Food and Chemical Toxicology 126 (2019) 7–14

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Crocus sativus by-products as sources of bioactive extracts: Pharmacological and toxicological focus on anthers

Giuseppe Chichiriccò[b], Claudio Ferrante[a], Luigi Menghini[a,*], Lucia Recinella[a], Sheila Leone[a], Annalisa Chiavaroli[a], Luigi Brunetti[a], Simonetta Di Simone[a], Maurizio Ronci[c], Pierpaolo Piccone[d], Barbara Lanza[e], Stefania Cesa[f], Annamaria Poma[b], Giulia Vecchiotti[b], Giustino Orlando[a]

[a] Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100, Chieti, Italy
[b] Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio, 67010, Coppito, L'Aquila, Italy
[c] Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100, Chieti, Italy
[d] Regional Agency for the Protection of the Environment, Provincial District of L'Aquila, Caselle di, Bazzano, AQ, Italy
[e] Council for Agricultural Research and Economics (CREA), Research Centre for Engineering and Agro-food Processing (CREA-IT), Via Nazionale 38, 65012, Cepagatti, PE, Italy
[f] Department of Drug Chemistry and Technologies, Sapienza University of Rome, P.le A. Moro 5, 00185, Rome, Italy

ARTICLE INFO

Keywords:
Crocus sativus L.
Anthers
Oxidative stress
Lipid peroxidation
Wound healing
Genotoxicity

ABSTRACT

Multiple studies revealed the potential application of high quality saffron byproducts as cheap sources of bioactive compounds endowed with antioxidant activity. In the present study, we analyzed the total fatty acids of the anthers, and explored the pharmacological and toxicological potential of anthers, by evaluating genotoxic and protective effects in multiple cell lines, brine shrimps and isolated rat tissues.

The phytochemical analyses showed that anthers are rich in long chain fatty acids most of which are unsaturated (80.51%). Particularly, anther water extract revealed to be well tolerated by multiple cell lines, and able to modulate reactive oxygen species (ROS) levels, without exerting either genotoxic or cytotoxic effects. The same extract was also able to blunt lipopolysaccharide (LPS)-induced nitrite and malondialdehyde (MDA) in isolated rat tissues. On the other hand, considering the concomitant null effect on HCT116 cell migration, in wound healing experimental paradigm, our findings suggest the efficacy of water anther extract as protective agent without any direct reverting effects on lesioned tissues.

Concluding, the promising results, deriving from the pharmacological and toxicological evaluations, support the valorization of saffron anthers as a strategy to optimize and develop the productive chain of Abruzzo saffron.

1. Introduction

Micro-macroelements, proteins, fatty acids and antioxidants are important factors for human nutrition. The male gametophyte of angiosperms, pollen, is a source of these components, so it is used by man, particularly that of bees, for dietary (Frias et al., 2016; Locatelli et al., 2018) and therapeutic applications (Komosinska-Vassev et al., 2015). The components are produced and stored by the pollen inside its cells and, in the case of entomophilous pollen, they are also produced and deposited from the sporophytic tissues of the anther on the outer pollen wall forming a sticky lipid layer called pollenkitt. This layer plays key roles in protecting and dispersing pollen, as well as in the pollen stigma interaction (Pacini and Hesse, 2005; Lin et al., 2013).

Saffron (*Crocus sativus* L.) is well known throughout the world for the expensive saffron spice that is obtained from dried stigmatic lobes of the pistil. In addition to its organoleptic properties, saffron spice is known for its therapeutic applications in many diseases and its potential arises from the antioxidant and anti-inflammatory properties of its components such as carotenoid pigments and its derivatives (Poma et al., 2012). In the last decades the cultivation of saffron in Italy was subjected to renewed attention and the total cultivation is increasing. This is due, albeit partially, to appropriate crop techniques, such as lifespan and plant density that could significantly improve quantitative characteristics of saffron and economic value of plant production

Abbreviations: LPS, lipopolysaccharide; MDA, malondialdehyde; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

* Corresponding author. Dipartimento di Farmacia, Università "G. D'Annunzio" Chieti-Pescara, Via dei Vestini 31, 66100, Chieti, Italy.
E-mail address: luigi.menghini@unich.it (L. Menghini).

https://doi.org/10.1016/j.fct.2019.01.040
Received 20 July 2018; Received in revised form 11 January 2019; Accepted 20 January 2019
Available online 11 February 2019
0278-6915/

(Temperini et al., 2009). 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"). Given the high cost of the saffron spice there is growing interest for possible profitable uses of the floral parts such as the tepals and anthers, after the removal of the stigmatic lobes from the saffron flowers. In saffron, the anther consists of a wall of 4 monolayered tissues enclosing two techae each containing 2 pollen sacs where pollen develops, which is typically rich in pollenkitt (Chichiriccò, 1999).

A number of biological activities such as antioxidant and metal chelating and high quality byproducts have been reported for saffron flower wastes (Termentzi and Kokkalou, 2008; Argento et al., 2010; Serrano-Diaz et al., 2012, 2013; Sani et al., 2013; Zeka et al., 2015). These studies point out that anthers are rich in proteins, sugars, and less in lipids, and they contain useful microelements and antioxidant compounds. We also pointed out (Chichiriccò et al., 2016) the dietary properties of saffron anthers resulting from the analysis of the micro-macroelements of pollen, and fatty acid content present both in the pollenkitt and on the inner cell layer of the anther wall. Petals and tepals 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 (Li et al., 2004; Tirillini et al., 2006; Zeka et al., 2015; Ahrazem et al., 2018). Particularly, saffron tepals revealed to be very effective as antioxidant and antimicrobial agents, with promising industrial applications in Pacific white shrimp aquaculture (Abbasvali et al., 2016). Additionally, Hosseinzadeh and Younesi (2002) described anti-inflammatory and antinociceptive effects related to saffron petals. Whereas, Moshiri et al. (2006) observed clinical efficacy of saffron tepals in the treatment of mild-to-moderate depression. We recently showed the protective effects of high quality byproducts such as tepals and anthers in *in vitro* and *ex vivo* pharmacological models of inflammation and oxidative stress (Menghini et al., 2018).

Multiple studies (Tuberoso et al., 2016; Lahmass et al., 2017; Lamass et al., 2018) 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.

Considering these findings, in the present study we aimed to further characterize saffron byproducts from a phytochemical point of view. Particularly, we analyzed the total fatty acids of the anthers, distinguishing those of the pollen from those of the anther devoid of pollen, and the micro-macroelements content of tepals. Furthermore, we explored the pharmacological and toxicological potential of saffron anthers, by evaluating genotoxic and protective effects in multiple cell lines, brine shrimps and isolated rat tissues challenged with lipopolysaccharide (LPS).

2. Material and methods

Anthers with pollen and the tepals of *Crocus sativus* L. (saffron) derived from cultivation of Navelli plateau, in the district of L'Aquila (Italy) (GPS: 42°14′9.580" N – 13°43′42.390" E), and they were collected after the flower picking and the stigmas separation carried out by the farmers. The identification of plant was confirmed by the corresponding author Prof. Luigi Menghini, Associate Professor in Botany at the Department of Pharmacy of "G. d'Annunzio University". Qualitative fingerprint has been previously reported (Menghini et al., 2018), and data are consistent with the findings of Mangal et al. (2018). Anthers were removed from tepals and air-dried at room temperature for some days and then a third of them were separated from pollen grains with a brush so that the final samples consisted of: 1) anthers with pollen, 2) anthers devoid of pollen and 3) pollen grains. Tepals were dried with a stove at 40 °C and then were ventilated to remove residual pollen.

2.1. Fatty acids in anthers and pollen

To analyze the fatty acids, 0.9 g of anther devoid of pollen and 0.5g of pollen grains were frozen at −80 °C, crushed separately in two mortars with pestle, and then each was treated with n-hexane for 30 min at room temperature. The next procedures for obtaining the solution with the extract to be analyzed, the methods used for analysis with the gas chromatography system and the calculation of the fatty acid percentages are those already described in our previous study (Chichiriccò et al., 2016). The analyses were in duplicate for each sample.

2.2. Micro-macroelements in tepals

Samples consisted of 0.9 g of dried tepals and they were analyzed by the Optima 8300 ICP-OES spectrometer and by the PerkinElmer AAnalyst 800 atomic absorption spectrometer according to the methods described in our previous article mentioned above.

2.3. Skin fibroblasts (Hs27): cell culture

The human skin fibroblasts Hs27 (ATCC® CRL-1634™) were purchased from American Type Culture Collection. They have been grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100UI/ml) and streptomycin (100 µg/ml), and maintained at 37 °C in a humidified atmosphere (95%) under 5% CO_2. Cells were seeded at a density of about 10,000 cells/cm^2 and amplified with an interval of 3–4 days, as necessary. The cells were removed to the plates with 0.05% trypsin, 0.02% EDTA solution and resurfaced 24 h before exposure to the treatment. All cellular materials were purchased from Sigma-Aldrich.

2.4. Skin fibroblasts (Hs27): cytokinesis-block micronucleus (CBMN) assay

CBMN assay was carried out with slight modifications according to the protocol of Fenech et al. (2007) and OECD guideline, 2010. A total of 2.5×10^5 cells were seeded in each flask and after 24 h of culture, the cells were used for controls and treatments. Cells were exposed to three different concentrations of plant extract, 10, 100, 500 µg/mL for 24 h, as a positive control colchicine was used at the concentration of 5 µg/ml. After the treatment, cytochalasin B (antimitotic, optimal for blocking the cells at the metaphase stage) at a final concentration of 5 µg/ml was added to the cell cultures. After 24 h, the cells of each flask were detached with 2.5 mL of trypsin, for 5 min and were neutralized with 2.5 mL of medium. Cells were then centrifuged for 8 min at 1100 g, the supernatant was removed and a hypotonic solution of 0.4 N KCl was added at a concentration of 75 mM. The cells are treated with the hypotonic solution in order to improve their subsequent observation under an optical microscope. At this point the cells were washed in PBS, resuspended (about 5×10^6 cells/ml) and re-centrifuged; immediately afterwards they are prefixed with 1 mL of methanol/acetic acid solution in a 3:5 ratio and distributed on slides (150 µl of cell suspension for slide). After air drying, the cells were fixed with a solution of methanol/glacial acetic acid (6:1, v:v) for 10 min and stained with a Giemsa solution (1:20, v:v) at 5% for 8 min. All procedures were conducted at room temperature. Using the Leitz optical microscope with 400× and 1000× magnification and following the criteria of the OECD guideline (2010), 1000 cells were analyzed for each condition. Three technical replicates were made for each condition, used for the CBMN analysis. The Hs27 cells were treated for 24 h with the anther extracts at 10, 100 and 500 µg/ml in order to evaluate effects on the induction of micronuclei (MN). Replication Index (RI) was calculated as follows (Anton et al., 2017):

((No. Binucleated cells) + (2 × No. multinucleated cells)) ÷ (Total No. of cells)$_{Treated}$

RI = ((No. Binucleated cells) + (2× No. multinucleated cells)) ÷ (Total No. of cells)$_{Control}$

2.5. . Artemia salina lethality bioassay

Artemia salina cysts were hatched in oxygenated artificial sea water (1 g cysts/L). After 24 h, brine shrimp larvae were gently transferred with a pipette in 6 well plate containing 2 ml of anther extract at different concentrations (0.1–20 mg/mL) in artificial sea water. Ten larvae *per* well were incubated at 25–28 °C for 24 h (Taviano et al., 2013). After 24 h the number of living napulii were counted under light microscope and compared to control untreated group. Results were expressed as percentage of mortality calculated as: ((T– S)/T)*100. T is the total number of incubated larvae and S is the number of survival napulii. Living nauplii were considered those exhibiting light activating movements during 10 s of observation. For each experimental condition two replicates per plate were performed and experimental triplicates were performed in separate plates.

2.6. C2C12, MCF7 and HCT116 cell lines: cultures, viability assay, ROS generation and wound healing assay

MCF7 (ATCC® HTB-22™), C2C12 (ATCC® CRL-1772™) and HCT116 (ATCC® CCL-247™) cells were cultured in Dulbecco's modified eagle medium (DMEM) (Euroclone) supplemented with 10% (*v:v*) heat-inactivated fetal bovine serum and 1.2% (*v:v*) penicillin G/streptomycin in 75 cm^2 tissue culture flask (n = 5 individual culture flasks for each condition), as previously described (Menghini et al., 2016). Morphology of cells was carefully examined under an inverted phase-contrast microscope. To assess the basal cytotoxicity of anther extract, human tumoral breast (MCF7) and colon (HCT116) cell lines were incubated on 96 microwell plates with extracts (ranging concentration 10–100 μg/mL) for 24 h, using 3-(4,5-dimethylthiazol-2-yl)-2,5-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 × 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 (Locatelli et al., 2018). Finally, we tested anther extract on HCT116 cell lines, in wound healing experimental paradigm. Cell migration was determined using the scratch wound healing assay with slight modification (Ju, 2012). HCT116 cells (6 × 103 cells/well) were seeded on 6-well plastic plates. Cell monolayer was preliminarily treated with a proliferation inhibitor mitomycin C (sigma-Aldrich) at the non-toxic concentration of 5 μM, in order to exclude the effect of cell proliferation (Taniguchi et al., 2018). After 2 h, on cells in the confluence interval 85–90%, a wound was generated by scratching the cell monolayer using a 0-200 μl pipette tip. Two gentle washes with PBS were performed in order to remove suspended and damaged cells. Cells were incubated in serum free media supplemeted with saffron extract at the non-toxic concentration of 100 μg/ml. Cell migraton was followed capturing at least 3 microscope images/well at time 0, 24, 48 and 72 h. An inverted light microscope Leika equipped with Nikon 5100 camera was used to capture image at 4 × magnification. The quantification of scratch area with no cells were quantified using Image-J software (NIH). Using GraphPad software, mean data at T0, 24, 48 and 72 h were calculated for untreated control and saffron group and expressed as percentage variation with reference to relative 100% of at 0 h.

2.7. Pharmacological ex vivo studies

Eighteen male adult Sprague-Dawley rats (200–250 g) were housed in Plexiglass 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. According to the recognized ethical principles of "Replacement, Refinement and Reduction of Animals in Research", bladder, cortex, esophagus, hypothalamus, kidney, lung, prostate and stomach specimens were obtained as residual material from vehicle-treated rats randomized in our previous experiments approved by Local Ethical Committee (University "G. d'Annunzio" of Chieti-Pescara) and Italian Health Ministry (Approval Number: 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 (Locatelli et al., 2017). During the incubation period, tissues were treated with a sub-toxic concentration of anther extract (125 μg/mL). Malondialdehyde (MDA) and nitrite levels were determined by thiobarbituric acid reactive substances (TBARS) method (Ferrante et al., 2017; Zengin et al., 2017).

2.8. 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. Phytochemical analysis

Table 1 reports the total fatty acids of either the pollen, i.e. those present in the pollen cells plus those in the pollenkitt, or the anther devoid of pollen. The data show a variety of fatty acids in the pollen with high predominance of acids with long chain most of which are unsaturated (80.51%) so that the ratio between unsaturated and saturated reaches a very high value (4.1). Among the unsaturated there are the omega acids (3,6,7,9) with a predominance of the linolenic acid. This acid well known for its health benefits for the man amounts to 50% in the pollen and this high value never reported so far for pollen enhances the quality of saffron pollen. From the comparison between the total fatty acids of the pollen reported in Table 1 and those that make up only the pollenkitt (Chichiriccò et al., 2016), it emerges that pollen cells contribute both to reduce the relative percentage of saturated acids and to increase that of polyunsaturated. The wealth of fatty acids can be related to the triploidy of saffron implying a surplus of chromosomes in the sporophyte and gametophyte cells (Chichiriccò, 1984, 1987).

Anthers devoid of pollen show a variety of fatty acids similar to pollen but with a smaller ratio between unsaturated and saturated (3.2). Small differences result by comparing with previous data (Chichiriccò et al., 2016) concerning the fatty acids spread out on the inner cell layer of the anther wall after the anther dehiscence. Additionally, both pollen and anther wall showed a trend to absorb doses of some heavy metals from soils, despite there being no alarm of metal contamination (Chichiriccò et al., 2016). On the other hand, our

Table 1
Percentage fatty acid content in the pollen and anther fraction of saffron crocus.

Fatty acids	% in pollen	s.d.	% in anther	s.d.	without pollen
Butyric	C4:0	0.44	0.31	0.63	0.01
Capronic	C6:0	0.65	0.25	0.84	0.23
Caprylic	C8:0	0.03	0.01	0.07	0.01
Caprinic	C10:0	0.03	0.01	0.10	0.01
Undecanoic	C11:0	0.00	0.00	0.02	0.00
Lauric	C12:0	1.32	0.23	2.97	0.11
Tridecanoic	C13:0	0.00	0.00	0.00	0.00
Myristic	C14:0	0.00	0.00	0.00	0.00
Myristoleic	C14:1	0.57	0.13	1.04	0.04
Pentadecanoic	C15:0	0.09	0.01	0.12	0.04
Palmitic	C16:0	15.09	0.69	15.13	0.33
Palmitoleic ω7	C16:1	0.05	0.00	0.30	0.03
Heptadecanoic	C17:0	0.06	0.01	0.14	0.01
Heptadecenoic	C17:1	0.03	0.01	0.26	0.30
Stearic	C18:0	0.68	0.30	2.42	0.28
Oleic ω9	C18:1	4.66	0.13	4.26	0.15
Vaccenic	C18:1	0.64	0.02	2.03	0.08
Linoleic ω6	C18:2	19.16	0.21	28.04	0.48
Arachic	C20:0	0.27	0.01	0.25	0.00
Linolenic ω3	C18:3	50.34	0.97	28.85	0.49
Eicosenoic ω9	C20:1	0.92	0.13	1.54	0.59
11,14-Eicosadienoic	C20:2	0.15	0.00	0.35	0.00
Behenic	C22:0	0.71	0.09	0.39	0.01
Erucic	C22:1	2.73	0.06	6.09	0.22
Lignoceric	C24:0	0.18	0.00	0.67	0.25
Eicosapentaenoic	C20:5	0.72	0.01	1.07	0.41
Docosapentaenoic	C22:5	0.43	0.01	1.96	0.20
Docosahexaenoic	C22:6	0.11	0.04	0.51	0.23
Total SFA		19.52		23.73	
Total MUFA		9.59		15.50	
Total PUFA		70.90		60.78	
ω6/ω3		0.38		0.97	

present data (See Table in supplementary materials) show that saffron tepals contain appreciable concentrations of lead and cadmium. Concerning these metals which are listed in the EEC No. 1881/2006 (See supplementary material), the maximum permitted levels in vegetable are 0.10–0.30 mg/kg and 0.05–0.20 mg/kg, respectively, when referred to wet weight. Our concentrations, although determined in dehydrated samples are relatively high and could suggest a trend of saffron tepals to accumulate some heavy metals (See Table in supplementary materials). This could explain, albeit partially, the narrow concentration range of tolerability and effectiveness related to the use of tepal + anther extract, in experimental models of inflammation and oxidative stress (Menghini et al., 2018). Considering the past and recent phytochemical and pharmacological studies (Chichiriccò et al., 2016; Menghini et al., 2018) we further deepened the pharmacological and toxicological aspects of saffron anthers. Particularly, we evaluated both protective and genotoxic effects of anther extracts in multiple experimental models *in vitro* and *ex vivo*.

3.2. Toxicological and pharmacological studies

Table 2 shows the cytokinesis block proliferation index (CBPI), index of evaluation of cell proliferation and therefore of cytostatic and cytotoxic effects with respect to the control. Compared to the control, the CBPI increases for all experimental conditions, therefore there is no significant decrease in the rate of proliferation.

In experiments conducted with cytochalasin B, the cytostasis/cytotoxicity can be quantified from the Replication Index (RI): we used the RI index determination to assess cell proliferation from at least 500 cells per culture (Fig. 1) and to estimate cytotoxicity by comparing values in the Hs27 treated and control cultures. The RI showed a not significant increase in the presence of 10,100 and 500 μg of anther extracts. So we can assume that anther extract was not cytotoxic for Hs27cultures according to RI values. Fig. 2 shows the numbers of Micronuclei (MN)/

Table 2
Micronuclei at different concentrations of plant extracts. CBPI ((No. of mononuclear cells) + (2 × No. of binuclear cells) + (3 × No. of multinucleated cells)) ×500/(total number of cells). BNMN/1000: micronuclei/1000 cells. Positive control (Ctrl+) was evaluated with colchicine at the concentration of 5 μg/mL.

	CBPI	BMNM/1000
CTRL (-)	1,11	4.33
10 μg/mL	1,141	10.66
100 μg/mL	1,141	18.33
500 μg/mL	1,18	12.33
CTRL (+)	1,2	

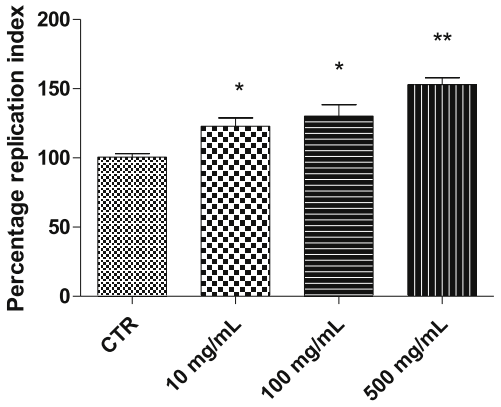

Fig. 1. Replication Index in Hs27. Induction of cytotoxicity according to RI in Hs27 cells in both the conditions (control and saffron anther extract at 10, 100 and 500 μg/ml exposure). Significance values were determined according to the t-Student: $*p < 0.05$, $**p < 0.005$ *vs* Control (CTR)-treated group.

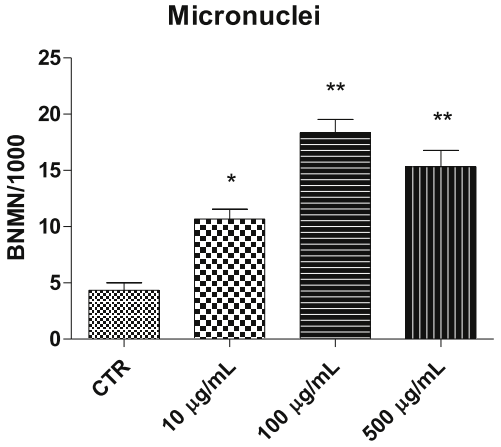

Fig. 2. Micronuclei induction in Hs27 at different concentrations of saffron anther extract (10–500 μg/mL). Micronuclei were evaluated *vs* negative control; BNMN/1000: micronuclei/1000 cells. Significance values were determined according to the t-Student: $*p < 0.05$, $**p < 0.005$ vs Control (CTR)-treated group.

1000 binucleated cells (BNMN). As regards the induction of micronuclei, we observed an increase at the different concentrations of treatment. To this end, we can conclude that, despite an induction of the formation of Micronuclei in Hs27 cell, the extract we have tested did not provide statistically significant results, as regards genotoxic potential. As a preliminary approach to evaluate potential toxicity, anther extract in the concentration range (0.1–20 mg/mL) was tested on brine shrimp mortality. It is a typical and general bioassay that could give information on bioactivity of complex plant extracts evaluated as lethality induced on the brine shrimp, Artemia salina Leach (Taviano

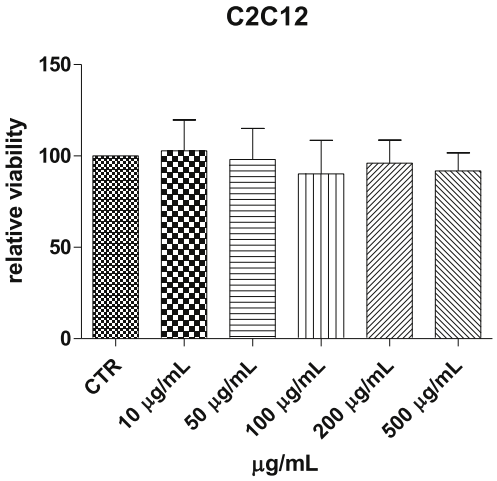

Fig. 3. Effect of saffron anther extract (10–500 µg/mL) on C2C12 cell line viability.

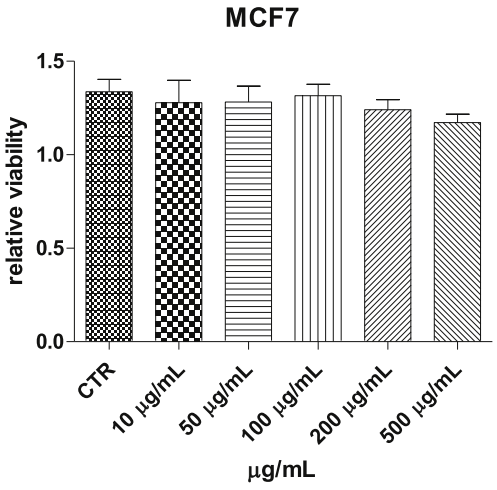

Fig. 4. Effect of saffron anther extract (10–500 µg/mL) on MCF7 cell line viability.

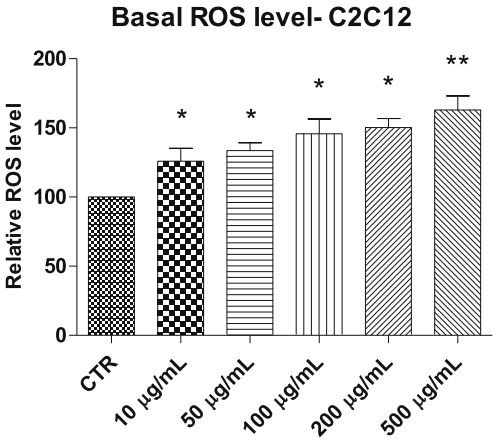

Fig. 5. Effect of saffron anther extract (10–500 µg/mL) on basal ROS production in C2C12 cell line. ANOVA, $p < 0.01$, *post hoc* $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* Control (CTR)-treated group.

et al., 2013). This organism is commonly used to investigate a variety of biological and toxicological activities of plant extracts and is considered, at least partially, predictive of cytotoxicity (Ohikhena et al., 2016). Experimental procedure was conducted following previous

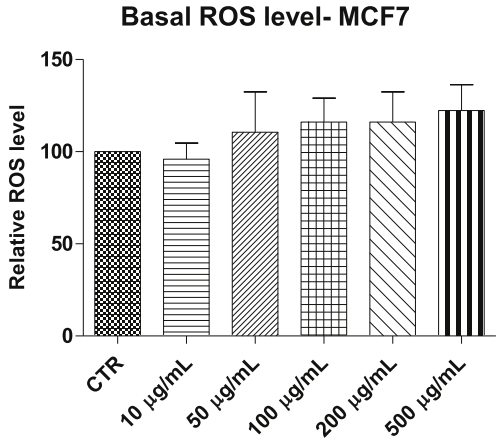

Fig. 6. Effect of saffron anther extract (10–500 µg/mL) on basal ROS production in MCF7 cell line.

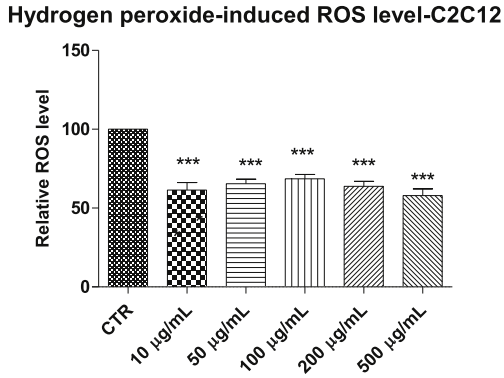

Fig. 7. Effect of saffron anther extract (10–500 µg/mL) on hydrogen peroxide-induced ROS production in C2C12 cell line. ANOVA, $p < 0.0001$, *post hoc* $^{***}p < 0.001$ *vs* hydrogen peroxide-treated group.

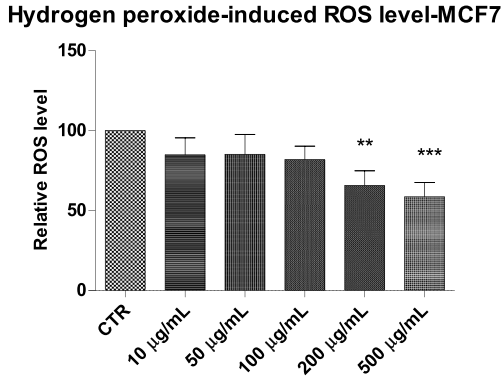

Fig. 8. Effect of saffron anther extract (10–500 µg/mL) on hydrogen peroxide-induced ROS production in MCF7 cell line. ANOVA, $p < 0.001$, *post hoc* $^{**}p < 0.001$, $^{***}p < 0.001$ *vs* hydrogen peroxide-treated group.

published data, with slight modification (Taviano et al., 2013). The anther extract did not reveal any toxicity in the concentration range (0.1–20 mg/mL), with a LC50 ≥ 10.17 mg/mL. In agreement with the null cytostatic, cytotoxic and genotoxic effects in Hs27 cell line, in the concentration range (10–500 µg/mL), and the lack of any toxicity, in Artemia salina lethality bioassay, at extract concentrations ≤5 mg/mL, anther extract also revealed to be well tolerated by MCF7 and C2C12 cell lines in the concentration range (10–500 µg/mL), as showed by the results of MTT test (Figs. 3–4). On the basis of viability test results, we further tested the effects of anther on basal and hydrogen

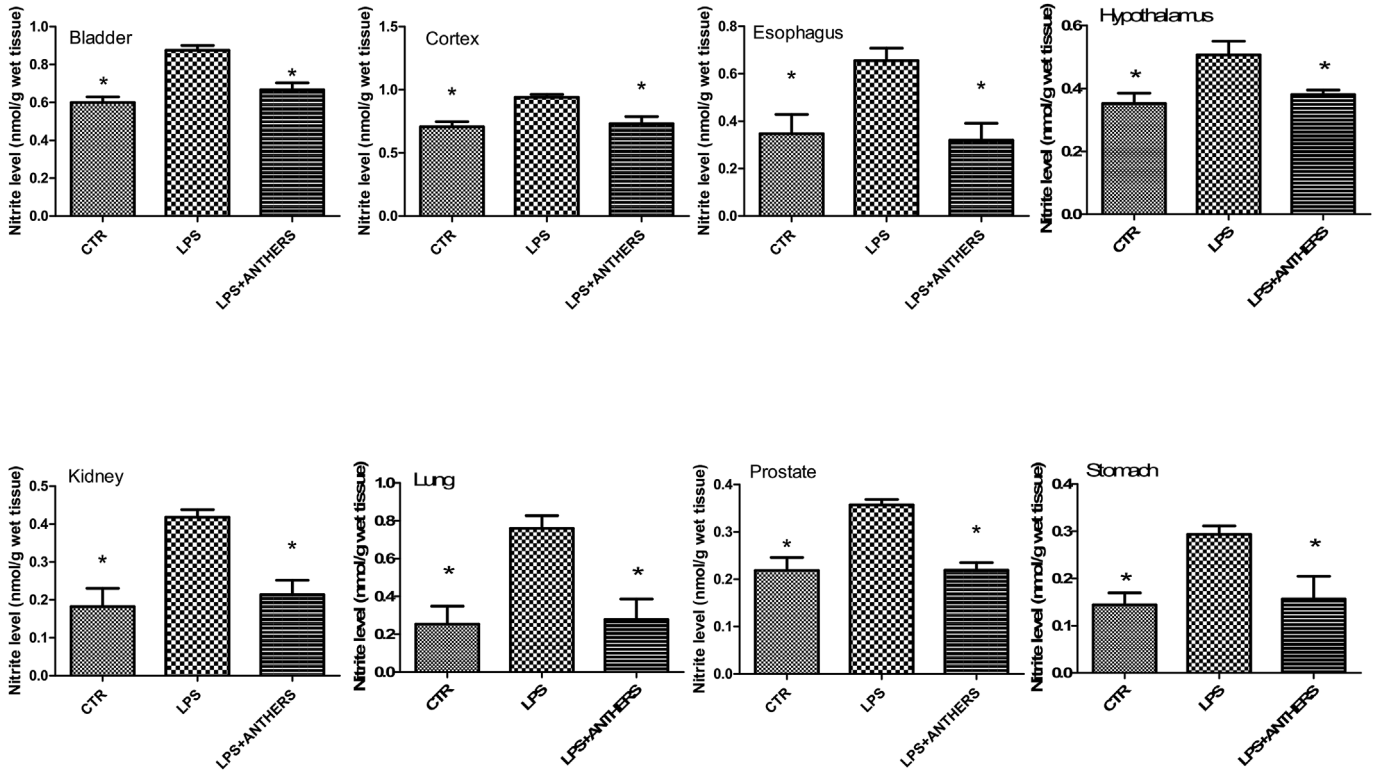

Fig. 9. Effect of saffron anther extract (125 μg/mL) on LPS-induced nitrite level (mmoL/g wet tissue) in multiple rat tissue specimens (Panel A-H). ANOVA, $p < 0.001$; post-hoc, $^{*}p < 0.01$ vs. LPS.

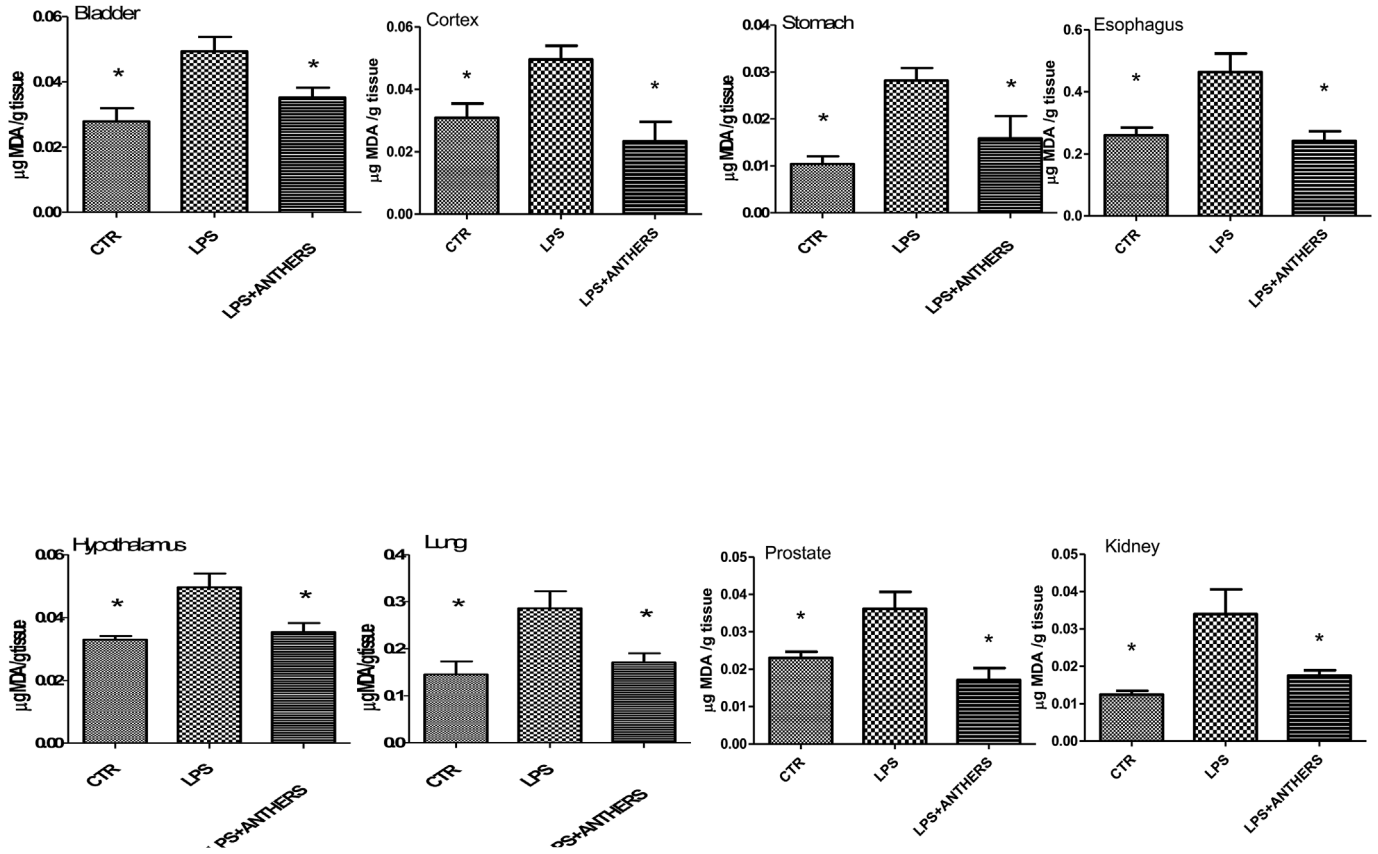

Fig. 10. Effect of saffron anther extract (125 μg/mL) on LPS-induced MDA level (μg/g wet tissue) in multiple rat tissue specimens (Panel A-H). ANOVA, $p < 0.001$; post-hoc, $^{*}p < 0.01$ vs. LPS.

peroxide-induced ROS production, in both C2C12 and MCF7 cell lines. We observed that C2C12 cell lines were more sensitive to the presence of the extract. Particularly, basal ROS production was increased only in C2C12 (Figs. 5–6), which is a non-tumoral cell line, while hydrogen peroxide-induced ROS levels were blunted by the extracts in both cell lines (Figs. 7–8), despite being the extracts more potent in C2C12 cells. These discrepant effects are consistent with more than one speculation. Anthers are rich in antioxidant compounds such as unsaturated fatty acids and flavonoids (Table 2; Menghini et al., 2018), and it is well known that antioxidants in the cell medium could exert pro-oxidative effects, by generating hydrogen peroxide and thus activating adaptive responses of cells to mild oxidative stress (Haliwell et al., 2000). In this context, the findings of increased basal and blunted hydrogen peroxide-induced ROS levels, in C2C12 cells treated with anther extract, could be related to improved cell antioxidant defense system. On the other hand, tumoral cell lines, such as MCF7, have increased production of ROS and reduced capacity to remove ROS due to altered antioxidant defense systems (Abrahim et al., 2012). This could explain, albeit partially, the null effect of anther extract treatment on basal ROS production and the reduced potency in blunting hydrogen peroxide-induced ROS concentration, in MCF7 cells. Based on this evidence, we tested anther extracts (125 μg/mL) on *ex vivo* rat peripheral and central tissues, such as bladder, kidney, stomach, esophagus, lung, prostate, cortex and hypothalamus challenged with LPS, a toxicity model for the evaluation of the efficacy of herbal extracts and drugs involved in inflammatory and oxidative stress modulation (Menghini et al., 2016, 2018; Locatelli et al., 2017, 2018). These evaluations add valuable visibility to recent investigations about the protective role of stigmas and byproducts extracts as antioxidant, anti-inflammatory and enzyme inhibitory agents (Menghini et al., 2018; Hasanapur et al., 2018). In the present study, we have chosen a 5-fold higher concentration of anther extract to treat rat specimens, compared to the previous study (Menghini et al., 2018). This choice was made considering that anther extract was tolerated by C2C12 cells up to the concentration of 500 μg/mL, while the anther + tepal extract was tolerated by the same cells up to a 5-fold lower concentration (100 μg/mL) (Menghini et al., 2018). Actually, this discrepancy in the tolerability between anther and anther + tepal extracts could be related, albeit partially, to the capability of tepals to accumulate heavy metals, at higher levels compared to anthers (See Table in supplementary materials and Chichiriccò et al., 2016). The supplementation of tissue medium with anther extract revealed able to blunt the increased levels of biomarkers such as nitrites and MDA, in all tested tissues (Figs. 9–10). Estimation of nitrite level is a useful marker of the synthesis of nitric oxide (NO) and can potentially be used as an indicator of disease activity in chronic inflammatory conditions (Goggins et al., 2001). NO is a well known free radical which can react with a variety of biomolecules in body fluids and tissues. These interactions produce a number of oxidation products including nitrite, nitrate, nitrosyl (NO-heme) species, and S- and N-nitroso products. The level of these NO-related substances, in fluids and tissues, is assumed to reflect the activity of NO-synthases, including the inducible NO synthase (iNOS) which is expressed at high levels during inflammation (Saijo et al., 2010). The reduced nitrite tissue level is consistent with the possible improvement of antioxidant defense system observed in C2C12 cells, following anther extract treatment. MDA is a recognized marker of lipid peroxidation, and lipid peroxidation may be closer related to nitrosative stress. The common link between MDA and nitrite could be myeloperoxidase (Tsikas et al., 2017), which could be upregulated in all tested tissue challenged with LPS (Menghini et al., 2016). Our findings of reduced MDA level following anther extract treatment further suggest protective effects exerted by anther extract in both peripheral and central tissues. Finally, we explored the effects of anther extract in an experimental model of wound healing in HCT116, a well-known gut tumoral cell line. We observed that the extract did not alter HCT116 cell migration in the 72 h following stimulation (Fig. 11).

Taken together, our findings suggest that anther extract could display preventive tissue damage effects, as revealed by the decreased levels of nitrites and MDA, in LPS-stimulated tissues, without no direct reverting effects on lesioned tissues, as suggested by the null effect in wound healing test.

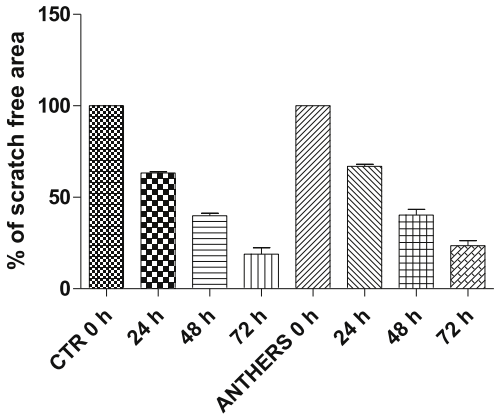

Fig. 11. Effect of saffron anther extract (125 μg/mL) on HCT116 migration.

4. Conclusions

The present study emphasizes that saffron flower is a nutritional source with therapeutic potentiality useful for the human diet and human health. The advantage is double if one considers that saffron flower deprived of stigmatic lobes is a waste for growers. In saffron anthers and pollen, some valuable fatty acids of the omega series are prevalent on the others. These, together with their derivatives, could play a wide plethora of therapeutic activities as antimicrobial, immunomodulatory, neuromodulatory, anti-aging of the skin and of course as anti-inflammatory (Cornara et al., 2017). Seeds of a few plants are the most qualitatively safe source for the human supply of omega fatty acids. The counterparty represented by the fish oil involves the risks due either to teratogenic, mutagenic, carcinogenic activities of marine pollutants or to the use of antibiotics in aquaculture (Abedi and Sahari, 2014). Regarding saffron, an added value is the multiple source of nutrients, namely pollen cells, pollenkitt and anther wall. This precious source is ready for use as food through the easy removal of the stamens from freshly picked flowers and possibly during the removal of stigmatic branches by the growers, thus preventing any contamination due to exposure and manipulation. This is a key point to preserve the nutritional value and quality of saffron pollen (Campos et al., 2008), and the primary one is to avoid saffron cultivation in contaminated soils, in particular by heavy metals which tend to concentrate in stamens and tepals.

Additionally, the promising results, deriving from the pharmacological and toxicological evaluations, suggest the valorization of saffron anthers, which are usually discarded, as potential protective agents. In agreement with the accepted principle of “Circular Economy”, our findings further support an intriguing approach to innovatively improve the high quality byproduct fraction with the final goal to optimize and develop the productive chain of Abruzzo saffron.

Conflicts of interest

Authors declare no financial/commercial conflicts of interest.

Acknowledgements

This work was supported by grants from the Italian Ministry of University (FAR 2015/2016).

The experimental work supports the Abruzzo District project “Filiera Regionale delle Piante Officinali”.

Authors would also express their gratitude to Dr. Lamberto Formiconi of “Terra Italica “ Company and the Consortium of L’Aquila Saffron (Consorzio dello Zafferano dell’Aquila) for providing plant material and technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.040.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.040.

References

Abbasvali, M., Ranaei, A., Shekarforoush, S.S., Moshtaghi, H., 2016. The effects of aqueous and alcoholic saffron (Crocus sativus) tepal extracts on quality and shelf-life of pacific white shrimp (Litopeneous vannamei) during iced storage. J. Food Qual. 39, 732–742.

Abedi, E., Sahari, M.A., 2014. Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. Food Sci. Nutr. 2 (5), 443–463.

Abrahim, N.N., Kanthimathi, M.S., Abdul-Aziz, A., 2012. Piper betle shows antioxidant activities, inhibits MCF-7 cell proliferation and increases activities of catalase and superoxide dismutase. BMC Complement Altern. Med. 12, 220.

Ahrazem, O., Argandoña, J., Fiore, A., Aguado, C., Luján, R., Rubio-Moraga Á, Marro, M., Araujo-Andrade, C., Loza-Alvarez, P., Diretto, G., Gómez-Gómez, L., 2018. Transcriptome analysis in tissue sectors with contrasting crocins accumulation provides novel insights into apocarotenoid biosynthesis and regulation during chromoplast biogenesis. Sci. Rep. 8 (1), 2843. https://doi.org/10.1038/s41598-018-21225-z.

Alonso, G.L., Zalacain, A., Carmona, M., 2012. Saffron. In: second ed. In: Peter, K.V. (Ed.), Handbook of Herbs and Spices 1. Woodhead Publishing, Cambridge, pp. 469–498.

Anton, C., Deng, J., Wong, Y.S., Zhang, Y., Zhang, W., Gabos, S., Yu Huang, D., Jin, C., 2017. Modeling and simulation toxicity assessment. Math. Biosci. Eng. 14 (3), 581–606.

Argento, S., Branca, F., Siracusa, L., Strano, T., Napoli, E.M., Ruberto, G., 2010. Reevaluation of saffron floral wastes: analysis of saffron flowers defatted hydroalcoholic extracts. Acta Hort 350, 251–260.

Campos, M.G.R., Bogdanov, S., de Almeida-Muradian, L.B., Szczesna, T., Mancebo, Y., Frigerio, C., Ferreira, F., 2008. Pollen composition and standardisation of analytical methods. J. Apicult. Res. 47, 156–163.

Charan, J., Kantharia, N.D., 2013. How to calculate sample size in animal studies? J. Pharmacol. Pharmacother. 4 (4), 303–306.

Chichiriccò, G., 1984. Karyotype and meiotic behavior of the triploid Crocus sativus L. Caryologia 37, 233–239.

Chichiriccò, G., 1987. Megasporogenesis and development of embryo sac in *Crocus sativus* L. Caryologia 40, 59–69.

Chichiriccò, G., 1999. Developmental stages of the pollen wall and tapetum in some *Crocus* species. Grana 38, 31–41.

Chichiriccò, G., Lanza, B., Piccone, P., Poma, A., 2016. Nutrients and heavy metals in flowers and corms of the Saffron Crocus (*Crocus sativus* L.). Med. Aromatic Plants 5, 254–258.

Cornara, L., Biagi, M., Xiao, J., Burlando, B., 2017. Therapeutic properties of bioactive compounds from different honeybee products. Front. Pharmacol. 8, 412.

Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. Nat. Protoc. 2 (5), 1084–1104.

Ferrante, C., Recinella, L., Locatelli, M., Guglielmi, P., Secci, D., Leporini, L., Chiavaroli, A., Leone, S., Martinotti, S., Brunetti, L., Vacca, M., Menghini, L., Orlando, G., 2017. Protective effects induced by microwave-assisted aqueous harpagophytum extract on rat cortex synaptosomes challenged with amyloid β-peptide. Phytother Res. 31 (8), 1257–1264.

Frias, B.E.D., Barbosa, C.D., Lourenco, A.P., 2016. Pollen nutrition in honey bees (*Apis mellifera*): impact on adult health. Apidologie 47, 15–25.

Goggins, M.G., Shah, S.A., Goh, J., Cherukuri, A., Weir, D.G., Kelleher, D., Mahmud, N., 2001. Increased urinary nitrite, a marker of nitric oxide, in active inflammatory bowel disease. Mediat. Inflamm. 10, 69–73.

Halliwell, B., Clement, M.V., Ramalingam, J., Long, L.H., 2000. Hydrogen peroxide. Ubiquitous in cell culture and in vivo? IUBMB Life 50 (4–5), 251–257.

Hasanpour, M., Ashrafi, M., Erjaee, H., Nazifi, S., 2018. The effect of saffron aqueous extract on oxidative stress parameters and important biochemical enzymes in the testis of streptozotocin-induced diabetic rats. Physiology and Pharmacology 22 (1), 28–37.

Hosseinzadeh, H., Younesi, H.M., 2002. Antinociceptive and anti-inflammatory effects of Crocus sativus L. stigma and petal extracts in mice. BMC Pharmacol. 2, 7.

Ju, J., Kwak, Y., Hao, X., Yang, C.S., 2012. Inhibitory effects of calcium against intestinal cancer in human colon cancer cells and Apc(Min/+) mice. Nutr Res Pract 6 (5), 396–404.

Komosinska-Vassev, K., Olczyk, P., Kaźmierczak, J., Mencner, L., Olczyk, K., 2015. Bee pollen: chemical composition and therapeutic application. Evid Based Complement Alternat Med 2015, 297425.

Lahmass, I., Lamkami, T., Delporte, C., Sikdar, S., Van Antwerpen, P., Saalaoui, E., Megalizzi, V., 2017. The waste of saffron crop, a cheap source of bioactive compounds. Journal of Functional Foods 35, 341–351.

Lahmass, I., Ouahhoud, S., Elmansuri, M., Sabouni, A., Elyoubi, M., Benabbas, R., Choukri, M., Saalaoui, E., 2018. Determination of antioxidant properties of six by-products of crocus sativus L.(Saffron) plant products. Waste and Biomass Valorization 9 (8), 1349–1357.

Li, C.Y., Lee, E.J., Wu, T.S., 2004. Antityrosinase principles and constituents of the petals of Crocus sativus. J. Nat. Prod. 67 (3), 437–440.

Lin, H., Gomez, I., Meredith, J.C., 2013. Pollenkitt wetting mechanism enables species-specific tunable pollen adhesion. Langmuir 29, 3012–3023.

Locatelli, M., Ferrante, C., Carradori, S., Secci, D., Leporini, L., Chiavaroli, A., Leone, S., Recinella, L., Orlando, G., Martinotti, S., Brunetti, L., Vacca, M., Menghini, L., 2017. Optimization of aqueous extraction and biological activity of harpagophytum procumbens root on ex vivo rat colon inflammatory model. Phytother Res. 31 (6), 937–944 67.

Locatelli, M., Macchione, N., Ferrante, C., Chiavaroli, A., Recinella, L., Carradori, S., Zengin, G., Cesa, S., Leporini, L., Leone, S., Brunetti, L., Menghini, L., Orlando, G., 2018. Graminex pollen: phenolic pattern, colorimetric analysis and protective effects in immortalized prostate cells (PC3) and rat prostate challenged with LPS. Molecules 23 (5), E1145.

Mangal, A.K., Tewari, D., Shantha, T.R., Bansal, S., Mangal, M., 2018. Pharmacognostical standardization and HPTLC fingerprinting analysis of Crocus sativus L. Indian Journal of Traditional Knowledge 17 (3), 592–597.

Menghini, L., Ferrante, C., Leporini, L., Recinella, L., Chiavaroli, A., Leone, S., Pintore, G., Vacca, M., Orlando, G., Brunetti, L., 2016. An hydroalcoholic chamomile extract modulates inflammatory and immune response in HT29 cells and isolated rat colon. Phytother Res. 30 (9), 1513–1518.

Menghini, L., Leporini, L., Vecchiotti, G., Locatelli, M., Carradori, S., Ferrante, C., Zengin, G., Recinella, L., Chiavaroli, A., Leone, S., Brunetti, L., Orlando, G., 2018. Crocus sativus L. stigmas and byproducts: qualitative fingerprint, antioxidant potentials and enzyme inhibitory activities. Food Res. Int. 109, 91–98.

Moshiri, E., Basti, A.A., Noorbala, A.A., Jamshidi, A.H., Hesameddin Abbasi, S., Akhondzadeh, S., 2006. Crocus sativus L. (petal) in the treatment of mild-to-moderate depression: a double-blind, randomized and placebo-controlled trial. Phytomedicine 13, 607–611.

OECD, 2010. Guidelines for the testing of chemicals, section 4:Health effects. Test No. 487. In: Vitro Mammalian Cell Micronucleus Test, pp. 1–23.

Ohikhena, F.U., Wintola, O.A., Afolayan, A.J., 2016. Toxicity assessment of different solvent extracts of the medicinal plant, phragmanthera capitata (sprengel) balle on brine shrimp (Artemia salina). Int. J. Pharmacol. 12 (7), 701–710.

Pacini, E., Hesse, M., 2005. Pollenkitt- its composition, forms and f unction. Flora 200, 399–415.

Poma, A., Fontecchio, G., Carlucci, G., Chichiriccò, G., 2012. Anti-inflammatory properties of drugs from saffron crocus. Antiinflamm. Antiallergy Agents Med. Chem. 11, 37–51.

Saijo, F., Milsom, A.B., Bryan, N.S., Bauer, S.M., Vowinkel, T., Ivanovic, M., Andry, C., Granger, D.N., Rodriguez, J., Feelisch, M., 2010. On the dynamics of nitrite, nitrate and other biomarkers of nitric oxide production in inflammatory bowel disease. Nitric Oxide 22 (2), 155–167.

Sani, A.M., Kakhki, A.H., Moradi, E., 2013. Chemical composition and nutritional value of saffron's pollen (*Crocus sativus* L.). Food Sci. Nutr. 43, 490–496.

Serrano-Díaz, J., Sánchez, A.M., Maggi, L., Martínez-Tomé, M., Murcia, M.A., Alonso, G., 2012. Increasing the applications of *Crocus sativus* flowers as natural antioxidants. J. Food Sci. 77 (11), 1162–1168.

Serrano-Díaz, J., Sanchez, A.M., Martinez, T.M., Winterhalter, P., Alonso, G.L., 2013. A contribution to nutritional studies on *Crocus sativus* flowers and their value as food. J. Food Compos. Anal. 31, 101–108.

Taniguchi, M., Ochiai, A., Namae, T., Saito, K., Kato, T., Saitoh, E., Tanaka, T., 2018. The antimicrobial and anti-endotoxic peptide AmyI-1-18 from rice α-amylase and its [N3L] analog promote angiogenesis and cell migration. Peptides 104, 78–84.

Taviano, M.F., Marino, A., Trovato, A., Bellinghieri, V., Melchini, A., Dugo, P., Cacciola, F., Donato, P., Mondello, L., Güvenç, A., De Pasquale, R., Miceli, N., 2013. Juniperus oxycedrus L. subsp. oxycedrus and Juniperus oxycedrus L. subsp. macrocarpa (Sibth. & Sm.) Ball. "berries" from Turkey: comparative evaluation of phenolic profile, antioxidant, cytotoxic and antimicrobial activities. Food Chem. Toxicol. 58, 22–29.

Temperini, O., Rea, R., Temperini, A., Colla, G., Rouphael, Y., 2009. Evaluation of saffron (Crocus sativus L.) production in Italy: effects of the age of saffron fields and plant density. J. Food Agric. Environ. 7, 19–23.

Termentzi, A., Kokkalou, E., 2008. LC-DAD-MS (ESI^+) analysis and antioxidant capacity of *Crocus sativus* petal extracts. Planta Med. 74 (5), 573–581.

Tirillini, B., Pagiotti, R., Menghini, L., Miniati, E., 2006. The volatile organic compounds from tepals and anthers of saffron flowers (Crocus sativus L.). J. Essent. Oil Res. 18, 298–300.

Tsikas, D., 2017. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: analytical and biological challenges. Anal. Biochem. 524, 13–30.

Tuberoso, C.I.G., Rosa, A., Montoro, P., Fenu, M.A., Pizza, C., 2016. Antioxidant activity, cytotoxic activity and metabolic profiling of juices obtained from saffron (Crocus sativus L.) floral by-products. Food Chem. 199, 18–27.

Zeka, K., Ruparelia, K.C., Continenza, M.A., Stagos, D., Vegliò, F., Arroo, R.R.J., 2015. Petals of *Crocus sativus* L. as a potential source of the antioxidants crocin and kaempferol. Fitoterapia 107, 128–134.

Zengin, G., Locatelli, M., Stefanucci, A., Macedonio, G., Novellino, E., Mirzaie, S., Dvorácskó, S., Carradori, S., Brunetti, L., Orlando, G., Menghini, L., Ferrante, C., Recinella, L., Chiavaroli, A., Leporini, L., Mollica, A., 2017. Chemical characterization, antioxidant properties, anti-inflammatory activity, and enzyme inhibition of Ipomoea batatas L. leaf extracts. Int. J. Food Prop. 20, 1907–1919.