DPPH solution without sample solution was used as control. Ascorbic acid was used as positive control.

This activity was given as % DPPH radical scavenging, calculated by the following equation:

$$\%\ \text{DPPH radical scavenging} = (\text{absorbance control} - \text{absorbance sample})/\text{absorbance control} \times 100.$$

For the realization of the β-carotene bleaching method (BCB) method,[25] 2 mL of a β-carotene 0.5 mg/mL solution in chloroform are added to 0.04 mL of linoleic acid and 0.4 mL of Tween 20. The mixture was then evaporated at 40ºC for 10 min through a rotary evaporator, in order to remove the chloroform, and immediately diluted with 150 mL of distilled water. Water was slowly added to the mixture and vigorously stirred to form an emulsion. Them, 5 mL of the emulsion were added to 0.2 mL of samples at different concentrations (100, 50, 25, 10, 5, 1 mg/mL). The tubes were stirred slowly and kept at 45°C in a water bath. The absorbance was read at a wavelength of 470 nm at an initial time (t=0) and subsequently at 30 min. The antioxidant activity (AA) was measured using the following equation.

$$AA = [1 - (A_0 - A_t) / (A_0^{\circ} - A_t^{\circ})] \times 100$$

where A_0 and A°_0 are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and A°_t are the absorbance values measure in the samples/standard and control at t=30 min, respectively.

Determination of pancreatic lipase activity

A water solution (3 mg/mL) was prepared from type II crude porcine pancreatic.[27] Then a 7.5 mmol/L solution of 4-nitrophenyl octanoate (NPO) in dimethyl sulfoxide was prepared. The composition of the reaction mixture was the following: 100 µL of 7.5 mmol/L NPO, 4 mL of Tris-HCl buffer (pH = 8.5), 100 µL of extract (concentration 430, 215, 107.5, 57.33, 23.60, 12.5, 5, 2.5, 1, 0.5, 0.25, 0.065 mg/mL) and 100 µL of enzyme solution. The mixture was incubated at 37°C. In the control, the extract was replaced with the same volume of dimethyl sulfoxide (DMSO). The absorbance was measured at 412 nm. A blank sample without the enzyme was prepared for each extract. Orlistat was used for comparison.

Determination of pancreatic α-amylase activity

The inhibition of the enzyme α-amylase was evaluated using a modified version of the method of Kwon and others.[28] An amount of 100 µL of a solution of the sample (concentration 430, 215, 57.33, 12.5, 5, 2, 1.36, 0.32, 0.16, 0.08 mg/mL) was added to 500 µL of 0.5 mg/mL enzyme solution in cold distilled water and to 500 L of 1% (w/v) starch solution in 0.01 M phosphate buffer at pH 7.0. The reaction mixture was incubated at 37 °C for 5 min; the reaction was stopped after the addition of 1 mL of the reagent dye DNS (3,5-dinitrosalicylic acid and 1% potassium sodium tartrate in 2% NaOH 0.4 M). The reaction mixture was incubated at 100°C for 5 min and the absorbance measured at 540 nm.

Cell culture and MTT assay

Human breast adenocarcinoma (MCF-7) cell line were obtained from the non-profit association American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) (Gibco BRL, San Francisco, CA, USA) at 37°C in a humid atmosphere containing 5% CO_2. Raw (RB) or boiled (BB) bulb extracts were dissolved in DMSO and further diluted in DMEM without FBS to obtain the desired final concentrations (1, 10, 50, 100, 200, 400 and 800 µg/mL). Appropriate controls (vehicle) containing 0.5% DMSO were performed. MCF-7 cells were seeded in 48-well plates at a density of 20×10^3 cells $well^{-1}$ and cultured in complete medium overnight. Before treatment, culture medium was switched into DMEM F-12 supplemented with 1% FBS and cells were untreated (vehicle) or treated with different concentrations of raw or cooked bulb extracts for 72 h. DMSO was used as vehicle control. Cell viability was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA). The optical density was measured spectrophotometrically at 570 nm.

Statistical analysis

Data were analyzed using SPSS r.11.0.0 statistical software (SPSS, Inc., Chicago, IL, USA). All measurements were carried out in replicates (n=3). Significant differences were calculated at P≤0.05 level among means by one-way ANOVA, using Tukey's test.

The values of IC_{50} (half maximal inhibitory concentration) for each measured parameter was calculated by means of scatter charts (where the X-axis indicates the concentration and the Y axis is the % activity or % inhibition). Trend lines were plotted and IC_{50} calculated by a linear trendline (Y = a X + b) by the formula IC_{50} = (0.5 – b) / a.

Results and Discussion

Total phenol and flavonoid contents

Polyphenols are a class of naturally-occurring phytochemicals that have been shown to modulate physiological and molecular pathways that are involved in energy metabolism, adiposity, and obesity.[29] These compounds have redox properties, which allow them to act as antioxidants.[11,17,18] As their ROS-scavenging ability is facilitated by their hydroxyl groups, the total phenol concentration is used as a basis for a screening of antioxidant activity. Our results show that the total phenol content in bulb extracts was 92.47±0.020, 49.80±0.012, and 39.53±0.027 mg chlorogenic acid equivalents (CAE) *per* g FW in RB, SB and BB, respectively (Table 1). The total polyphenol content of *M. comosum* is particularly high enough, compared to other plant species, to justify its important antioxidant activity observed.[24] From a nutritional point of view, it is interesting to observe how the polyphenols content decreased significantly after a normal cooking operation. In fact, in the cooked bulbs, the total polyphenols content was significantly reduced (–46 and –47% in SB and BB, compared to RB).

Among phenols, flavonoids, including flavones, flavanols and condensed tannins, are important plant secondary metabolites, the antioxidant activity of which depends on the presence of free hydroxyl groups, especially those at the C3 position. In our experiment, the values of total flavonoid content were 4.57±0.003, 1.63±0.010, and 0.635±0.026 mg quercetin equivalents (QE) *per* g in RB, SB and BB, respectively (Table 1). Total flavonoids appeared to be about 5% of the total polyphenols in RB and their value and this proportion significantly decreased after cooking (3 and 2% of total polyphenols in SB and RB, respectively).

Antioxidant activity

Plants are rich in secondary metabolites, including phenols, flavonoids and carotenoids, which have antioxidant activity due to their redox properties and chemical structures. Particularly, *Muscari* spp. has been demonstrated to have a strong antioxidant activity and a high phenol content.[24] It was also demonstrated that chemical constituents of *Muscari* spp. are homoisoflavonoids, that confer a strong antioxidant capacity and have antimutagenic properties.[30]

The effect of antioxidants on the scavenging activity of DPPH radical is due to their hydrogen-donating ability, with the reduction of the stable free radical DPPH to the yellow-colored 1,1-diphenyl-2-picrylhydrazyl free radical. This method offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. The highest free radical scavenging activity (DPPH) was exerted by RB, with an IC_{50}=1.34±0.19 mg/mL (r^2=0.992) followed by SB (IC_{50}=3.5880±0.09 mg/mL; r^2=0.982) and BB (IC_{50}=9.63±0.04 mg/mL; r^2=0.978). Moreover, a significant correlation was observed between phenol content and the scavenging of DPPH radical in raw bulbs (r^2=0.991, P<0.5), indicating that the radical scavenging capacity of the extracts could be related to the concentration of phenol compounds.

The results on lipid peroxidation inhibitory activity of the bulbs of *M. comosum*, assessed by the *β*-carotene bleaching (BCB) test are shown in Table 2. This method is based on the loss of the yellow color of *β*-carotene due to its reaction with radicals which are produced by linoleic acid oxidation (inhibition of lipid peroxidation), the addition to the reaction mixture (*β*-carotene + linoleic acid) of an antioxidant inhibits the oxidation of *β*-carotene. The results obtained from BCB assay are similar to the data obtained from DPPH test, indeed raw bulbs presented a higher antioxidant activity (IC_{50}=9.13±1.31 mg/mL; r^2=0.917) compared to the cooked ones (IC_{50}=17.37±0.91 and 14.81±1.14 mg/mL in SB and BB, with r^2=0.985 and 0.980, respectively).

Inhibition of lipase and α-amylase activities

The management of obesity and prevention of diseases related to diet is nowadays, in the clinical, an ordinary activity required for containing public spending and reducing the consumption of drugs paid by the national health systems. Increasing the

Table 1. Total polyphenol and flavonoid content and antioxidant activity evaluated by ascorbic acid method of extracts of raw and cooked lampascioni (*Muscari comosum*).

Treatment	Total polyphenols (mg CAE/g FW)	Total flavonoids (mg QE/g FW)	DPPH (IC_{50})[a] (mg/mL)
Raw bulbs	92.47±0.020[a]	4.57±0.003[a]	1.34±0.19[c]
Steamed bulbs	49.80±0.012[b]	1.63±0.010[b]	3.59±0.09[b]
Boiled bulbs	39.53±0.027[c]	0.64±0.026[c]	9.63±0.04[a]

Total phenolics are expressed as chlorogenic acid equivalents (CAE) per g of fresh material (FW). Total flavonoids are expressed as quercetin equivalents (QE) per g of fresh material (FW). Means (n=3) ±SD with different letters within the same column are significantly different at P<0.05. Positive reference DPPH: ascorbic acid.

Table 2. Antioxidant activity evaluated by β-carotene bleaching method of extracts of raw and cooked lampascioni (Muscari comosum).

Treatment	Concentration (mg/mL)	Inhibition (%)	IC_{50} (mg/mL)
Raw bulbs	25	100.00±0.01	9.13±1.31[b]
	10	65.41±0.08	
	5	45.59±0.71	
	1	17.06±1.14	
	0.5	7.21±0.06	
Steamed bulbs	25	83.26±0.72	17.37±0.91[a]
	10	41.05±0.02	
	5	13.73±1.52	
	1	0.22±0.01	
Boiled bulbs	25	71.12±1.20	14.81±1.14[a]
	10	34.02±0.93	
	5	11.27±0.06	
	0.5	0.08±0.01	

Means (n=3) ±SD with different letters within the same column are significantly different at P<0.05.

prevalence of obesity and type 2 diabetes mellitus and the negative clinical outcomes observed with the commercially available anti-diabetic and anti-lipolytic drugs have led to the investigation of new therapeutic approaches focused on controlling post-prandial lipid absorption and glucose levels. At this purpose, inhibition of lipid- and carbohydrate-hydrolyzing enzymes is so an emerging and useful tool.

Several natural products extract can provide a vast pool of pancreatic lipase inhibitors that can possibly be developed into clinical products. In our experiment, raw bulb extracts showed a significant inhibitory action on pancreatic lipase. The lipase inhibitory effects were indicated by the IC_{50} values of 0.28±0.07 (r^2=0.912), 2.14±0.01 (r^2=0.966), and 3.22±0.03 (r^2=0.989) mg/mL in RB, SB and BB, respectively (Table 3).

The inhibition of α-amylase by bulb extracts also showed significant differences among cooking methods, with IC_{50} values of 0.16±0.03 (r^2=0.939), 0.73±0.13 (r^2=0.984), and 0.69±0.02 (r^2=0.980) mg/mL in RB, SB and BB, respectively (Table 4). These findings highlighted the importance of *M. comosum* in a diet rich of carbohydrates, as bulb extracts could reduce glucose absorbance, which in turn affects blood sugar levels.

Inhibition of the growth of breast adenocarcinoma cells

The analysis of the effects of different concentrations of raw or cooked bulb extracts on breast adenocarcinoma MCF-7 cells growth by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay revealed that treatment with raw bulb extracts exerted a dose-dependent inhibition on MCF-7 cell proliferation compared to vehicle-treated cells (Figure 1). By contrast, cooked bulb extracts also reduced MCF-7 cells viability but only at higher concentration (Figure 1). These results clearly indicate that cooking practice clearly influence the anti-proliferative effect of *M. comosum*, lowering their efficacy. This consideration is also supported by the clear difference between the IC_{50} value of raw bulb extracts (10.27 µg/mL; 95% interval confidence: 9.214-11.25 µg/mL), respect to that of cooked extracts (669.3 µg/mL, 95% interval confidence: 517.6-865.6 µg/mL).

Conclusions

The antioxidant activity and the inhibitory activity of pancreatic lipase and α-amylase of the wild onion *lampascioni*

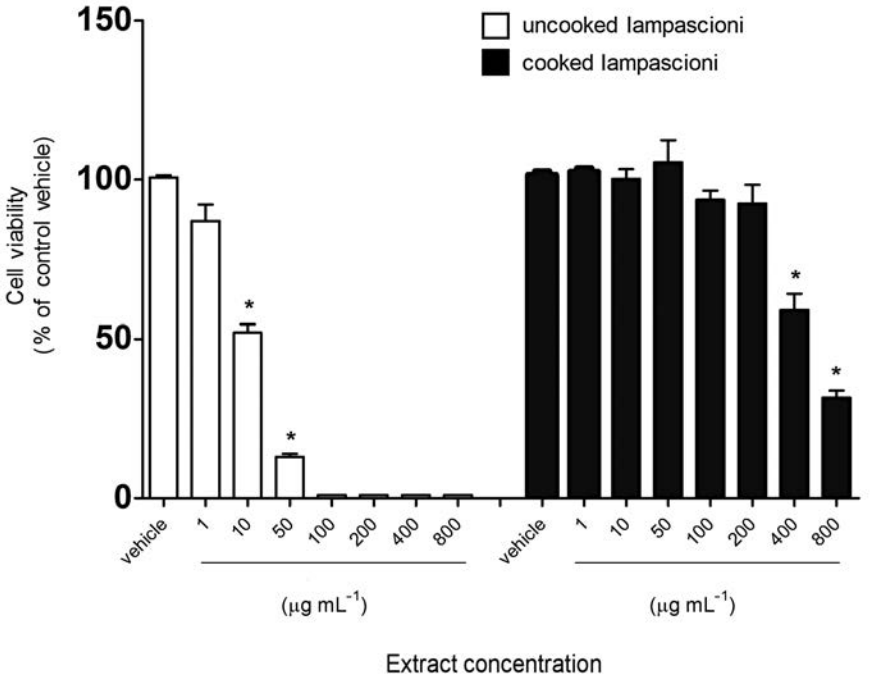

Figure 1. Cell viability of tumoral MCF-7 cells treated of extracts of raw and cooked (boiled) lampascioni (*Muscari comosum*). Asterisks indicate significantly difference at P<0.05, compared to the control (vehicle).

Table 3. Lipase inhibitory activity of extracts of raw and cooked lampascioni (Muscari comosum).

Treatment	Concentration (mg/mL)	Inhibition (%)	IC_{50} (mg/mL)
Raw bulbs	1	99.62±1.12	0.28±0.07[c]
	0.25	58.82±1.09	
	0.065	21.79±1.11	
Steamed bulbs	5	91.80±0.09	2.14±0.01[b]
	2.5	24.75±1.23	
	0.5	0.92±1.21	
Boiled bulbs	2.5	55.70±1.01	3.22±0.03[a]
	1	34.86±1.11	
	0.5	16.81±0.09	

Orlistat IC_{50} (positive control) = 0.082 mg/mL. Means (n=3) ±SD with different letters within the same column are significantly different at P<0.05.

Table 4. α-amylase inhibitory activity of extracts of raw and cooked lampascioni (*Muscari comosum*).

Treatment	Extract concentration (mg/mL)	Inhibition (%)	IC_{50} (mg/mL)
Raw bulbs	0.32	93.52±0.12	0.16±0.03b
	0.21	82.75±1.00	
	0.07	23.60±1.31	
Steamed bulbs	1.36	87.97±0.05	0.73±0.13a
	0.32	24.35±0.23	
	0.16	9.16±1.81	
Boiled bulbs	1.36	93.48±1.11	0.69±0.02a
	0.32	27.65±0.11	
	0.16	9.17±0.23	

Means (n=3) ±SD with different letters within the same column are significantly different at P<0.05.

(*M. comosum*) can increase antioxidant defenses, and at the same time reduce the absorption of fats and carbohydrates (Tables 1-4). These phytochemical components were closely related to the antitumor activity against breast adenocarcinoma cells. This study also demonstrated that the action of bioactive components of "lampascioni" extracts decreases as a function of the cooking mode, being both lower in the cooked bulbs. This means that traditional cooking can partially deplete food biological properties, compared to the raw product.

Prevention is a more effective strategy than treatment of chronic diseases. This wild dietary plant could be an excellent source of compound that can regulate the absorption of fats and sugars, so being useful for the control of obesity and some of the risk factors of the cardio-metabolic syndrome associated with obesity. The use of raw *M. comosum*, presenting higher biological activities than the cooked bulbs, is to be associated to its transformation into food supplement. Considering that the potential of natural products for the treatment of obesity are under exploration, *M. comosum* could be an excellent plant for the development of future effective, safe, anti-obesity drugs, also able to prevent cancer.

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