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Crocus sativus L. stigmas and byproducts: qualitative fingerprint, antioxidant potentials and enzyme inhibitory activities

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Abbreviations: CST, saffron stigmas; CTA, byproducts (tepals+anthers); IBD, inflammatory bowel disease; LPS, lipopolysaccharide; MDA, malondialdehyde; LDH, lactate dehydrogenase; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ROS, reactive oxygen species; DPPH, α,α-diphenyl-β-picrylhydrazyl; CUPRAC, CUPric Reducing Antioxidant Capacity; FRAP, ferric reducing ability of plasma; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid.).

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Running head: Antioxidant and enzyme inhibitory effects of saffron byproducts

Abstract

Saffron (*Crocus sativus* L.) has been previously reported to be active as a protective agent in multiple experimental models of oxidative stress, inflammation and cancer. These findings refer to the protective effects of stigmas, not byproducts such as tepals and anthers. In this context, the aims of the present work were to characterize the phytochemical profile of saffron stigmas (CST) and high quality byproducts (tepals + anthers - CTA) extracts. Additionally, we studied the antioxidant and chelating effects of CST and CTA extracts by preliminary *in vitro* assay. The antioxidant activity was further investigated through the evaluation of reactive oxygen species (ROS) levels and lactate dehydrogenase (LDH) activity on mouse myoblast (C2C12) and human colon cancer (HCT116) cell lines. Additionally, we evaluated CST and CTA extract treatment on cholinesterases, α-glucosidase and α-amylase activity, in vitro. Finally, we studied the effects of CST extract on malondialdehyde (MDA) level in rat colon specimens challenged with *E. coli* lipopolysaccharide (LPS).

We observed that water CST extracts are rich in phenolic content, whereas for CTA the olive oil was the elective extraction solvent. As expected, water CST extracts were the most effective in reducing hydrogen peroxide-induced oxidative stress in both cell lines and *in vitro* assays. Furthermore, both CST and CTA water extracts reduced the LDH activity in HCT116 cells

challenged with hydrogen peroxide and LPS-induced MDA levels in rat colon specimens. Concluding, the present findings showed protective effects exerted by CST and CTA extracts in *in vitro* and *ex vivo* models of inflammation and oxidative stress.

1. Introduction

Saffron (*Crocus sativus* L.) is cultivated to obtain the precious spice that, represented by stigmas, is considered as the most expensive all over the world. In the last decades the cultivation of saffron in Italy was subjected to renewed attention and the total cultivation is increasing. The traditional production of Abruzzo saffron (Zafferano dell'Aquila), which has been awarded Protected Designation of Origin (PDO) status in Italy, is characterized by huge amount of manual work, which is the main factor influencing the final cost (Alonso et al., 2012; Supplementary Material: Official Registration of PDO brand, 2004 in "Gazzetta Ufficiale Italiana"). On the other hand, the manipulation of each flower to separate floral parts allows producing a very high quality main products (stigmas) as well as high quality byproducts. In collaboration with local farmers and cosmetic laboratories of L'Aquila District (Italy), an experimental research was started to investigate a new market for stigmas and to valorize byproducts, such as tepals and anthers. In addition to its elevated value as a spice, saffron has been previously reported to be active as a protective agent in multiple experimental models of oxidative stress, inflammation and cancer (Corso et al., 2016; D'Alessandro et al., 2013; Maccarone et al., 2016). Preclinical and clinical studies also demonstrated the antidepressant efficacy of saffron extracts (Shafiee et al., 2018). These pharmacological effects could be related, in most cases, to stigma major compounds, such as

crocin, safranal and kaempeferol (Kim & Park, 2018; Hosseinzadeh & Noraei, 2009). Nevertheless, high quality byproducts of saffron such as petals have been previously reported to be rich in crocin and kaempferol, thus representing a significant source of bioactive compounds for the development of potential functional foods and cosmetic formulations (Tirillini et al., 2006; Zeka et al., 2015). Particularly, saffron petals revealed to be very effective as antioxidant and antimicrobial agent, with promising industrial applications in Pacific white shrimp aquaculture (Abbasvali et al., 2016). Multiple studies (Tuberoso et al., 2016; Lahmass et al 2017) also revealed the potential application of high quality byproducts such as spaths, leaves, corms and floral-derived juices as cheap sources of bioactive compounds endowed with antioxidant activity.

Additionally, Hosseinzadeh and Younesi (2002) described anti-inflammatory antinociceptive effects related to saffron petals. Whereas Moshiri et al (2006) observed clinical efficacy of saffron petals in the treatment of mild-to-moderate depression.

In this context, the aims of the present work were to characterize the phytochemical profile of extracts obtained with different solvents (water, ethanol, olive oil) in order to investigate new potential therapeutic tools from saffron stigmas (CST) and high quality byproducts (tepals + anthers - CTA). In addition, we performed *in vitro* studies on non-tumoral mouse myoblast (C2C12) and human colon cancer (HCT116) cell lines, in order to evaluate the effects of the tested extracts on cell viability and reactive oxygen species (ROS).

Additionally, we performed biological investigations on rat colon specimens challenged with *E. coli* lipopolysaccharide (LPS), a validated experimental model of inflammatory bowel disease (IBD) (Locatelli et al., 2017; Menghini et al., 2011). The protective effect was evaluated by measuring the level of malondialdehyde (MDA) and the activity of lactate dehydrogenase (LDH), following extracts treatment. Finally, we evaluated the effects of CST and CTA extracts on the activity of α-glucosidase, α-amylase, acetylcholinesterase (AChE), butyrylcholinesterase (BChE) in order to investigate potential use in the treatment of Alzheimer's disease (AD) and type 2 diabetes (T2D).

2. Materials and methods

98 *2.1. Chemicals and standards*

99 Commercial standards of crocin and safranal (>88%) were purchased from Sigma-Aldrich (Italy).
100 Safranal was further purified by column chromatography on silica gel (230-400 mesh, G60 Merck),
101 using ethyl acetate:*n*-hexane (1:3) as eluent. ^{1}H, ^{13}C NMR and IR spectra of the purified compound
102 were in agreement with those reported in the literature (De Monte et al., 2014). 4-Nitroaniline, used
103 as an internal standard (IS), was purchased from Sigma-Aldrich (Italy). Phenolics as chemical
104 standards (gallic acid, catechin, chlorogenic acid, *p*-hydroxy-benzoic acid, vanillic acid,
105 epicatechin, syringic acid, 3-hydroxy-benzoic acid, isovanillin, *p*-coumaric acid, rutin, sinapinic
106 acid, *t*-ferulic acid, naringin, 2,3-dimethoxy benzoic acid, benzoic acid, *o*-coumaric acid, quercetin,
107 *t*-cinnamic acid, naringenin (all purity >98%) were purchased from Sigma Aldrich (Milan, Italy).
108 HPLC grade (>99.9%) methanol and ethanol were purchased from Carlo Erba (Italy). Ultrapure
109 water generated by the MilliQ system (Millipore, Bedford, MA) was used.

110 *2.2. Plant material*

111 Plant material was kindly furnished by local farmers in the area of "Zafferano dell'Aquila PDO"
112 consortium, Navelli, AQ (Italy). The collection of saffron flowers is traditionally handmade.
113 Briefly, in October 2016, the flowers were collected in early morning and carefully handled in order
114 to recover the stigmas and separate the byproducts that result the most abundant fraction of flower.
115 According to local tradition, immediately after collection, saffron stigmas (CST) were dried on
116 wood embers and stored in a glass bottle, in the dark. The drying method is crucial for saffron
117 quality. For instance, safranal is not present in fresh stigmas, its concentration in saffron depends
118 strongly on both drying and storage conditions. As regards the official drying method of Abruzzo
119 saffron (Supplementary data: Official Registration of PDO brand, 2004 in "Gazzetta Ufficiale
120 Italiana"), stigmas are laid out to dry over the wood charcaol for twenty minutes in order to preserve
121 their fragrance, aroma and purple red colour. Waste products, represented by anthers and tepals
122 (CTA), were carefully collected and dried on air oven at 40 °C, in the dark until constant weight,
123 then powdered and stored in vacuum bag, in the dark.

Samples of 30 fresh flowers were collected and after manual separation of stigmas were weighted in order to define the relative weight of stigmas and byproducts.

2.3. Extraction

Extractions were performed by maceration in a sonicator bath at a frequency of 30 kHz [2 consecutive extractions of 30 min each at room temperature (20 °C)] with distilled water (W), ethanol 95° (EtOH) or extra virgin olive oil (EVO) obtained from local growth plants by cold processing. The selection of water as elective solvent was carried as previously suggested (Lahmass et al., 2017). On the other we chose ethanol and olive oil as comparative organic solvents approved for cosmetic and food use. The plant material/solvent ratio was 1/20 (*w*/*w*). Each extract was freshly prepared and centrifuged at 5000 *g*. Finally, supernatant was 5-fold diluted in *n*-hexane for the following phytochemical analyses.

2.4. Phytochemical analysis

2.4.1. Preliminary antioxidant assays, total phenolic and total flavonoid content

We selected six different chemical assays (DPPH and ABTS radical scavenging, FRAP, CUPRAC, phosphomolybdenum and metal chelating tests) for providing the full antioxidant abilities of the tested extracts. Trolox and EDTA (for metal chelating assay) were used as positive controls. Total phenolic and flavonoid contents were also quantified spectrophotometrically and the results were expressed as gallid acid (mg GAE/g extract) and rutin (mg RE/g extract) equivalents. The experimental procedures for all these preliminary assays were comprehensively described in our previous paper (Zengin et al., 2016).

2.4.2. Polyphenol fingerprint by HPLC-PDA

HPLC-PDA analyses of safranal and crocin were performed as already described (Nescatelli et al., 2017). Briefly, the HPLC-DAD Thermo Quest Spectrasystem LC (Thermo Fisher Scientific, Waltham, MA) was equipped with a P4000 pump, an automatic UV6000 UV-Vis Diode Array Detector, and the SN4000 interface. Separation of compounds was performed on Eclipse XDB-C18 analytical column (4.6 × 250 mm, 5 μm particle size; Agilent Technologies, Santa Clara, CA)

protected by a guard cartridge of the same packing, operating at 25 °C. A gradient elution with a mixture of water (A):acetonitrile (B) (30–70% B in 20 min) at a flow rate of 0.9 mL min^{-1} was used. Injection volumes were 10 µL for all samples and reference standards. Multi-wavelength detection was in the range of 200-550 nm and quantification was carried out by integration of the peak areas.

HPLC-PDA fingerprint of the main phenolics was obtained by means of a validated method using a reversed phase HPLC-PDA in gradient elution mode (Locatelli et al., 2017; Zengin et al., 2016). Analyses were carried out by using a Waters liquid chromatograph equipped with a photodiode array detector, a C18 reversed-phase column (Prodigy ODS (3), 4.6 × 150 mm, 5 µm; Phenomenex, Torrance, CA), an on-line degasser (Biotech 4-CH degasi compact, LabService, Anzola Emilia, Italy) and a column oven set at 30 °C (± 1 °C). The gradient elution was achieved by a solution of water–acetonitrile (93:7 ratio, with 3% of acetic acid) as initial conditions. The complete separation was performed in 60 min. The detailed method was reported as *Supplementary data.*

2.5. Biological studies

2.5.1. Cholinesterase inhibition

Cholinesterase (ChE) inhibitory potential was detected by Ellman's method. According to this method, the sample solution (2 mg/mL, 50 µL) was mixed with Ellman's reagent (DTNB (5,5-dithio-bis(2-nitrobenzoic) acid, 125 µL) and acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) in buffer (Tris-HCl, pH 8.0, 25 µL). Successively, this mixture was incubated (15 min, at 25 °C). Then, the substrates (acetylthiocholine iodide for AChE and butyrylthiocholine chloride for BChE, 25 µL) were added in this mixture. Blank solution was prepared without the corresponding enzyme. These solutions were incubated for 10 min at 25 °C and then the absorbances were measured at 405 nm in a 96-well microplate reader. Galantamine was used a positive inhibitor and the results were expressed as galantamine equivalents (mg GALAE/g extract) (Zengin et al., 2016).

2.5.2. α-Amylase inhibition

175 The inhibitory effects of the tested extracts against α-amylase were evaluated by a common method,
176 namely Caraway-Somogyi iodine/potassium iodide (IKI). In brief, sample solutions (2 mg/mL, 25
177 µL) were premixed with the α-amylase solution (50 µL) in phosphate buffer (pH 6.9) After 10 min
178 at 37 °C, a starch solution (50 µL, 0.05%) was added into these mixtures. The final mixtures were
179 left to stand for 10 min at 37 °C and HCl solution was used to stop the reaction (1 mM, 25 µL).
180 Blank solution was prepared without the enzyme. At last, the iodine-potassium iodide reagent was
181 added and the absorbances were read at 630 nm in a 96-well microplate reader. Acarbose was used
182 as positive inhibitor and the results were evaluated as acarbose equivalents (mmol ACAE/g extract)
183 (Zengin et al., 2016).

184 *2.5.3. α-Glucosidase inhibition*

185 α-Glucosidase inhibition was evaluated by using PNPG (4-nitrophenyl β-D-glucopyranoside) as
186 substrate. The extracts (2 mg/mL, 50 µL) were mixed with 50 µL of glutathione (2 mg/mL), 50 µL
187 of enzyme (in phosphate buffer, pH 6.8) and 50 µL of substrate (PNPG, 10 mM). After 15 min at 37
188 °C, 50 µL of sodium carbonate were added to stop the reaction. Blank solution was prepared
189 without the enzyme. The final absorbances were measured at 400 nm in a 96-well microplate
190 reader. Acarbose was used as positive inhibitor and the results were evaluated as acarbose
191 equivalents (mmol ACAE/g extract) (Zengin et al., 2016).

192 *2.5.4. Cell cultures, ROS generation and LDH activity*

193 C2C12 and HCT116 cells were cultured in Dulbecco's modified eagle medium (DMEM)
194 (Euroclone) supplemented with 10% (*v*:*v*) heat-inactivated fetal bovine serum and 1.2% (*v*:*v*)
195 penicillin G/streptomycin in 75 cm^2 tissue culture flask (n = 5 individual culture flasks for each
196 condition), as previously described (Menghini et al., 2011). Morphology of cells was carefully
197 examined under an inverted phase-contrast microscope (Sintiprungrat et al., 2010). To assess the
198 basal cytotoxicity of all saffron extracts, cells were incubated on 96 microwell plates with extracts
199 (ranging concentration 10-100 µg/mL) for 24 h, using 3-(4,5-dimethylthiazol-2-yl)-2,5-
200 diphenyltetrazolium bromide (MTT) test. 10 µL of MTT solution (5 mg/mL) were added to each

well and incubated for 3 h. Effects on cell viability were evaluated in comparison to untreated controls.

ROS generation was measured through a well-known ROS-sensitive fluorescence indicator, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). To this regard, cells were seeded in a black 96-well plate (1.5 x 10^4 cells/well) in medium containing 25 µg/mL extracts and stimulated for 1 h with H_2O_2 (1 mM). After the incubation period (30 min) with DCFH-DA (20 µM), the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

LDH activity was measured by evaluating the consumption of NADH in 20 mM HEPES-K^+ (pH 7.2), 0.05% bovine serum albumin, 20 µM NADH and 2 mM pyruvate using a microplate reader (excitation 340 nm, emission 460 nm) according to manufacturer's protocol (Sigma-Aldrich). Extracts were tested at 25 µg/mL. Data were from triplicate test and expressed as relative variations compared to vehicle-treated cells (Menghini et al., 2011).

2.5.5. Ex vivo studies

Eighteen male adult Sprague-Dawley rats (200-250 g) were housed in Plexiglas cages (40 cm × 25 cm × 15 cm), placed in climatized colony rooms (22 ± 1 °C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h). Rats were fed ad libitum a standard laboratory diet (chow; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the EU Directive 63/2010/EU. Colon specimens were obtained as residual material from vehicle-treated rats randomized in our previous experiments approved by Italian Health Ministry (Project N. 880/2015). Rats were sacrificed by CO_2 inhalation and colon specimens were maintained in humidified incubator with 5% CO_2 at 37 °C for 4 h, in DMEM buffer with added bacterial LPS (10 µg/mL) (incubation period), as previously described (Chiavaroli et al., 2017). During the incubation period, tissues were treated with scalar sub-toxic concentrations of stigmas extracts (25 µg/mL). Finally, MDA level was determined by thiobarbituric acid reactive substances (TBARS) method (Mihara et al., 1980).

2.6. Statistical Analysis

GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) was used as statistical analysis software. Experiments were performed at least in triplicate and results are presented as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was employed to assess significant differences ($p<0.05$). As regards the animals randomized for each experimental group, the number was calculated on the basis of the "Resource Equation" N=(E+T)/T (Charan and Kantharia, 2013).

3. RESULTS AND DISCUSSION

3.1. Extraction and phytochemical characterization

As reported in Figure 1, over 90% of total fresh weight corresponds to byproducts consisting in tepals and anthers (CTA), which are usually discarded. CST extract contains a high crocin and safranal content, with the highest and statistically significant recovery in the following order: water>olive oil>ethanol as the solvent, whereas for CTA the olive oil was the elective extraction solvent for crocin (Table 1). On the other hand, total phenolic and flavonoid content was higher in water CTA extract, compared CST (Table 2). This finding is consistent with the study by Serrano-Díaz and collaborators (2012). More recently, Cusano and colleagues (2018) demonstrated significant amounts of anthocyanins in saffron tepals, from samples cultivated in multiple areas, including Abruzzo (Italy). The quantitative data, compared to those of CST are 40-130 times lower in CTA. Focusing on the main quality markers for commercial saffron, such as crocin and safranal, the highest and statistically significant concentrations are, as expected, in CST extracts (Table 1). On the other hand, experimental data attest that a small amount is present in byproducts and particularly in their olive oil extract. This result suggests that extraction of CTA in olive oil could represent a promising strategy to obtain low cost products containing active metabolites and could suggest further studies to investigate applicative use of saffron lipophilic extracts. From all the floral parts, the most abundant phenolic derivative resulted to be catechin (Table 1). In agreement with the study by Serrano-Díaz and collaborators (2012), the total phenolic content results higher in

CTA (Table 2), while CST is the main site of accumulation of safranal and carotenoids such as crocin (Table 1).

3.2. Antioxidant and protective effects

Antioxidant measurements (in phosphomolybdenum, FRAP, CUPRAC, ABTS, DPPH, and metal chelating assays) indicated a solvent effect with higher efficacy related to water extracts compared to olive oil extracts. As reported in Table 2, the water extracts displayed higher radical scavenging activity, compared to oil extracts, as revealed by DPPH and ABTS tests. Both tests are common methods for evaluating *in vitro* antiradical activity. On the other hand, these radicals have different characters. From this perspective, ABTS assay is superior to the DPPH assay when applied to samples containing hydrophilic, lipophilic, and highly pigmented antioxidant compounds (Kim et al., 2002). FRAP, CUPRAC and phosphomolybdenum assays evaluated the reducing potential of the extracts, as previously reported (Zengin et al., 2018; Uysal et al., 2018). Similarly, these assays confirmed the best reducing abilities exerted by water extracts, compared to oil extracts (Table 2). Our results also demonstrated that water extracts were more active for metal chelating ability (Table 2). Finally, the tested water extracts exhibited the best antioxidant properties in these assays (Table 2). To this regard, obtained results could be related to higher amounts of total phenols and flavonoids in the water extracts, compared to olive oil extracts (Bahadori et al., 2017).

Water, olive oil and alcohol extracts from all floral parts were also tested *in vitro* to evaluate their modulatory effects on cell viability and oxidative stress. We observed that saffron extracts (10-100 µg/mL) were well tolerated by C2C12 cells, while viability of HCT116 was reduced at concentration ranging 50-100 µg/mL (data not shown). As previously reported, the sensitivity of HCT116 line to cytotoxic effects of the herbal extracts (Locatelli et al., 2017) could depend on the low grade of differentiation. Considering the results of viability test, the effects on ROS production were evaluated at the sub-toxic concentration of 25 µg/mL extracts. In both cell lines the supplementation of extracts significantly reduced the ROS production induced by hydrogen peroxide (Figures 2A-D). CST extracts resulted more effective compared to CTA extracts.

Additionally, water CST extract showed a better inhibitory trend on ROS production, with respect to olive oil and ethanol CST extracts, despite there being no statistical difference (Figures 2A-D). On the other hand, extracts from CTA also reduced hydrogen peroxide-induced ROS level, with the highest activity expressed by water extract. Also for CST extracts, water extract was the most effective in reducing hydrogen peroxide-induced oxidative stress in both cell lines (Figures 2C-D). Furthermore, experimental data showed that the supplementation of both water CST and CTA extracts reduced the LDH activity in HCT116 cells challenged with hydrogen peroxide (Figure 3). This measurement further confirmed the higher activity of CST extract compared to CTA (Figure 3). LDH could be considered a marker of tissue damage, especially in the gut, and reduced LDH activity following herbal extracts treatment has been related to protective effects in IBDs (Kannan & Guruvayoorappan, 2013; Nagarjun et al., 2017). Despite the lower content on total phenolic and flavonoid compounds, water CST extract displayed high crocin levels which could explain, albeit partially, the major blunting effect on ROS and LDH activity compared to CTA extract, in the tested cell lines (Bharti et al., 2012; Lv et al., 2016; Cusano et al., 2018).

Based on this evidence, we tested CST water extract in isolated colon specimens treated with LPS, observing a significant reduction of LPS-induced increase in MDA levels, a recognized biomarker of lipid peroxidation (Ferrante et al., 2017; Mancuso et al., 2012). The reduced level of MDA, in colon specimens (Figure 4), is consistent with an antioxidant effect by water CST extract, as revealed by the blunting effect on hydrogen peroxide-induced ROS levels. The imbalance in the pro-oxidant/antioxidant homeostasis is characterized by overproduction of ROS, that could lead to the onset of peroxidation reactions on cellular biomolecules such as proteins, lipids, and nucleic acids (Chiavaroli et al., 2017). Particularly, lipid peroxidation has long been involved in tissue damage related to several chronic disease states, including IBDs (Achitei et al., 2013). Macrophages and neutrophils are the main production sites of ROS whose effects include neutrophil recruitment at the inflamed epithelial colon tissue. Previously, we observed that LPS stimulus was effective in inducing multiple pathways of oxidative stress and inflammation in the colon (Locatelli et al., 2017;

Menghini et al., 2011). Actually, this effect is consistent with the elevated concentration of phenolic compounds found in the extract (Table 1). Phenolics and particularly flavonoids were found to be effective in reducing the burden of oxidative stress, in the colon of mice intraperitoneally injected with LPS, via suppressing phosphorylation in mitogen-activated protein kinases (MAPKs) pathway, which is crucial for macrophage activation and the production of inflammatory mediators (Lin, Huang, Chu, & Lin, 2010; Tao, Wei, & Hu, 2016).

3.3. Enzyme inhibitory activity

AD and T2D are chronic diseases that often occur together in aged individuals. AD is characterized by neurodegeneration associated with progressive behavioral, cognitive and memory functions decline (Wang et al., 2017). On the other hand, T2D is a chronic metabolic disease characterized by peripheral insulin resistance associated with pancreatic β-cell and insulin deficit. Epidemiological studies revealed close relationships between AD and T2D onset (Fei, Yan Ping, Ru Juan, Ning Ning, & Lin, 2013; Garcia-Casares et al., 2014), consistently with the possible involvement of common physiopathological mechanisms, including inflammation, insulin resistance and oxidative stress (Golpich et al., 2017; Mittal & Katare, 2016). Considering both the antioxidant effects exerted by saffron extracts in our *in vitro* tests, and the enzyme inhibition theory, which is considered as one of the most promising tool to contrast these diseases (Mocan, Zengin, Crişan, & Mollica, 2016; Mocan et al., 2017), we further investigated the protective effects of the extracts by evaluating their effect on AChE, BChE, α-glucosidase and α-amylase activity (Table 3). In agreement with the observed antioxidant effects, both water CST and CTA extracts, which displayed the highest content in phenolic compounds, revealed to be the most effective in inhibiting cholinesterases and α-glucosidase (Ademosun, Oboh, Bello, & Ayeni, 2016; Martinez-Gonzalez, Díaz-Sánchez, Rosa, Vargas-Requena, & Bustos-Jaimes, 2017), despite there being no significant difference in the inhibitory effects exerted by olive oil and water extracts on α-amylase activity. Additionally, the major α-glucosidase activity inhibition, compared with α-amylase, is consistent with previous studies (Llorent-Martínez et al., 2017). These results further support a major

inhibitory selectivity of phenolic compounds toward α-glucosidase, rather than α-amylase through the formation of non-covalent interactions with the target enzyme (Martinez-Gonzalez et al., 2017).

4. Conclusions

Concluding, the present findings showed protective effects exerted by CST and CTA extracts, in *in vitro* and *ex vivo* models of inflammation and oxidative stress. Additionally, the CTA extracts also revealed to be as promising enzyme inhibitory agents. The results support further studies for the valorization of high quality byproducts of saffron, which are usually discarded. Keeping in mind the findings of this work, we suggest an intriguing approach to recycle the flower waste fraction in order to optimize and develop the production of Abruzzo saffron.

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CONFLICT OF INTEREST

Authors declare no financial/commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at

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TABLE AND FIGURE CAPTIONS

Table 1. Phenolic fingerprint of CST and CTA extracts. Values are expressed in µg/mg dry extract. Data represent the mean ± S.D. (n = 3). In each row, different letters mean significant differences ($p<0.05$).
Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers.

Table 2. Antioxidant properties of CST and CTA extracts. Values expressed are means ± S.D. of three parallel measurements. Data represent the mean ± S.D. (n = 3). In each row, different letters mean significant differences ($^{a}p<0.05$; $^{b}p<0.01$).
Abbreviations: TE: trolox equivalents; EDTAE: EDTA equivalents. GAE: gallic acid equivalents; RE: rutin equivalents; na: not active; CST: *Crocus sativus* stigmas; CTA: tepals+anthers.

Table 3. Enzyme inhibitory effects of CST and CTA extracts. Values expressed are means ± S.D. of three parallel measurements. Data represent the mean ± S.D. (n = 3). In each row, different letters mean significant differences ($^{a}p<0.05$; $^{b}p<0.01$).
Abbreviations: AChE: acetylcholinesterase; BChE. butyrylcholinesterase; GALAE. galantamine equivalents; ACAE. acarbose equivalents; na: not active; CST: *Crocus sativus* stigmas; CTA: tepals+anthers.

Figure 1. Mean weight of each part of *Crocus sativus* fresh flower.

Figure 2A. Effects of CST and CTA extracts (25 µg/mL) supplementation on ROS production in HCT116 cell line. ANOVA $p<0.05$; as regards post-hoc test the letter "a" indicates significant differences compared to CTR group. $^{a}p<0.05$ *vs*. CTR group.
Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers; CTR: control.

Figure 2B. Effects of CST and CTA extracts (25 µg/mL) supplementation on ROS production in C2C12 cell line. ANOVA $p<0.05$; as regards post-hoc test the letter "a" indicates significant differences compared to CTR group. $^{a}p<0.05$ *vs*. CTR group.
Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers; CTR: control.

Figure 2C. Protective effects of CST and CTA extracts (25 µg/mL) on hydrogen peroxide-induced ROS production in HCT116 cell line. ANOVA $p<0.05$; as regards post-hoc test the letter "a" indicates significant differences compared to H_2O_2 group. $^{a}p<0.05$ *vs*. H_2O_2 group.
Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers; CTR: control.

Figure 2D. Protective effects of CST and CTA extracts (25 µg/mL) on H_2O_2-induced ROS production in C2C12 cell line. ANOVA $p<0.05$; as regards post-hoc test the letter "a" indicates significant differences compared to H_2O_2 group. $^{a}p<0.05$ *vs*. H_2O_2 group.
Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers; CTR: control.

Figure 3. Effects of CST and CTA water extracts (25 µg/mL) on H_2O_2-induced LDH activity in HCT116 cell line. ANOVA $p<0.0001$; as regards post-hoc test different letters indicate significant differences compared to H_2O_2 group. $^{a}p<0.05$, $^{b}p<0.01$ *vs*. H_2O_2 group.
Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers; CTR: control; LDH, lactate dehydrogenase.

545 **Figure 4**. Effects of CST water extract (25 µg/mL) on LPS-induced MDA production in rat colon
546 tissues, *ex vivo*. ANOVA $p<0.05$; the letter "a" indicates significant differences compared to LPS
547 group. $^{a}p<0.05$ *vs*. LPS group.
548 Abbreviations: CST: *Crocus sativus* stigmas; CTA: tepals+anthers; CTR: control; LPS:
549 lipopolysaccharide; MDA: malondialdehyde.