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RESEARCH ARTICLE

Crocus cancellatus subsp. *damascenus* stigmas: chemical profile, and inhibition of α-amylase, α-glucosidase and lipase, key enzymes related to type 2 diabetes and obesity

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Abstract

Spices are appreciated for their medicinal properties besides their use as food adjuncts to enhance the sensory quality of food. In this study, *Crocus cancellatus* subsp. *damascenus* was investigated for its antioxidant activities employing different *in vitro* systems. Stigma extract demonstrated a radical scavenging activity against both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals with IC_{50} values of 34.6 and 21.6 μg/mL and a good ferric reducing ability (53.9 μM Fe(II)/g). In order to clarify the potential functional properties of this spice, the carbohydrate-hydrolysing enzymes and pancreatic lipase inhibitory properties were investigated. *Crocus cancellatus* subsp. *damascenus* extract inhibited α-amylase and α-glucosidase with IC_{50} values of 57.1 and 68.6 μg/mL, respectively. The bioactivity was discussed in terms of phytochemicals content. The obtained results may be of interest from a functional point of view or as food additive and to promote the revalorization of this species.

Keywords

α-amylase, α-glucosidase, antioxidant activity, lipase, phytochemical profile, saffron

History

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Introduction

Spices have been in use for thousands of years in cooking to enhance the sensory quality of food. In recent years, the physiological functionality of food spice used in traditional cooking has received much attention due to the increasing interest in human health and has been studied *in vitro* and *in vivo* by many research groups[1–4].

Saffron spice is a member of the *Iridaceae* family. The stigmas must be hand-picked from the delicate blossoms upon opening to preserve the desirable volatile components[5]. With its unmatched signature bitter-like taste, slightly metallic sub-notes and pungent hay-like aroma, saffron was used as both flavoring and coloring agent in food[6]. Interest in the impact of saffron on human health is growing due to their proved health properties[7]. Iran has a long history in saffron production and there are a lot of established Iranian saffron populations, which are cultivated since ancient times[8].

Among species distributed in Iranian country *Crocus cancellatus* subsp. *damascenus* was first described by W. Herbert but other names have been bestowed upon it over the years including *Crocus edulis*, which refer to its use as a food[9].

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Oxidative stress is initiated by reactive oxygen species (ROS). ROS can easily damaging various cellular macromolecules. However, human cells have developed a series of protecting mechanisms to prevent the production of free radicals and oxidative damage. Natural products have been proposed as a substitute of synthetic antioxidants since they are related to negative health effects[10]. Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance[11]. Three hundred and sixty-six million people had diabetes in 2011 and by 2030 this will have risen to 552 million[12]. Type 2 diabetes is a heterogeneous disease resulting from a dynamic interaction between defects in insulin secretion and insulin action. Patients with type 2 diabetes are insulin-resistant and often have a metabolic syndrome, a multifactorial intervention including arterial hypertension and dyslipidemia. One therapeutic approach for treating in the early stage diabetes is to decrease post-prandial hyperglycaemia by retarding the absorption of glucose through the inhibition of α-amylase and α-glucosidase[13]. However, these drugs have some adverse effects like causing hypoglycemia at higher doses and other side effects. For these reasons several research groups addressed their activity on the discovery of natural products with inhibitory potential on key enzymes related to type 2 diabetes[14].

Abnormalities in plasma lipid and lipoprotein concentrations in patients with diabetes are outlined[15]. Obesity rates increased to 82% globally in the past two decades[16].

Pancreatic lipase is a key enzyme for the absorption of dietary triglycerides. Interference with fat hydrolysis results in the reduced utilization of ingested lipids, therefore, inhibition of lipases decreases fat absorption that is useful for obese patients.

The objective of the present study was to investigate the chemical composition of *C. cancellatus* subsp. *damascenus* stigmas extract and to correlate the chemical profile with the antioxidant activity, carbohydrate-hydrolyzing enzymes and pancreatic lipase inhibitory properties.

Materials and methods

Chemicals and reagents

Solvents analytical grade were purchased from VWR International s.r.l. (Milan, Italy).

Ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution, potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-carotene, linoleic acid, Tween 20, propyl gallate, tripyridyltriazine (TPTZ), $FeCl_3$, $FeSO_4$, butylated hydroxytoluene (BHT), potato starch, sodium phosphate, sodium chloride, α-amylase from porcine pancreas (EC 3.2.1.1), α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), maltose, sodium acetate, sodium potassium tartrate, 3,5-dinitrosalicylic acid, *o*-dianisidine color reagent (DIAN), glucose oxidase peroxidase enzyme solution (PGO), lipase Type II, crude from porcine pancreas (EC 3.1.1.3), 4-nitrophenyl octanoate, orlistat, catechin hydrate, caffeic acid, ferulic acid, diphenylborinic acid aminoethylester, Folin–Ciocalteu reagent, $AlCl_3$ were purchased from Sigma-Aldrich S.p.a. (Milan, Italy). Acarbose was obtained from Serva (Heidelberg, Germany) and *p*-anisaldehyde from Alfa Aesar (Karlsruhe, Germany).

Samples and extraction procedures

Stigmas of *C. cancellatus* subsp. *damascenus* were bought in January 2006 in a local market in Mashhad (Iran) and were identified by Dr. Farsad Nadjafi, Ferdowsi University of Mashhad, Iran. A voucher specimen is deposited at the herbarium of School of Agriculture, Ferdowsi University of Mashhad. The dried stigmas of *C. cancellatus* subsp. *damascenus* (500 g) were extracted with ethanol 70% through maceration (48 h × 3 times). The resultant solutions were dried to give 28.9 g.

Determination of total phenol and flavonoid content

The amount of total phenols of *C. cancellatus* subsp. *damascenus* ethanol extract was determined by the Folin–Ciocalteu method[17]. Chlorogenic acid was used as a standard and the total phenol content was expressed as chlorogenic acid equivalents in mg per g of extract. The total flavonoid content was determined spectrophotometrically using a method based on the formation of a flavonoid–aluminium complex[17]. Quercetin was chosen as a standard and the levels of total flavonoid content were determined in triplicate and expressed as quercetin equivalents in mg per g extract (Table 1).

Determination of total carotenoid content

The total carotenoid content was determined by measuring the absorption of the stigmas extract using the methodology proposed by Gao et al[18]. β-Carotene was used as a standard. The total carotenoid content was determined in triplicate and expressed as β-carotene equivalents in mg per g of extract (Table 1).

Table 1. Extraction yield (%) and phytochemicals content of *C. cancellatus* subsp. *damascenus stigmas* extract.

	C. cancellatus subsp. *damascenus*
Extract yield (%)	5.8
Total phenols*	146.8 ± 2.5
Total flavonoids†	57.5 ± 1.2
Total carotenoids‡	93.5 ± 1.6

Data are mean ± SD ($n = 3$).
*Data are expressed as mg chlorogenic acid equivalents per g extract.
†Data are expressed as mg quercetin equivalents per g extract.
‡Data are expressed as mg β-carotene equivalents per g extract.

Table 2. Main constituents tentatively identified in *C. cancellatus* subsp. *damascenus* stigmas extract.

Compound	R_t (min)	Abundance (%)
2-Furancarboxaldehyde, 5-methyl-	7.46	0.8
4H-pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl-	10.37	3.2
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	11.41	29.5
Methyl palmitate	17.44	1.6
Palmitic acid	17.75	1.1
Xanthotoxin	18.54	1.3
Methyl oleate	18.72	10.7
Methyl stearate	18.88	5.5
Osthol	19.15	5.4
Isopimpinellin	19.84	1.1
Methyl arachidate	20.17	0.8

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analyses

The *C. cancellatus* subsp. *damascenus* extract was analyzed by using a Hewlett–Packard 6890 gas chromatograph equipped with an HP-5 MS capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and interfaced with a Hewlett Packard 5973 Mass Selective (Agilent Technologies, Cernasco sul Naviglio, Milan, Italy). Ionization of the sample components was performed in electron impact mode (EI, 70 eV). Helium was used as carrier gas. The analytical conditions were as follows: oven temperature was 5 min isothermal at 50 °C, then 50–250 °C at a rate of 5 °C/min; then held isothermal for 10 min. Constituents were tentatively identified by comparison of their retention times with those of the literature or with those of authentic compounds available in our laboratory. Further tentative identification was made by comparison of their mass spectra with those stored in Wiley 138 library. The extract was analyzed also by a Shimadzu GC17A gas chromatograph system (Shimadzu, Milan, Italy). An SE-52 capillary column (30 m with an internal diameter of 0.25 mm and a film thickness of 0.25 μm) was used with nitrogen as the carrier gas. GC oven temperature and conditions were as described above. The quantification of the components was performed on the basis of their GC peak areas and the percentages of the characterized components are as given in Table 2. Component relative concentrations were calculated based on GC peak areas without using correction factors.

HPTLC analysis

The high performance thin layer chromatography (HPTLC) system (CAMAG, Muttenz, Switzerland) consisted of a Linomat 5 sample applicator using 100 μL syringes and connected to a nitrogen tank and a Camag TLC Visualizer linked to winCATS software. Analyses were conducted by means of normal phase glass plates 20 cm × 10 cm (VWR International s.r.l., Milan, Italy) with glass-backed layers silica gel 60 (2 μm thickness) prewashed with

methanol and carefully dried for 3 min at 100 °C. The syringe delivery speed was 150 nL/s and the other operating conditions were: injection volume, 1 μL; band width, 8 mm; distance from bottom, 15 mm; solvent front position, 90 mm. The HPTLC plates were developed with the mobile phase ethyl acetate/dichloromethane/acetic acid/formic acid/water (100:25:10:10:11; v/v/v/v). For the identification of ferulic acid the mobile phase ethyl acetate/dichloromethane/acetic acid/formic acid/water (100:31.25:1.25:1.25:1.25; v/v/v/v) was instead utilized. The developed layers were allowed to dry and then derivatized with Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) and anhysaldehyde (1.5 mL *p*-anisaldehyde, 2.5 mL H_2SO_4, 1 mL AcOH in 37 mL EtOH). All plates were inspected under a UV light at 254 or 366 nm or under white light upper and lower (WRT) before and after derivatization by means of a Camag TLC visualizer. Band stability was checked by keeping the resolved peaks and inspecting at intervals of 12, 24 and 48 h. Repeatability was determined by running a minimum of three analyses. R_f (retardation factor) values for main selected compounds varied less than 0.02%.

The quantification of catechin hydrate and ferulic and caffeic acids was done. Working stock solutions were prepared by dilution with methanol (or ethanol for caffeic acid) to give final concentrations ranging from 0.5 to 10 mg/mL. Standard solutions of each compound were spotted on HPTLC plate to give absolute amounts in the range 0.5–10 μg/band. The calibration curves were prepared using absolute amount (μg/band) as independent variable (X) and the peak area of standards as dependent variable (Y). Regression analyses test of the compound were performed using GraphPad Prism Software (GraphPad Software, San Diego, CA). The curves confirmed linear relationship between the working concentration and the peak areas.

DPPH radical scavenging activity assay

Radical scavenging capacity was determined using DPPH assay[3]. The DPPH radicals scavenging activity was calculated according to the following equation: Scavenging activity $= [(A_0 - A_1/A_0) \times 100]$, where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance in the presence of the extract.

Antioxidant capacity determined by radical cation ABTS$^+$

This assay was based on the method previously described[17]. The scavenging ability of sample was calculated according to the following equation: ABTS scavenging activity $(\%) = [(A_0 - A)/A_0] \times 100$, where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

β-Carotene bleaching test

The bleaching β-carotene was measured and expressed as antioxidant activity (AA)[17]: $[1-(A_0-A_t)/(A_0^\circ - A_t^\circ)] \times 100$, where A_0 and A_0° are the absorbance values measured at the incubation $t = 0$ min for samples and control, respectively, while A_t and A_t° are the absorbance values measured in the samples and control, respectively, at $t = 30$ min and $t = 60$ min.

FRAP assay

The ferric-reducing ability power (FRAP) method measures the absorption change that appears when the TPTZ (2,4,6-tripyridyl-*s*-triazine)–Fe^{3+} complex is reduced to the TPTZ–Fe^{2+} form in the presence of antioxidant compounds[19]. The FRAP value represents the ratio between the slope of the linear plot for reducing Fe^{3+}–TPTZ reagent by extracts compared to the slope of the plot for $FeSO_4$.

Fe^{2+} chelating activity assay

The chelating activity of *C. cancellatus* subsp. *damascenus* extract for ferrous ions Fe^{2+} was measured according to the method previously described[20]. The chelating activity of the extract for Fe^{2+} was calculated using equation: chelating rate $= (A_0 - A_1)/A_0 \times 100$, where A_0 was the absorbance of the blank and A_1 was the absorbance in the presence of the extract.

Carbohydrate-hydrolyzing enzymes inhibitory activity

The α-amylase bioassay method adopted was previously reported[4]. The α-amylase inhibition was expressed as percentage of inhibition and calculated by the following equations: % reaction = [(maltose) test/(maltose) control] × 100; % inhibition = 100 − % reaction ± SD.

The α-glucosidase inhibition was measured through a modified Sigma-Aldrich bioassay method[4]. The enzyme inhibition was expressed as percentage of inhibition and calculated by the following equations: % reaction = [(glucose) test/(glucose) control] × 100; % inhibition = 100 − % reaction ± SD.

Measurement of pancreatic lipase activity

The inhibition of pancreatic lipase was determined as previously described[21]. A water solution of type II crude porcine pancreatic lipase was prepared. A reaction mixture (100 mL of 5 mM 4-nitrophenyl octanoate, 4 mL of Tris-HCl buffer (pH 8.5), 100 mL of extract and 100 mL of enzyme solution) was prepared and incubated at 37 °C for 25 min before the substrate was added. The absorbance was measured at 412 nm. Orlistat (20 μg/mL) was used as positive control.

Statistical analysis

The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use of Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). The concentration–response curve was obtained by plotting the percentage inhibition versus concentration. Differences within and between groups were evaluated by one-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test compared with the positive controls.

Results and discussion

Even though spices by themselves do not significantly contribute to the nutritive value of diet, by virtue of their health benefits they have been extensively investigated[1–4,21,22]. In this context, we decided to investigate the radical scavenging, the inhibition of lipid oxidation under accelerated conditions, the metal chelating and reducing ability of *C. cancellatus* subsp. *damascenus* dried stigmas.

Chemical composition

The ethanol extract of *C. cancellatus* subsp. *damascenus* showed a total amount of phenols with a value of 146.8 mg chlorogenic acid equivalent per g of extract and a total flavonoid content of 57.5 mg quercetin equivalent per g of extract.

Our results about the total flavonoid content are in agreement with those reported by Acar et al.[23] for *Colchicum baytopiorum*, *Crocus flavus* subsp. *dissectus* and *Cenchrus biflorus* (values of 36, 71 and 32 mg quercetin equivalent per g of extract). Karimi et al.[24] determined the total phenol and flavonoid content of *C. sativus* from Iran founding for the ethanol extract a value of 6.0 mg gallic acid equivalent per g dried weight (DW) and 2.9 mg rutin equivalent per g DW, respectively, while a total phenols content of 3.42 mg gallic acid per g DW phenolics was found in

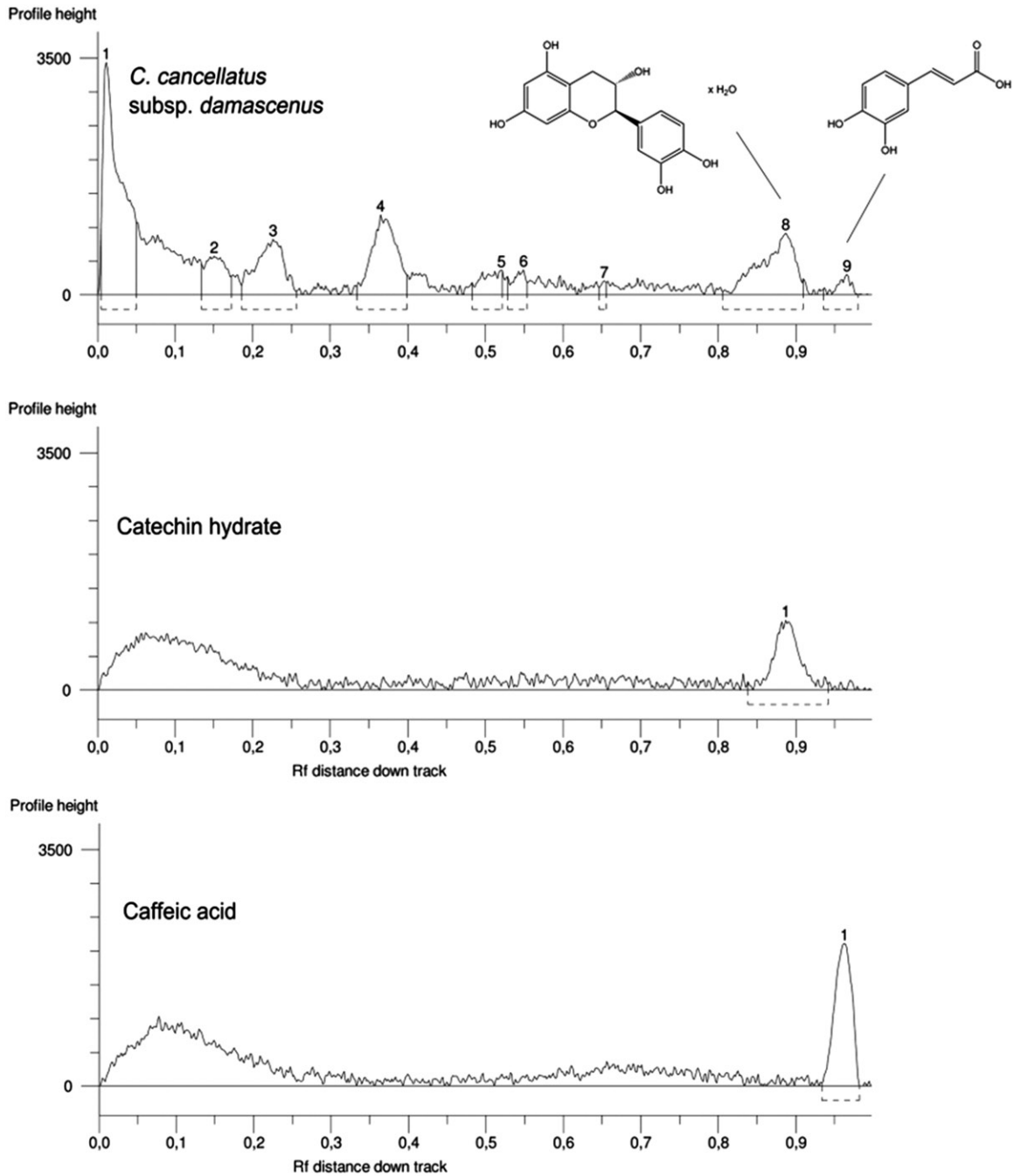

Figure 1. HPTLC chromatograms of *C. cancellatus* subsp. *damascenus* extract and standards.

the methanolic extract of saffron petal[25]. The total carotenoid content of our sample was estimated as 93.5 mg β-carotene equivalent per g of extract. GC–MS analysis identified in the extract of *C. cancellatus* subsp. *damascenus* a total of 11 compounds among them 5-(hydroxymethyl)-2-furancarboxaldehyde, (29.5%), methyl oleate (10.7%), methyl stearate (5.5%) and osthol (5.4%) are the most abundant.

Catechin hydrate, caffeic acid and ferulic acid were identified by comparing their R_f values obtained from the peaks with those of standards (Figures 1 and 2).

Quantification of catechin hydrate, caffeic and ferulic acids was performed using regression equations. The correlation coefficients (R^2) were found to be >0.98. Among identified compounds, the most abundant one was catechin hydrate, with an amount of 55.2 mg per g of extract. The contents of ferulic and caffeic acids were found to be 13.9 and 10.01 mg per g of extract, respectively.

Antioxidant activity

The extract of *C. cancellatus* subsp. *damascenus* was tested for its antioxidant activities employing various established *in vitro* systems (Table 3). Stigmas extract exhibited concentration–response relationship in all tests. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of food. Stigmas ethanol extract demonstrated DPPH radical scavenging activity with an IC_{50} value of 34.6 μg/mL. This activity is 6.2-fold lower than the positive control ascorbic acid (IC_{50} value of 5.0 μg/mL).

To confirm the radical scavenging activity, ABTS test was applied founding an IC_{50} value of 21.6 μg/mL. The extract was also able to inhibit the discoloration of β-carotene with IC_{50} values of 104.5 and 41.0 μg/mL after 30 and 60 min of incubation, respectively. The sample was also tested to observe its influence

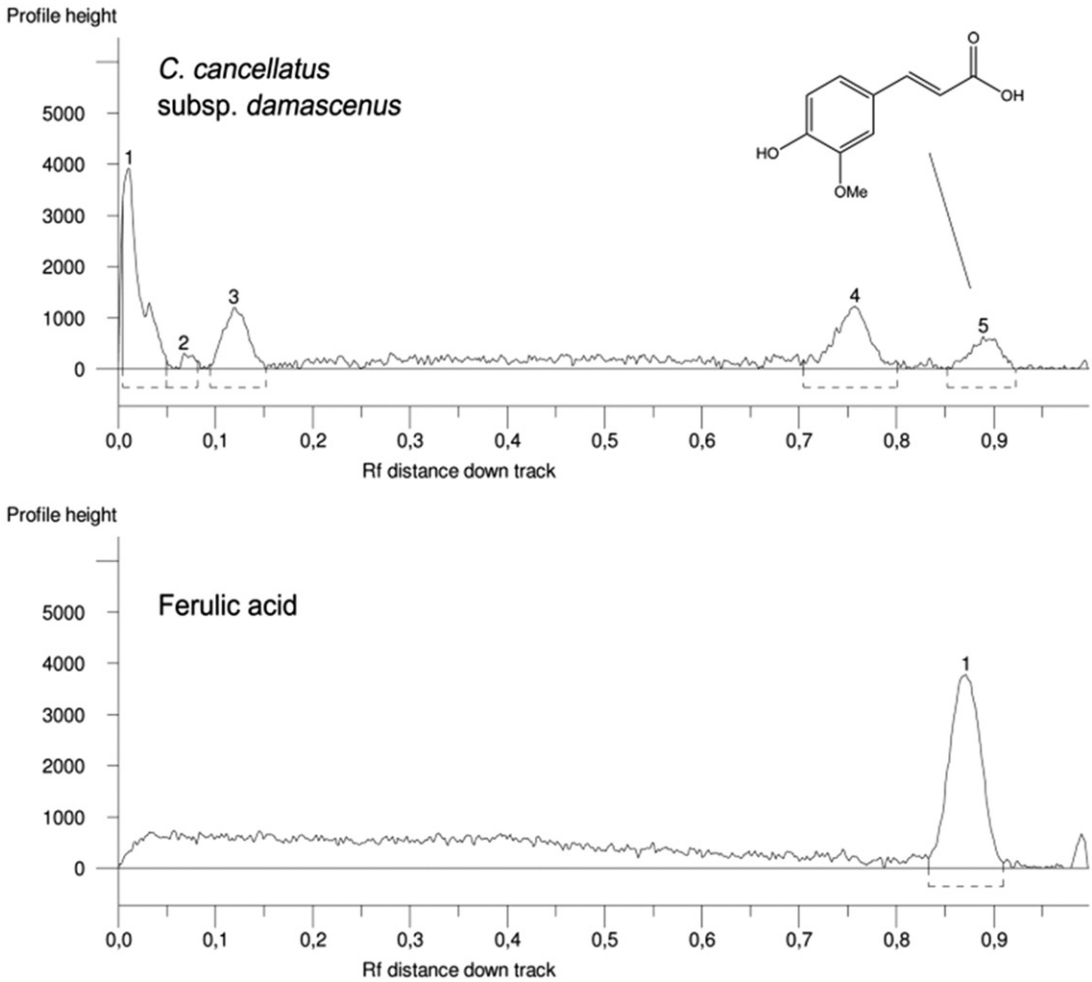

Figure 2. HPTLC chromatograms of *C. cancellatus* subsp. *damascenus* extract and standard.

Table 3. Radical scavenging activity, antioxidant property, metal reducing and chelating activity of *C. cancellatus* subsp. *damascenus* stigmas extract.

			β-Carotene bleaching test (μg/mL)			
C. cancellatus subsp. *damascenus*	DPPH test (μg/mL)	ABTS test (μg/mL)	30 min	60 min	FRAP test μM Fe(II)/g)	Fe^{2+} chelating activity assay
Extract	34.6 ± 1.6**	21.6 ± 2.3**	104.5 ± 3.8**	41.0 ± 1.3**	53.9 ± 2.1**	24.6 ± 2.2**
Ascorbic acid	5.0 ± 0.8	1.7 ± 0.3				
Propyl gallate			1.0 ± 0.041	1.0 ± 0.034		
BHT					63.2 ± 4.3	
EDTA						1.27 ± 0.05

Data are expressed as mean ± S.D. ($n = 3$); DPPH radical scavenging activity assay; antioxidant capacity determined by radical cation ($ABTS^+$); ferric reducing antioxidant power (FRAP). Ascorbic acid was used as positive control in ABTS and DPPH test; propyl gallate was used as positive control in β-carotene bleaching test BHT was used as positive control in FRAP test; EDTA was used as positive control in Fe^{2+} chelating activity assay. Differences within and between groups were evaluated by one-way analysis of variance test ***$p < 0.0001$ followed by a multicomparison Dunnett's test: **$p < 0.01$ compared with the positive controls.

on metal transition ion iron. In FRAP assay that measures the reducing ability of antioxidant compound on ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex, *C. cancellatus* subsp. *damascenus* showed a reducing potency of 53.9 μM Fe(II)/g that is greater than positive control BHT (62.2 μM Fe(II)/g). The reducing ability of the sample could be correlated with the phenolic level as reported in literature since Soong and Barlow[26] demonstrated that phenolics could act as reducing agents, hydrogen donators and singlet oxygen quenchers. Moreover, stigmas extract is able also to chelate iron. In fact, in Fe^{2+}-chelating activity assay stigmas extract showed an IC_{50} value of 24.6 μg/mL. The obtained results clearly evidenced that this spice can inhibit the iron-mediated lipid peroxidation of low-density lipoprotein, an effect ascribed to their capacity to form complexes with reduced metals and act as hydrogen donors.

Previously, Assimopoulou et al.[27] reported the strong radical scavenging activity (above 2000 ppm) of *C. sativus* growing in Greece while Gismondi et al.[28] described the antioxidant properties of Civitaretenga *C. sativus* from Italy. In DPPH test stigmas extract showed an IC_{50} value of 3.76 mg DW while FRAP assay

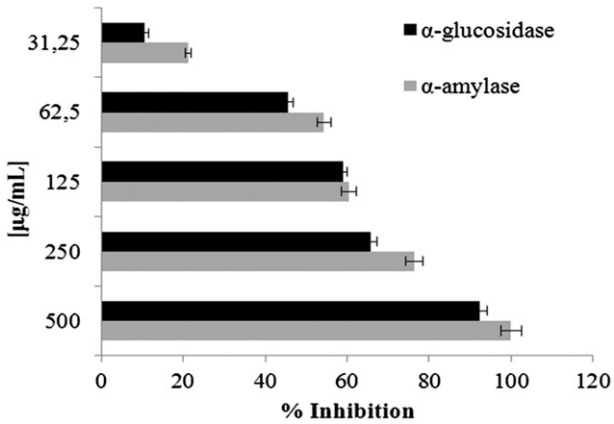

Figure 3. Concentration–response curve of *C. cancellatus* subsp. *damascenus* against α-glucosidase and α-amylase enzymes. Data are mean ± SD ($n = 3$).

indicated that one gram of dried sample presented the same antioxidant power of 2.5 mM $FeSO_4$. Iranian *C. sativus* ethanol extract demonstrated an IC_{50} value of 299.4 µg/mL at tested concentration of 300 µg/mL against DPPH radical while a ferric reducing power of 53.1% was obtained testing the same concentration[24]. *Crocus flavus* subsp. *dissectus* at 2 mg/mL exhibited antioxidant activity measured by DPPH and β-carotene bleaching test equal to in the concentration of 0.8 mg/mL of BHA used as standard antioxidant[23]. Moreover, *C. cancellatus* subsp. *damascenus* showed also a high flavonoids content that could contribute to the antioxidant activity. In particular, several studies demonstrated that diet flavonoids are absorbed from the gut after consumption and significantly increase the antioxidant capacity of the blood. These beneficial effects of increased antioxidant capacity in the body may be the reduction of oxidative damage to important molecules[29,30].

Carbohydrate-hydrolyzing enzyme inhibitory activity

Recently, several studies demonstrated the hypoglycemic properties of spice and their constituents[14]. α-Amylase and α-glucosidase hydrolyzed glucosidic bonds in order to convert food containing starch into digestible carbohydrate food so inhibition of these enzymes could reduce the high post-prandial blood glucose peaks in diabetics. *Crocus cancellatus* subsp. *damascenus* showed a concentration–response relationship in both test with IC_{50} values of 57.1 and 68.6 µg/mL for α-amylase and α-glucosidase, respectively (Figure 3). Acarbose was used as positive control (IC_{50} values of 50.0 and 35.5 µg/mL against α-amylase and α-glucosidase, respectively).

A perusal analysis of literature revealed that *C. sativus* injected in alloxan-diabetic rats determined a significant increase of fasting blood glucose and HbA1c levels, but a decrease of blood insulin levels[31]. The influence of saffron on glucose metabolism was confirmed by Kang et al.[32] that demonstrated how this spice increases glucose uptake and insulin sensitivity in muscle cells *via* multi-pathway mechanisms.

Pancreatic lipase inhibitory activity

Lipase inhibitory activity was measured by monitoring the hydrolysis of *p*-NPC. Orlistat showed an IC_{50} value of 0.018 mg/mL. *Crocus cancellatus* subsp. *damascenus* extract induced 50.39% of inhibition of lipase activity at 5 mg/mL. The potential of natural products for the treatment of obesity is still largely unexplored[33]. Natural sources could represent a starting point for further investigation in developing functional food and isolation of active compounds[34].

Conclusion

This study investigated the phytochemical composition and the potential biological activity for the treatment of dysmetabolic disorders, such as type 2 diabetes and obesity, of *C. cancellatus* subsp. *damascenus* extract. Based on the experimental results reported herein, *C. cancellatus* subsp. *damascenus* possess high levels of phenols and flavonoids.

Catechin hydrate, caffeic acid and ferulic acid were identified as main constituents. The investigation of biological properties was accomplished by four standard antioxidant assay procedures and α-amylase, α-glucosidase and pancreatic lipase inhibitory assays. Collectively, the results emphasize the role of this spice as a potential dietary nutraceutical supplement to keep human beings healthy. Furthermore, it holds promise for becoming a natural food additive as an antioxidant agent.

Declaration of interest

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