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Phytochemical analysis and effects on ingestive behaviour of a *Caralluma fimbriata* extract

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ABSTRACT

Caralluma fimbriata Wall. is currently used as a "natural slimming" food supplement, likely due to its content in pregnane glycosides. In the present study, a commercially available *Caralluma fimbriata* extract (Slimaluma®; CFE, 100 mg/kg) has been evaluated for its ability to affect the ingestive behaviour in female rats, also with reference to the modulation of the brain neuropeptides NPY and ORX.The interference of CFE with α-amylase and lipase enzymes has been investigated *in vitro*, as possible peripheral mechanism of action. Also, the chemical composition of CFE has been assessed by NMR and spectrophotometric analysis.

Results from *in vivo* study showed that CFE induced effects neither on blood parameters, nor on liver and gut histomorphology. Interestingly, a reduction in body weight gain with an increase in water intake and hypothalamic levels of NPY and ORX peptides were found. Phytochemical analysis, showed CFE contained about 12% of pregnane glycosides and 1.3% of polyphenols.

Present results suggest possible effects of *C. fimbriata* on ingestive behaviour, likely mediated by central and peripheral mechanisms.

1. Introduction

Obesity and overweight are growing public health problems and their management is becoming of great clinical importance (Karatsoreos et al., 2013). Preliminary approaches in the management of overweight patients are preventive measures (diet, eating habits, etc.) and lifestyle changes (e.g., physical activity, specific exercises). The pharmacological approaches intended to counteract overweight include two main classes of drugs: the appetite suppressants (sibutramine, amphetamine derivatives, etc.) and the inhibitors of nutrient absorption (i.e., orlistat). However, pharmacological treatments are often accompanied by serious adverse effects (Astell et al., 2013), so that it is not surprising that consumers commonly turn to herbal dietary supplements.

Among natural agents used as appetite suppressants, *Caralluma fimbriata* Wall. [syn. *Caralluma adscendens* var. *fimbriata* (Wall.) Gravely & Mayur. – Fam. Apocynaceae] is one of the less studied. It is an edible succulent cactus used for centuries by Western Indians as both a food and an appetite suppressant (Yuliana et al., 2011; Gooda Sahib et al., 2012). Nowadays it is used as a "natural" slimming food supplement, and a standardized extract of *C. fimbriata* (Slimaluma®) has been patented (Yuliana et al., 2011; Dutt et al., 2012). Pregnane glycosides seem to be responsible for the slimming properties of this plant (Kuriyan et al., 2007; Astell et al., 201). These compounds were also found in other medicinal plants such as *Hoodia gordonii*, that are reported to affect food intake (Gooda Sahib et al., 2012). Moreover, other phytochemicals, including flavonoids, saponins etc. have been found in *C. fimbriata* (Astell et al., 2013).

In spite of its traditional use, the scientific evidence on the *C. fimbriata* efficacy is poor, and the majority of the available

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literature should be examined critically since a high proportion of studies are sponsorized and/or funded just by the company involved in preparation and/or marketing of the *C. fimbriata* commercial extracts (Kuriyan et al., 2007; Odendaal et al., 2013; Lakshmi et al., 2014; Rajendran et al., 2014; Sudhakara et al., 2014).

Furthermore, taking into account the well-known problems of standardization of the herbal preparations marketed as food supplements (Wolsko et al., 2005; Garg et al., 2012), and considering that a poor quality can affect the safety and efficacy of herbal drugs (Chan, 2003), ascertaining the true composition of end products represents an important goal.

Based on the above considerations and without any conflict of interest, present study was aimed to characterize the chemical composition of a commercially available extract of *C. fimbriata* (CFE), by modern analytical techniques as suggested by EMA (EMEA/HMPC/253629/2007). Furthermore, in order to investigate the activity of this extract in feeding behaviour, we tested the effect of CFE on food and water intake in a non-obese rat model, after a sub-chronic treatment. Being pregnane glycosides recognized to have appetite-suppressant effects, probably via enhanced hypothalamic signaling (MacLean and Luo, 2004), and considering the key role of hypothalamus on the regulation of energy homeostasis (Williams et al., 2001), we also evaluated the effect of *C. fimbriata* on the hypothalamic expression of neuropetide Y (NPY) and orexin (ORX), two factors playing a crucial role on feeding attitude (Fick and Belsham, 2010). Finally, we assessed the capability of *C. fimbriata* extract to inhibit the α-amylase and lipase enzymes, as possible peripheral mechanisms of action.

2. Material and methods

2.1. Herbal extract

CFE (a dry ethanolic extract from the aerial parts of *C. fimbriata* named Slimaluma®, lot no. FAIT120503613) was purchased by the FAGRON Company (Bologna, Italy). It appears as a brown and water-soluble powder. According to the technical data sheet, the extract is reported to contain 27.5% of total pregnane glycosides. The quality assessment of the product, carried out by the Company, excluded the presence of germs (e.g., *Escherichia coli*, *Salmonella* spp, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), molds, fungi and pesticides, while the amount of unavoidable heavy metals (i.e., Pb, Cd, As, Hg) was lower than the limits allowed.

2.2. Chemicals

Tannic acid (CAS 1401-55-4; 99.9% purity), sodium carbonate (Na_2CO_3; CAS 497-19-8; 99.9% purity), Folin-Ciocalteu's phenol reagent, and aluminum chloride hexahydrate ($AlCl_3$ x 6 H_2O; CAS 7784-13-6; Ph. Eur. purity) were purchased from Merck (Darmstadt, Germany), while α-amylase, α-glucosidase, lipase, potato starch, 4-nitrophenyl a-D-glucopyranoside (PNG), *p*-nitrophenylpalmitate (PNP), polyvinylpyrrolidone (PVPP), 3,5-dinitrosalicylic acid (DNSA), acarbose, orlistat, quercetin, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP), ethanol (EtOH), deuterium dioxide (DO2), deuterated methanol (CD3OD), the polyclonal rabbit IgG antibodies against NPY and ORX, the biotinilated goat IgG and all other chemicals, included those for histomorphological study, were provided by Sigma-Aldrich (Milan, Italy). Deuterium oxide (D-99%; Cat. No. DLM4-100) was from Cambridge Isotope Laboratorie Inc., Andover, MA 01810 USA. Chemicals for ultrastructural study were: glutaraldehyde, uranyl acetate and lead citrate (SIC, Rome, Italy) osmium tetroxide (Agar Scientific, Stansted, UK), propylene oxide (BDH Italia, Milan, Italy) and epoxy resin (Electron Microscopy Sciences, Hatfield, PA, USA). Products for clinical chemistry, including GLUC3 4483/190, GGT2 2721/122, ASTLP 7493/190, ALTLP 7388/190, ALP2 3752/190, CHOL2 9773/190, HDLC3 9803/190, TRIGL 7107/322, AMYL2 3742/122, LIPC 9590/322, TP2 3734/190 and LDHI2 4732/122 were provided by Roche Diagnostics, CH. Insulin rat and streptavidin peroxidase conjugate were from Demeditec Diagnostics (Kiel, Germany) and GE Healthcare (UK), respectively.

2.3. Phytochemical analysis

Owing to the variability of commercial preparations, the first step of the present study was to chemically characterize the extract. At this aim, nuclear magnetic resonance (NMR) spectroscopy was employed to evaluate the total pregnane (Tomassini et al., 2014) content, while colorimetric assays were performed to determine the amount of polyphenol compounds.

2.3.1. NMR spectroscopy

10 mg of CFE extract were dissolved in 600 μl D_2O/CD_3OD mixture (2: 1 ratio, respectively) containing TSP at the final concentration of 2 mM, as internal standard for chemical shift. All the NMR experiments were performed on a Bruker Avance III spectrometer operating at a Larmor frequency of 400,13 MHz for ^1H and 100 MHz for ^{13}C. Signal assignments were achieved by standard homonuclear 1H−1H TOCSY and heteronuclear 1H−13C HSQC and HMBC bidimensional experiments. 1D (one-dimensional) ^1H NMR spectrum was acquired with a *presat* pulse sequence for solvent suppression, a spectral width of 15 ppm, 64 k data points, 5.5 s of acquisition time, 128 scans and a repetition delay of 9.5 s in order to achieve full relaxation for all protons. 2D (two-dimensional) $^1H-^1H$ TOCSY (Total Correlation Spectroscopy) spectrum was acquired with a data matrix of 8 k x 256 data points, a spectral width of 15 ppm in both dimensions, 80 scans and a mixing time of 110 ms. 2D $^1H-^{13}C$ HSQC (Heteronuclear Single Quantum Correlation) spectrum was acquired with a data matrix of 8 k x 256 data points for hydrogen and carbon respectively, a spectral width of 15 ppm for hydrogen dimension and 200 for the carbon one, 128 scans and an average coupling constant of 145 Hz. Spectra processing was performed using Bruker software TOPSPIN for 2D experiments and ACD 12.0 for 1D ones.

2.3.2. Colorimetric determination of total polyphenols, tannins and flavonoids

Total polyphenols were determined according to Rababah et al. (2005), with minor changes. Each sample (20 μl of a solution 1 mg/ml of *C. fimbriata* extract in EtOH 50% v/v) was mixed with 100 μl of the Folin-Ciocalteau reagent (10% v/v) and incubated for 5 min. After addition of 80 μl of a sodium carbonate solution (7.5% w/v), the mixture was shaken and incubated for 2 h, then the absorbance was measured at 765 nm by a microplate reader (Bio-Rad, Hercules, CA, USA). The content of total polyphenols was expressed as tannic acid equivalents (TAE) per milligram of sample.

To determine the amount of tannins, 100 mg PVPP were added to 1 ml of an extract solution (1 mg/ml in EtOH 50% v/v). PVPP induces the formation of an insoluble complex with tannins, which precipitate. 20 μl of supernatant were used to determine the polyphenol content, as described above, while the tannin amount was obtained by the difference between total polyphenols and the polyphenols contained in the supernatant, and was expressed as TAE per milligram of sample.

Flavonoids were measured by the spectrophotometric method described by Meda et al. (2005) with minor changes. Briefly, the extract (50 μl of a solution 1 mg/ml), aluminum trichloride (20 μl of a solution 10% w/v in methanol), $NaNO_2$ (10 μl of a solution 5% w/v in deionized water) and NaOH (60 μl of a solution 1 M) were added

to 70 μl of deionized water, then were mixed and incubated for 10 min. The absorbance was measured at 510 nm by a microplate reader and the total flavonoids were determined and expressed as quercetin equivalents (QE) per milligram of sample. Each assay was carried out twice and each sample was tested in triplicate. The results were expressed as mean ± SEM.

2.4. Animal treatment protocol

The protocol used in this study was approved by the Italian Ministry of Health. All experiments were performed in accordance with the International Animal Welfare Legislation (Directive 2010/63/EU, 2010).

The study has been carried out on 18 Sprague Dawley female rats (Charles River, Lecco, Italy) randomly assigned to two experimental groups: control group (drinking water) and treated group (100 mg/kg of CFE in drinking water); the treatment lasted 58 days. Female rats were chosen as women are the major users of the slimming products (Vitalone et al., 2011). The tested dose was chosen on the basis of previous published data obtained with a *C. fimbriata* extract (Kamalakkannan et al., 2010). During the 58-day treatment period, rat body weight, water consumption and food intake were recorded 3 times a week. At the end of the treatment, the animals were sacrificed in accordance with the approved guidelines and regulations. Biological samples were collected for biochemical, histological and immunohistochemical analysis. Particular attention has been devoted to the liver examination, considering that early studies on pregnane glycosides of *Hoodia gordonii* were discontinued due to liver toxicity (Dara et al., 2008).

2.5. Clinical chemistry

The blood samples were obtained by intra-cardiac puncture. The clotted blood was centrifuged at 3000 rpm for 20 min in a refrigerated centrifuge (4 °C). Serum was separated from plasma, and both were used to perform biochemical-clinical analysis, by using automated methods (COBAS 6000 system, Roche Diagnostics, CH), as previously published by our group (Mazzanti et al., 2009).

Particularly, the following parameters were measured: insulin, glycemia, total cholesterol, high-density lipoprotein (HDL), total triglycerides, total proteins, amylase, lipase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatise (ALP) and lactate dehydrogenase (LDH).

Insulin assay was carried out by ELISA with monoclonal antibodies against rat hormone.

The homeostasis model of assessment insulin resistance (HOMA-IR) index was calculated using the following international formula: [fasting serum glucose (mmol/l) x fasting serum insulin (mU/l)/22.5].

2.6. Histomorphological and ultrastructural analysis of gut and liver

Gut fragments were obtained from duodenum (immediately after the pylorus sphincter), jejunum (30 cm beyond the pylorus sphincter), ileum (immediately before the ileocecal valve), and colon (5 cm above the anus), and were immediately fixed in 10% buffered formalin, at room temperature for at most 48 h. Liver samples were obtained from the right lobe and fixed in buffered formalin for 24 h. The samples of intestinal segments were dehydrated with alcohol, cleared in xylene and embedded in paraffin wax (melting point 55–57 °C). Afterwards, the tissues were serially sectioned using a rotary microtome Pabisch Top Automat S-140, obtaining 3 μm thick sections that were stained with haematoxylin-eosin and Masson's trichromic. Histomorphological observations were carried out using a light microscope (Leica DM 4500B), connected to a Videocam (ProgRes C10 plus) and provided with an Image Analysis System, Delta Sistemi (Rome, Italy).

To perform the ultrastructural examination, the specimens were fixed by immersion in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer for not less than one week; then they were post-fixed in a buffered solution of osmium tetroxide 1,33% for 2 h, dehydrated in increasing concentrations of ethyl alcohol and then embedded in Epon 812. After resin polymerization in an oven at 60 °C for 48 h, semi-thin (about 1 μm thick) and thin (70–90 nm) sections were obtained using an EMUC 6 Leica ultramicrotome with glass and diamond knives. Semi-thin sections were mounted on glass slides and stained with toluidine blue. The observations were carried out with an optical microscope Zeiss Axioskop 40 using MRGrab 1.0 as image processing software. Ultrathin sections collected on 400 mesh copper nets, counterstained with lead citrate and uranyl acetate (Reynolds, 1963) were studied and photographed with a Zeiss EM 10 transmission electron microscope, equipped with a digital camera model 782 ES500W Erlangshen CCD (Gatan DigitalMicrograph, CA) for image acquisition.

2.7. Immunohistochemistry of rat brain and optical density analysis

Brain specimens were removed, rapidly transferred to frozen isopentane (- 40 °C) for 20 s and then stored at - 80 °C. The NPY immunoreactivity experiments were conducted on 15 μm cryostat rat brain sections using a conventional streptavidin-biotin technique. The coronal sections (- 2.8 bregma) were fixed in 4% of paraformaldehyde/0.1 M phosphate buffer for 15 min. Sections were stained using a polyclonal rabbit IgG antibody raised against NPY and against ORX at a dilution of 1: 400 and 1: 500 respectively, which was determined in preliminary experiments.

Briefly, the sections were incubated for 30 min firstly with 3% hydrogen peroxide, then with 10% goat serum, to block non-specific protein binding. Negative control sections were treated with non-immunized goat immunoglobulin serum under the same conditions. Following careful washing with PBS, biotinylated goat IgG (dilution 1: 1000) was applied for 2 h, followed by streptavidin peroxidase conjugate for 90 min. The color reaction was developed with a solution of 0.02% diaminobenzidine in 0.003% hydrogen peroxide for 15 min. The sections were mounted onto gelatin-chrome alum-coated glass slides and examined through a white light microscope (Leitz, Germany). The immunohistochemistry procedures were simultaneously carried out on a number of brain sections (at least 4 sections per animal) from each experimental group.

The positive NPY/ORX staining was evaluated as optical density (O.D.) values, according to the methods described by Nativio et al. (2012). The brain areas of interest (i.e. hypothalamus) were considered according to the stereotaxic coordinates of the rat brain atlas (Pellegrino et al., 1979). Standard outline digital images were captured using a SPOT-RT CCD video camera (SPOT-RT Image Software V 3.0 SPOT Diagnostic Instruments Inc., Sterling Height, Michigan) for each region of interest. A semiquantitative analysis of the positive immunoreactivity was performed on brain multiple sections/region. The final optical density values (O.D.) were calculated as a function of the specific signal relative to the external background measure (taken from outside the section). Constant optical conditions were maintained throughout the morphometric and densitometric evaluation.

2.8. In vitro metabolic enzyme inhibition

The ability of CFE to inhibit *in vitro* the α-amylase and lipase

enzymes was measured by spectrophotometric assays. For determining α-amylase activity, the DNSA method was applied, according to Oboh et al. (2012), with minor changes. To perform the assay, serial dilutions of the sample (250 μl) were pre-incubated with α-amylase (0.5 mg/ml in phosphate buffer solution, corresponding to 25 U/ml; 250 μl) for 10 min at 37 °C. Then, after addition of the potato starch solution (0.5% w/v in acetate buffer 0.1 M, pH = 4.5; 250 μl), the mixture was incubated for 10 min at 37 °C. The reaction was stopped by adding 500 μl of DNSA reagent (25 ml of 96 mM DNSA solution in water, 8 ml of 5.3 M sodium potassium tartrate solution in 2 M sodium hydroxide and 12 ml of water). The mixtures were then incubated in a water bath at 100 °C for 5 min, cooled to room temperature, thus plated in 96-multiwell microplates. For each treatment, the presence of reducing sugars was determined by the turning of the solution to red and the DNS absorbance was measured at 540 nm by a microplate reader (Epoch Microplate Spectrophotometer, BioTeK).

Lipase activity was assayed by measuring the enzymatic hydrolysis of *p*-nitrophenyl palmitate to *p*-nitrophenol, according to Costamagna et al. (2016), with minor changes. Serial dilutions of the samples (16 μl) were mixed with a lipase solution in water (5.0 mg/ml; 12 μl) and TRIS HCl (75 mM, pH 8.5; 162 μl). The mixture was supplemented with PNP (10 mM; 25 μl) and pre-incubated on ice for 5 min. For each treatment, the *p*-nitrophenol absorbance was determined at 405 nm by a microplate reader (Epoch Microplate Spectrophotometer, BioTeK).

Acarbose (250 μg/ml) and orlistat (25 μg/ml) were included in all the experiments as standard inhibitors (100% enzyme inhibition) for α-amylase and lipase, respectively, while the vehicle represented the maximum enzyme activity. Additional treatments, in which enzyme solution was replaced by buffer solution, were included in order to evaluate the interfering absorbance produced by the extract. The experiments were performed at least in triplicate and in each experiment about six replicates were prepared. Data obtained from at least two experiments were pooled in the statistical analysis. The inhibitory activity was calculated as percentage of inhibition with respect to the vehicle control.

2.9. Statistical analysis

Data are reported as mean ± SEM. The Student's t-test was used to compare paired data, while the one-way analysis of variance (ANOVA) followed by the Bonferroni Post-Test was applied to analyze differences from three or more experimental groups. The level of statistical significance was $p < 0.05$.

3. Results

3.1. Phytochemical analysis

The resonance assignment of ^{1}H NMR CFE spectra (Fig. 1) was performed by the analysis of 2D NMR experiments (TOCSY, HSQC and HHMBC; Supplementary material, Figs. 1S–5S) and by comparing the observed chemical shifts with ones from literature data (Abe and Yamauchi, 2000). From the analysis of NMR spectra, several molecule classes, including aminoacids (leucine, isoleucine, alanine, glutamine and tryptophan); organic acids (lactate, acetate and formate), carbohydrates (glucose and sucrose), trigonelline and pregnanes, were identified (Table 1).

Pregnanes were univocally identified on the basis of their diagnostic TOCSY correlations among the sterol moiety protons (8 resonances between 0.9 and 1.9 ppm as well as the hydrogens in position 3 at 3.52 ppm, 6 at 5.34 ppm and 12 at 4.06 ppm). They represent the main components of the hydro-alcoholic extract, as estimated on the basis of integral of the CH_3 protons at 21 position (resonance at 2,30 ppm; Table 1) compared to TSP and their amount was about 120 mg/g expressed as pregnane equivalents. The pregnanes differ from each other for the glycoside moiety in

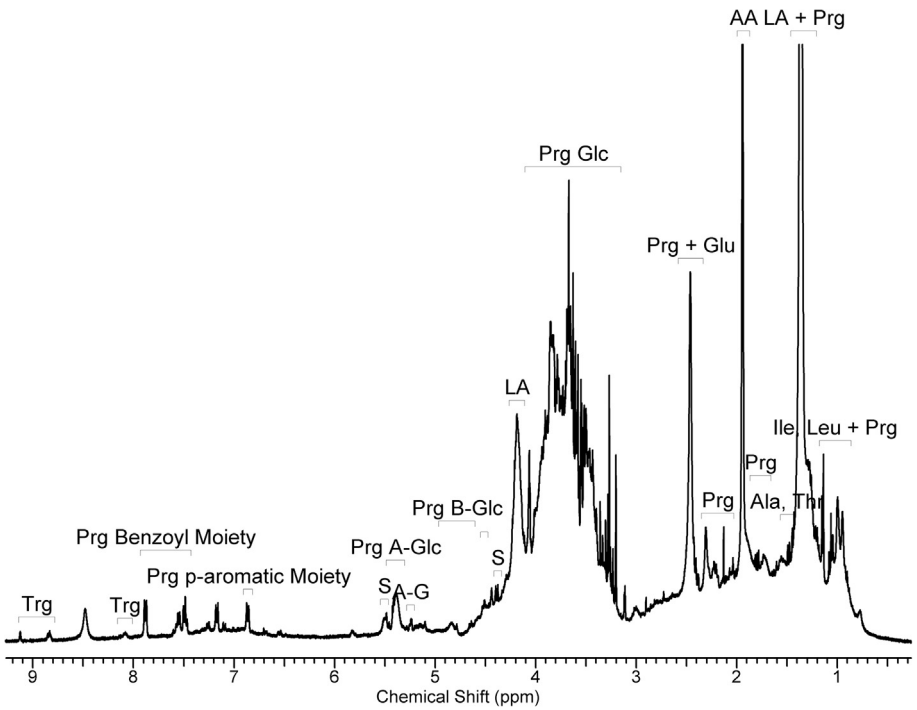

Fig. 1. 1D ^{1}H NMR spectrum of the *Caralluma fimbriata* hydroalcoholic extract. Key: Ile, isoleucine; Leu, leucine; Thr, threonine; Ala, alanine; Glu, glutamate; LA, lactic acid; AA, acetic acid; A-G, alpha-glucose; B-G, beta-glucose; S, sucrose; Trg, trigonelline; Prg, pregnane; Prg-Glc, pregnane glycoside; Prg A-Glc, pregnane alpha-glycoside; Prg B-Glc, pregnane beta-glycoside; Prg benzoyl moiety; Prg p-aromatic moiety.

Table 1
Metabolites identified in ^{1}H-NMR spectra of the *Caralluma fimbriata* extract.

Compound[a]	Assignment	^{1}H δ (ppm)	Multiplicity[b]	^{13}C δ (ppm)
Organic acids				
Acetic acid (AA)	CH_3	1.92	s	26.31
Lactic acid (LA)	CH_3	1.36	bs	23.39
	CH	4.16	m	72.22
Formic acid (FA)	CH	8.46	s	171.90
Amino acids				
Alanine (Ala)	β-CH_3	1.48	d	19.05
	α-CH	3.75	q	53.56
Glutamate (Glu)	γ-CH_2	2.05	m	29.31
	β,β′-CH_2	2.40	m	36.02
	α-CH	4.15	m	57.19
Isoleucine (Ile)	δ-CH_3	1.05	t	13.85
	γ-CH_3	1.12	d	17.38
	γ′-CH	1.25	m	27.01
	γ″-CH	1.49	m	27.01
	β-CH	1.99	m	38.71
	α-CH	3.69	m	63.04
Leucine (Leu)	δ,δ′-CH_3	0.89	m	23.85, 24.59
	γ-CH	1.72	m	26.81
	β-CH_2	1.73	m	42.60
	α-CH	3.74	m	56.21
Threonine (Thr)	γ-CH_3	1.33	d	22.15
	α-CH	3.60	m	63.46
	β-CH	4.27	m	68.94
Carbohydrates				
α-Glucose (α-G)	CH-1	5.22	d	93.10
	CH-2	3.55	m	72.49
	CH-3	3.72	m	73.84
	CH-4	3.42	m	70.67
	CH-5	3.84	m	72.52
	CH_2-6	3.73, 3.90	m	96.97
β-Glucose (β-G)	CH-1	4.65	d	96.97
	CH-2	3.26	dd	75.17
	CH-3	3.50	m	76.84
	CH-4	3.42	m	70.70
	CH-5	3.48	m	74.57
	CH_2-6	3.74, 3.91	m	61.80
Sucrose (S)	GLC CH-1	5.49	d	93.22
	CH-2	3.59	m	72.11
	CH-3	3.79	m	73.54
	CH-4	3.48	m	70.26
	CH-5	3.85	m	73.38
	CH_2-6	3.82	m	61.18
	FRU CH_2-1′	3.69	m	62.44
	CH-3′	4.22	m	77.45
	CH-4′	4.06	m	75.04
	CH-5′	3.90	m	82.44
	CH_2-6	3.82	m	63.38
Miscellaneous Metabolites				
Pregnane (Prg)	Aglycone moiety			
	CH_2-1	0.93, 1.58	m	37.19
	CH_2-2	1.51, 1.84	m	31.50
	CHOH-3	3.52	m	71.81
	CH_2-4	2.28	m	42.37
	CH-6	5.34	m	121.79
	CH_2-7	1.52, 2.28	m	31.98
	CH_2-12	4.06	m	77.02
	CH_2-15	1.57	m	24.25
	CH_2-16	1.26, 1.85	m	28.37
	CH_3-18	2.01	s	16.1
	CH_3-19	1.30	s	18.2
	CH_3-21	2.30	s	32.1
	Glycoside moieties			
	α−CH-1	5.35	bs	103.71
	β−CH-1	4.53	bs	106.32
	CH-2−CH-5	3.28−4.00	m	64−70
	$-CH_3$	1.28	bs	19.49
	Aromatic moieties			
	Benzyl moiety	7.48−7.87	m	130−135
	p-aromatic moiety CH-2,6	7.18	d	133.0
	p-aromatic moiety CH-3,5	6.85	d	119.0
Trigonelline (Trg)	CH-2	9.12	s	148.39
	CH-4,6	8.82	d	148.02
	CH-5	8.02	dd	132.77
	N-CH_3	4.43	s	51.06

Chemical shift are determined setting ^{1}H and ^{13}C δ of TSP to 0.00 ppm.
[a] Identified metabolites.
[b] s: singlet; d: doublet; dd: double doublet; t: triplet; q: quartet; m: multiplet; bs: broad signal.

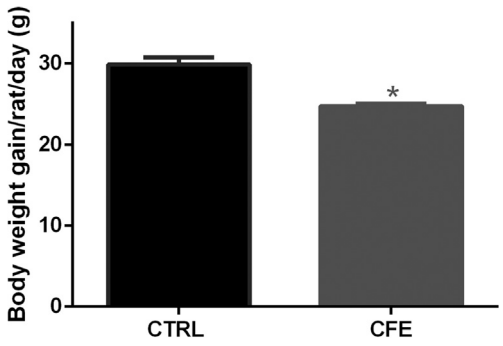

Fig. 2. Effect of the *Caralluma fimbriata* extract (CFE; 100 mg/kg/die) on body weight gain with respect to the vehicle group (CTRL). Data represent the mean ± SEM (n = 9 replicates). *p < 0.05 vs. vehicle.

position 3 and for the substitution on the OH in position 12. On average, there are 2 units of carbohydrate for each steroidal ring. Most (76%) pregnanes are substituted in position 12 with acetyl groups, while the remaining part with aromatic groups. Of the latter, about half are para-di-substituted with phenolic groups, while the remaining part contains benzyl groups.

The extract here analyzed by colorimetric assay showed to contain 13.25 ± 0.54 μg/mg (1.32% w/w) of total polyphenols, expressed as tannic acid equivalent, of them 7.22 ± 0.63 μg/mg (0.72% w/w) were constituted by tannins. The flavonoid content, expressed as quercetin equivalents, was 1.67 ± 0.54 μg/mg, corresponding to 0.17% of dry ethanolic extract.

3.2. Effects of CFE on ingestive behaviour

In our experimental conditions, CFE induced a slight reduction of body weight during treatment (Supplementary material, Fig. 6S), which was confirmed by a significant decrease of body weight gain (24.7 ± 0.3 g/day vs 29.9 ± 0.9 g/day of the vehicle group; $p < 0.05$) (Fig. 2). As regards the ingestive parameters, food intake was slightly increased (18.4 ± 0.1 g/rat/day vs 18.0 ± 0.1 of the vehicle group; $p < 0.05$) and water consumption was significantly increased (29.2 ± 0.5 g/rat/day vs 37.0 ± 0.3 g/rat/day of the vehicle group; $p < 0.001$) all over the 58-days experiments (Fig. 3A and B).

CFE treatment did not affect the blood parameters measured (glucose, AST, ALT, GGT, ALP, LDH, HDL, total cholesterol, triglycerides, and amylase), except for an increase of blood insulin and HOMA IR, and a slight decrease of lipase and total protein levels with respect to the vehicle group (Table 2).

Necroscopic examination did not highlight significant macroscopic differences in treated rats in comparison to controls, particularly as regards the abdominal cavity and the gut surface. No adherences to the large omentum, or ulcerated lesions were observed in the examined samples.

Table 2
Hematic parameters (mean ± SE) of Sprague Dawley female rats treated with the *Caralluma fimbriata* extract (CFE) (100 mg/kg/day) for 58 days. Extract was administered in drinking water; controls received only drinking water.

Hematic parameters	Units	Animal treatment	
		Control	CFE
Insulin	mU/l	4.94 ± 0.41	7.01 ± 1.18*
Glycemia	mmol/l	6.45 ± 0.15	6.69 ± 0.19
HOMA-IR		1.40 ± 0.10	2.12 ± 0.38*
AST	U/l	196.29 ± 21.17	151.89 ± 12.47
ALT	U/l	44.63 ± 2.34	39.22 ± 2.22
GGT	U/l	0.43 ± 0.13	0.19 ± 0.06
ALP	U/l	29.22 ± 2.54	35.67 ± 2.93
LDH	U/l	645.80 ± 68.92	713.38 ± 49.02
Total Cholesterol	mmol/l	2.34 ± 0.15	2.23 ± 0.17
HDL	mmol/l	0.71 ± 0.03	0.88 ± 0.08
Total Triglycerides	mmol/l	1.46 ± 0.27	1.52 ± 0.32
Amilase	U/l	1465.36 ± 107.90	1358.86 ± 39.29
Lipase	U/l	6.13 ± 0.13	5.67 ± 0.17*
Total Proteins	g/dl	7.38 ± 0.30	6.48 ± 0.10*

* $p < 0.05$ vs control (Student's t-test).
HOMA-IR, Homeostasis model of assessment of insulin resistance index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; HDL, High-density lipoprotein.

The histological examination of the intestine fragments from CFE-treated rats showed that the structure of intestinal mucosa did not differ from the controls. In particular, no histological alterations, including inflammatory infiltrate or neutrophilic exudate in the submucosa and in the muscle layer, were found in both experimental groups. Liver weight was similar in both control and CFE-treated animal groups and the light microscopy examination showed a normal hepatic structure (i.e. liver parenchyma with regular organization of hepatic cords and lack of vascular congestion) in all the analyzed specimens (data not shown). No evidence of hepatocellular necrotic areas, inflammatory cells infiltration in periportal or pericentral areas, hepatocellular swelling or cytoplasmatic vacuolization were found. The ultrastructural features of liver and gut samples confirmed the lack of CFE effects on liver parenchyma.

Analogously, a normal microarchitecture was found for the small intestine and the typical columnar epithelium, as well as classical tubular mucous glands and crypts, without signs of any inflammation within the intestinal wall, was highlighted for the large intestine (Figs. 4 and 5).

The immunohistochemical analysis of the brain samples showed, for CFE-treated animals, an increased expression (O.D. analysis) of NPY and ORX peptides, both in the hypothalamus

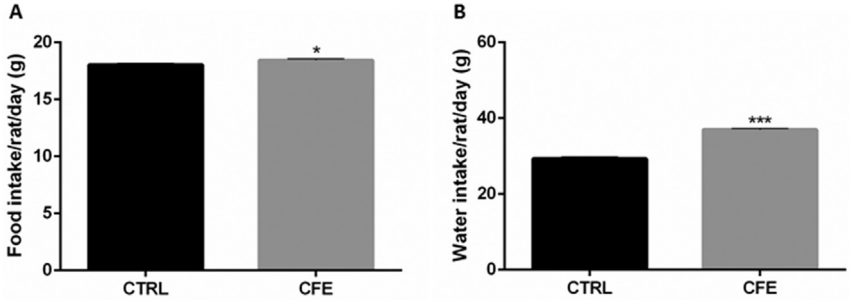

Fig. 3. Effect of the *Caralluma fimbriata* extract (CFE; 100 mg/kg/die) on food intake (A) and water consumption (B) with respect to the vehicle group (CTRL). Data represent the mean ± SEM (n = 9 replicates). * and ***p < 0.05 and p < 0.001 respectively vs. vehicle.

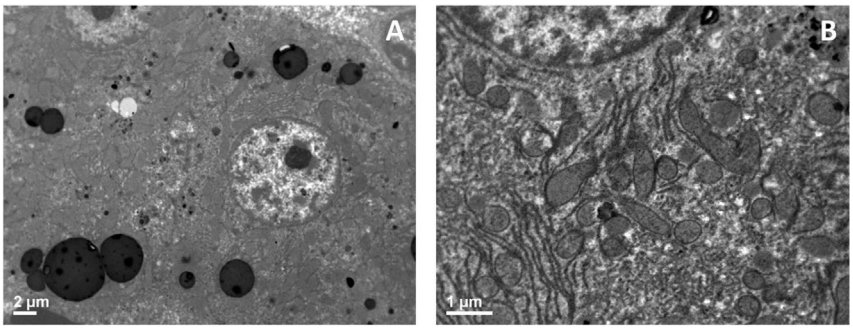

Fig. 4. Samples of liver from rats treated with the *Caralluma fimbriata* extract (CFE; 100 mg/kg/die) observed by Transmission Electron Microscopy (TEM). A) Low magnification of the hepatocyte. The image shows the nucleus with a prominent nucleolus; some electron dense lipid inclusions can be observed in the cytoplasm. B) High magnification of the hepatocyte. Part of the nucleus is observed. The cytoplasm shows numerous and somewhat dilated mitochondria and cisternae of the rough endoplasmic reticulum.

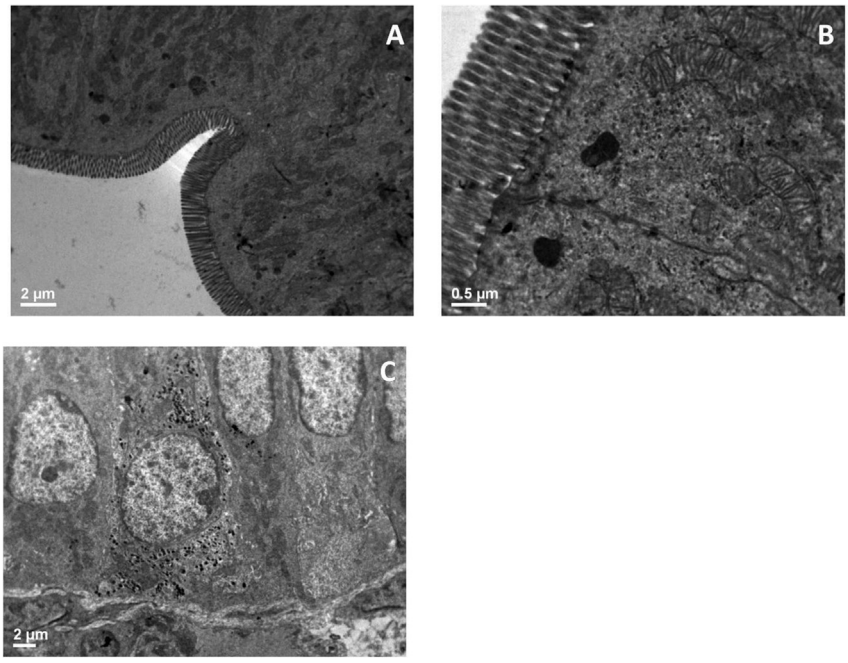

Fig. 5. Samples of small intestine from rats treated with the *Caralluma fimbriata* extract (CFE; 100 mg/kg/die) observed by Transmission Electron Microscopy (TEM). A) Low magnification of the duodenum mucosa. The microvilli are clearly observed at the apical pole of the enterocytes whereas several mitochondria are present in the cytoplasm. B) High magnification of the duodenal enterocytes. Microvilli are well preserved and classical junctional complexes are identified between two contiguous epithelial cells. C) Intestinal crypt. A typical neuroendocrine cell is observed among other epithelial cells. It is recognized by the numerous, small, electron dense secretory granules contained in the cytoplasm.

(34.2 ± 2.6% and 35.9 ± 4.2% respectively), particularly in the arcuate nucleus, and in the frontal cortex (37.0 ± 2.4% and 34.5 ± 2.1% respectively) (Figs. 6 and 7).

When assayed for the effects on the metabolic enzymes, CFE exhibited significant inhibitory effects only at high concentrations (5 and 10 mg/ml), being the maximum inhibition value of 65% and 75% for the α-amylase and lipase activity respectively (Supplementary material, Figs. 7S and 8S).

4. Discussion

The phytochemical study of CFE showed that the extract contains several typologies of pregnanes, some characterized by the presence of glycosides, other by aromatic moieties. The overall pregnane content, as measured by the CH_3 - 21 signal present in all pregnane backbones, was of about 12%; this amount corresponds to less than half of that reported in the technical sheet provided by the supplier (27.5%). It has to be underlined that in the present study, just a sample of CFE has been analyzed, so we cannot gauge the quality of commercial samples of *C. fimbriata* (quality control fell outside our purpose); nevertheless, this result should be taken into consideration, since the biological activity of *C. fimbriata* is ascribed to pregnane glycosides.

Our analysis also highlighted the presence of polyphenolic compounds, most of which are constituted by tannins, with a minor flavonoid content. The polyphenol amount here found is similar to that reported for other *Caralluma* species; for example, the yield of luteolin-4′−*O*-neohesperidoside was 0.5% in *Caralluma attenuata* and 0.015% in *Caralluma lasiantha* (Ramesh et al., 1999).

Results from *in vivo* experiments showed that CFE induced neither alterations of blood parameters nor inflammatory and/or ulcerative effects on intestinal mucosa and liver parenchyma. These data are in line with the results obtained by Odendaal et al. (2013), which highlighted the lack of any toxicity of CFE in a comprehensive GLP-compliant safety assessment.

In our experiments, the treatment with CFE induced a

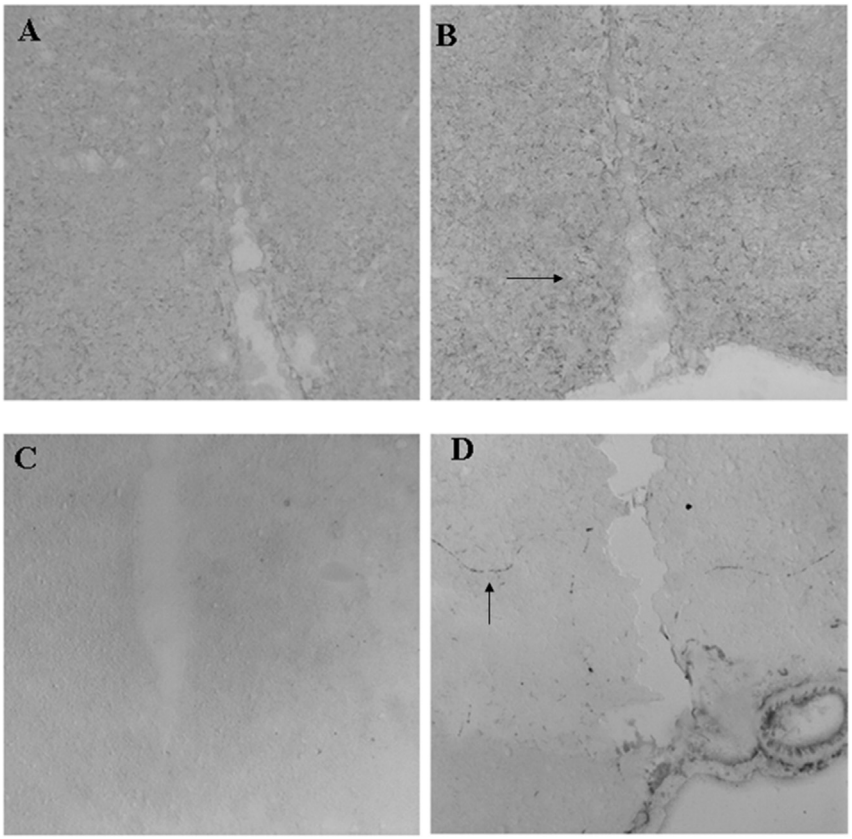

Fig. 6. Expression of NPY and ORX peptides in the arcuate nucleus from rats treated with vehicle (A, C) and *Caralluma fimbriata* extract (CFE; 100 mg/kg/die) (B, D).

statistically significant reduction in the body weight gain. This effect was not accompanied, as expected, by a decrease in food intake which, on the contrary was slightly increased, but, strangely, by a significant increase in water consumption. In addition, the CFE treatment increased the brain expression of the orexigenic factors NPY and ORX in hypothalamus and frontal cortex of treated rats.

On the basis of these evidences, our results suggest a possible role of CFE in the regulation of ingestive behaviour, probably due to pregnane glycosides, although the contribution of other components of the phytocomplex, cannot be excluded. Literature evidences report that pregnane glycosides are able to modulate the ingestive behaviour by possibly affecting the hypothalamic feeding circuits (MacLean and Luo, 2004; Komarnytsky et al., 2013). Particularly ikemagenin, a pregnane glycoside isolated from *Asclepias incarnata*, exhibited *in vivo* appetite-regulating effects by affecting the melanocortin signaling pathway and the secretion of brain-derived neurotropic factor (BDNF) in the hypothalamus, thus suggesting that the substance can be uptaken through the blood brain barrier, so acting at central level (Komarnytsky et al., 2013). On the other hand, the ability of the pregnane hoodigogenin A to penetrate the blood brain barrier has been highlighted in a MDR1-MDCK cell model (Madgula et al., 2010).

Hypothalamus represents one of the main nutrient sensor of brain and plays a pivotal role in the regulation of energy balance, by the involvement of different chemical messengers, among which NPY represents the earliest recognized and the most potent orexigenic one. NPY has been found overexpressed during fasting as well as in case of energy deprivation, energy demand or hyperphagic conditions, while a gradually decrease occurs during food intake (Mercer et al., 2011). There is a close interaction among NPY and other brain factors, both orexigenic (e.g. Agouti-related peptide, AgRP) and anorexigenic (e.g. α-melanocyte-stimulating, α-MSH; brain-derived neurotrophic factor, BDNF). Particularly, ORX seems to elicit increased levels of NPY mRNA expression in the hypothalamus, through the modulation of the CREB (cAMP response element) transcription factor, thus suggesting the pivotal role of orexin-NPY neuronal circuit on the feeding control (Kageyama et al., 2012; Álvarez-Crespo et al., 2013). Orexin neurons in the lateral hypothalamus and NPY neurons in the arcuate nucleus have reported to be stimulated by fasting and low glucose levels, thus activating feeding regulatory pathways (Muroya et al., 2004). NPY is also strictly involved in the regulation of water consumption (Pau et al., 1988; Pich et al., 1992). Analogously, ORX has been found to produce drinking stimulant effects by interacting with specific receptors (Karasawa et al., 2014).

The increased ingestive behaviour could represent a physiological response to the increased levels of NPY and ORX, but why in rats treated with CFE water consumption is increased more than food consumption? Furthermore, why rats who received CFE do not gain weight even though they eat as much or more than the vehicle treated?

The control of feeding behaviour also occurs at peripheral level. We can hypothesize that the CFE treatment could affect the peripheral absorption of some nutrients. In this regard, the inhibition of the metabolic enzymes α-amylase and lipase seems to poorly contribute to the CFE effects, due to the high concentrations effective in our *in vitro* experiments.

Venkatesh et al. (2003) found that extracts from *C. attenuata* were able to reduce the blood glucose levels in diabetic rats and hypothesized the involvement of extrapancreatic mechanisms. Furthermore, a *C. tuberculata* extract has been reported to improve the glucose utilization likely by increasing the insulin secretion and

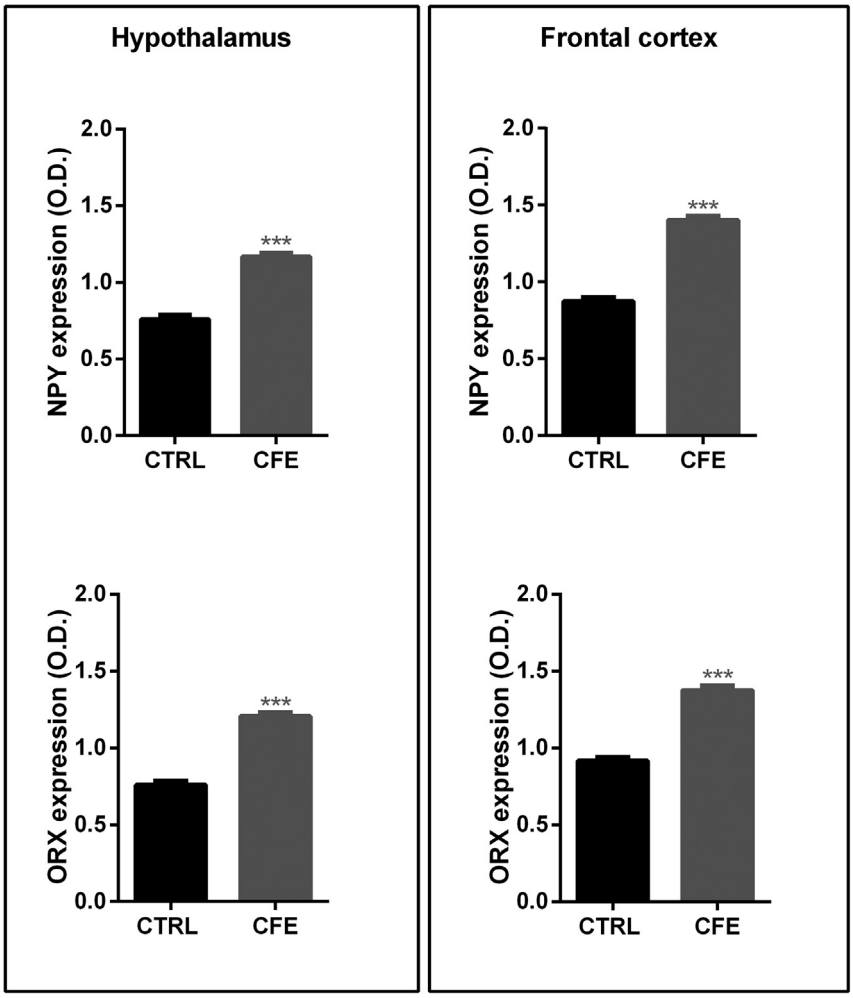

Fig. 7. Optical density (O.D.) analysis of NPY and ORX peptides in the hypothalamus from rats treated with vehicle (CTRL) and *Caralluma fimbriata* extract (CFE; 100 mg/kg/die). Data represent the mean ± SEM (n = 9 replicates). ***p < 0.001 vs. vehicle.

blood levels in diabetic rats: this effect could be due to both polyphenols and pregnane glycosides, for which similar properties have been reported (Yoshikawa et al., 1996; Venkatesh et al., 2003; Mathews et al., 2006; Abdel-Sattar et al., 2008). At this purpose, in our experiments high levels of blood insulin and HOMA IR have been found in CFE-treated rats, so suggesting a possible increased requirement of glucose by cells. Being the glucose blood levels unchanged between treated and untreated groups, a possible interference of CFE with the cellular nutrient uptake (e.g. inhibition of GLU transporters at membrane level) can be hypothesized, although further investigations are needed.

As a consequence of the reduced nutrient absorption, the hypothalamic activation of orexigenic signals can occur, with increased levels of NPY and ORX here found. This is in agreement with the results obtained by Komarnytsky et al. (2013) who found that the pregnane glycosides from *Asclepias incarnata* exerted appetite-regulating effects due to a decrease of Agouti-related protein and BDNF levels but also by peripheral mechanisms.

Finally, taking into account that the orexin/hypocretin transmission in the hypothalamus has been involved in the primary reinforcing effects of palatable foods, the increase in water consumption here observed could be due to the palatability of CFE. *C. fimbriata* has been found to contain bitter compounds (Bader et al., 2003), which can produce an intense taste sensing by activating specific taste receptors (Janssen et al., 2011). At hypothalamic level, this can lead to the upregulation of some hunger peptides, like NPY and ORX, and to the sensitization of the pathways involved in the fluid ingestion control (Erlanson-Albertsson, 2005; Bourque, 2008). At this purpose, water ingestion has been found regulated by food and beverage taste (Stanhewicz and Kenney, 2015), although the true mechanisms that coordinate the choice of different essential nutrients, such as sugar or water, remain to be clarified (Jourjine et al., 2016).

5. Conclusions

Present results highlight a possible activity of *C. fimbriata* in the modulation of ingestive behaviour, involving both food and water intake. The mechanism is complex and probably occurs at central and peripheral level. We can hypothesize an interference of the extract with the nutrient uptake at cellular level: this could explain the increase of both the insulin blood levels and the hypothalamic orexigenic signals found in treated animals, as a compensative requirement of nutrient ingestion or utilization. The extract taste can drive the animal to water intake rather than to food intake. A reduction of body weight gain can occur as a consequence of the increased water intake, likely induced by the hypothalamic orexigenic signals.

The actual effectiveness of *C. fimbriata* as a slimming agent, the mechanism of action and the specific contribution of pregnane

glycosides, polyphenols and other bitter compounds requires to be further investigated.

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Author contributions

Study design: Mazzanti G. and Vitalone A.; NMR and phytochemical analysis: Sciubba F, Di Sotto A.; *in vivo* and *in vitro* experiments: Mazzanti G., Vitalone A., Di Sotto A.; analysis of gut and liver specimens: Mammola C.L., Heyn R., Miglietta S.; analysis of brain specimens: Passarelli F. and Nativio P.; Clinical chemistry: Mariani P.; writing the manuscript: Mazzanti G., Vitalone A., Di Sotto A. Final approval: all authors.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.07.027.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.07.027.

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