

Biochemical, Antioxidant and Antineoplastic Properties of Italian Saffron (*Crocus sativus* L.)

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ABSTRACT

Saffron, the most expensive spice in the world, is got by *Crocus sativus* L. stigmas. The production of this substance has attracted human interest, since ancient cultures, for its medicinal and culinary properties. Because of saffron high economic value, sometimes, since Middle Ages, it is adulterated with other vegetal materials, dyes or synthetic molecules. Object of this work was the study of one of the best world saffron: Civitaretenga (AQ, Central Italy) *C. sativus*. Taste, color and aroma of Civitaretenga spice were determined, according to international standards (ISO/Technical Specification 3632), to define its high quality. A biochemical approach was then applied to obtain a secondary metabolite profile of this product. So, crocins, total phenolic content, flavonoids and phenolic acids were detected by HPLC-DAD and spectrophotometric analysis. Moreover, *in vitro* antioxidant properties and *in vivo* antineoplastic effects, on highly metastatic murine B16-F10 melanoma cell line, were successfully revealed in Civitaretenga *C. sativus* extract. All these data confirmed the elevated quality of Civitaretenga saffron and its highly reducing and chemopreventive activity.

Keywords: *Crocus sativus* L.; Saffron; Plant Secondary Metabolites; Antioxidant; Antineoplastic

1. Introduction

Crocus sativus L. is an herbaceous species of the Iridaceae family. It is a monocot plant very diffused in the Mediterranean Basin and Western Asia [1]. The infertility of this species has been associated to its triploid genome (3n), whose origin has not been clarified yet: literature data suppose that it would be generated from the evolution, or the hybridization, of other *Crocus* exemplars [2]. Consequently, *C. sativus* only propagates vegetatively [3-5]. Its flower is made up of six violet tepals, three yellow stamens and a single pistil whose stigma, defined by three red filaments, is the source of the saffron [6]. This spice is the most expensive in the world because *C. sativus* bloom occurs, in a very short period, only once a year and its harvest should be performed manually [7]. *C. sativus* stigmas are characterized by the presence of sugars, minerals, fats, vitamins and secondary metabolites: terpenes, flavonoids, anthocyanins and carotenoids. Between them, carotenoids are the most important molecules because they determinate color and taste of the spice. From this class of compounds, in *C. sativus*, we can essentially find lycopene, α - and β -carotene, zeaxanthin and crocetin that are liposoluble and the hydrosoluble crocins, derived by crocetin esterification

with sugars [8-10]. Principal saffron crocins are *trans*-crocetin di-(β -D-gentiobiosil) ester (named *trans*-4-GG), *trans*-crocetin (β -D-glucosil)-(β -D-gentiobiosil) ester (named *trans*-3-Gg), *trans*-crocetin (β -D-gentiobiosil) ester (named *trans*-2-G), *cis*-crocetin di-(β -D-gentiobiosil) ester (named *cis*-4-GG), *trans*-crocetin di-(β -D-glucosil) ester (named *trans*-2-gg) and *cis*-crocetin(β -D-glucosil)-(β -D-gentiobiosil) ester (named *cis*-3-Gg) [11]. High temperatures and humidity levels induce crocin oxidation and degradation and consequently the decrease of spice attributes [12]. Aroma, on the other hand, is determined by the amount of safranal, a terpenic aldehyde, and picrocrocin, a glycosidic form of the safranal [9-13]. Saffron has captured human attention since when past populations employed it as drug, perfume, dye and aroma [14-17]. Its adulteration, motivated by the high economic value, has been performed, since Middle Ages, with natural or synthetic substances [18,19]. For these reasons, international standards [20] were framed to scientifically determine saffron quality, to find out spice fraudulent alterations and for its valorization. According to these conventions, spectrophotometric and chromatographic investigations are able to determine spice color, taste and aroma [21-23]. Object of this work has been the identification of Civitaretenga (AQ, Central Italy) *C. sativus* biochemical components and the quality classification of the saffron

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derived from its stigmas, according to ISO/Technical Specification 3632 standards. As *C. sativus* extract is reported in literature showing a large amount of flavonoids, especially kaempferol, quercetin and naringenin [24,25], in this study, we also analyzed its antioxidant power and antiproliferative effects on murine B16-F10 melanoma cells.

2. Materials and Methods

2.1. Plant Material

Crocus sativus bulbs were obtained from Civitaretenga cultivars (AQ, Central Italy) and grown in the Botanical Garden of the University of Rome "Tor Vergata". For spice production stigmas of *C. sativus* were dehydrated at 103°C for 2, 8 or 16 hours.

2.2. Spice Quality Determination

Spice quality was established, in relation to its taste (picrocrocin content), aroma (safranal amount) and color (crocin level), with respect to ISO/TS 3632 standards [20]. Briefly, 5 mg of saffron were resuspended in 20 mL of ultrapure water at room temperature in the dark for 1 hour and then filtered by a 0.45 µm cellulose filter. Spice quality was analyzed by spectrophotometric method using the following formulas:

- spice taste- $E_{1\text{ cm}}^{1\%}$

$$= (257\text{ nm Abs} \times 20000) / (100\% \text{ moisture content}),$$
- safranal content- $E_{1\text{ cm}}^{1\%}$

$$= (330\text{ nm Abs} \times 20000) / (100\% \text{ moisture content}),$$
- color intensity- $E_{1\text{ cm}}^{1\%}$

$$= (440\text{ nm Abs} \times 20000) / (100\% \text{ moisture content});$$

where $E_{1\text{ cm}}^{1\%}$ is 1% molar extinction coefficient of the solution measured in 1 cm cuvette and 257 nm, 330 nm and 440 nm are absorption wavelengths respectively of picrocrocin, safranal and crocins.

2.3. Secondary Metabolite Extraction

Secondary metabolites extraction was performed on dried (16 h) stigmas according to [22] method. 50 mg of dried stigmas were powdered with liquid nitrogen. The extraction was performed in 10 mL of ddH₂O (or methanol) in agitation at 4°C, in the dark, for 24 hours. After centrifugation at 30.000 g for 20 minutes, the supernatant was filtered and analyzed.

2.4. Total Phenolic Content

Total polyphenol determination was carried out by Folin-

Ciocalteu assay [26]. 9 mL of ddH₂O (or methanol) and 1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich) were added to 1 mL of each secondary metabolite extract. After 5 minutes of incubation, 10 mL of Na₂CO₃ 7% (w/v) and 4 mL of ddH₂O (or methanol) were added. The solution was vortexed and incubated at room temperature for 1 hour in the dark. Absorption was determined at 760 nm by a UV-visible spectrophotometer Cary 50 (Bio Varian). Total polyphenol concentration was calculated with respect to a caffeic acid calibration curve (20 - 100 mg/L) and results were expressed as µg of caffeic acid equivalents (µg CAE/mg DW) of dried stigmas.

2.5. HPLD-DAD Analysis

Crocins, flavonoids and phenolic acids, extracted (in water) as described before, were identified and quantized by a high performance liquid chromatography (HPLC, Shimadzu) instrument associated with DAD detector (SPD-M20A), multisolvent delivery system (LC-10AD), auto-sampler (SIL-10AD), controller module (SCL-10A) and Class VP 5.02 software. Crocin analysis was performed according to Alonso *et al.* [27] with some modifications. A Hamilton PRP-10 column (10 µm, 4.6 × 150 mm) was used and the elution gradient was performed with 10% (v/v) acetonitrile (A) and methanol (B). The gradient system was 100% A to 90% A in 10 minutes and then 0% A to 50 minutes, at flow rate of 1 mL/min. Column re-equilibration between runs was of 10 minutes. The column was kept at 30°C. Crocins (*trans*-4-GG, *trans*-3-Gg, *cis*-4-GG, *trans*-2-G, *cis*-3-Gg and *trans*-2-G) were measured at 440 nm. Flavonoids and phenolic acids were analyzed according to Canini *et al.* [28] with a Phenomenex Gemini NX C18 column (5 µm, 4.6 × 150 mm). Flavonoids (myricetin, quercetin and kaempferol) were monitored at 280 nm while phenolic acids (gallic, chlorogenic and caffeic acids) were monitored at 320 nm. The elution gradient was performed with trichloroacetic acid pH 2.5 (A) and acetonitrile (B). The gradient system was 85% A to 65% A in 20 minutes and then 20% A in 8 minutes and maintained at this concentration for 5 minutes, at flow rate of 1 mL/min. The column was kept at 35°C. Column re-equilibration between runs was of 10 minutes. For each analysis, 20 µL of each sample was injected. Peak identification was assured according to their retention times and by co-elution with authentic standards (Fluka).

2.6. DPPH Radical Scavenging Test

Aqueous saffron extract antioxidant activity was determined on the basis of its scavenging activity on the stable DPPH free radical (Merck). According to Brand-Williams *et al.* [29], we monitored absorbance decrease of a 100 µM DPPH methanolic solution, at 517 nm, after 30

minutes of sample addition. Sample antiradical activity was calculated by the following ratio:

$$(\text{Abs control} - \text{Abs sample} / \text{Abs control}) \times 100 ;$$

where Abs control is DPPH solution absorption and Abs sample is DPPH solution absorption after sample addition. Antioxidant activity was expressed as IC₅₀ (sample concentration producing 50% of DPPH activity decrease with respect to the control).

2.7. FRAP Assay

FRAP assay measures absorbance change at 593 nm owing to the formation of a blue-colored Fe II—tripirydyl-triazine compound (Merck), from the colorless oxidized Fe III form, by the action of electron donating antioxidants [30]. 200 µL of sample was added to 1.8 mL freshly prepared and pre-warmed (37°C) FRAP reagent (10 mM TPTZ in 40 mM HCl, 20 mM FeCl₃, 0.3 M acetate buffer pH 3.6; 1:1:10 v/v/v) and incubated at 37°C for 10 minutes. Fe II standard solution (50 - 500 µM) was obtained from ferrous sulphate (FeSO₄ in ddH₂O). Results were expressed as mM ferric ions reduced to ferrous form per gram of sample dried weight, according to Woidylo *et al.* [31].

2.8. Cell Cultures and Proliferation Assays

Highly metastatic murine B16-F10 melanoma cell line was propagated under standard culture conditions [32]. Briefly, cells were cultured in Dulbecco's modified Eagle's medium (D-MEM), supplemented with 10% fetal calf serum (FCS), 200 mM glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin, and maintained in humidified atmosphere with 5% CO₂ at 37°C. To test *C. sativus* antineoplastic property, B16-F10 cells were seeded and grown in 35-mm dishes and treated with aqueous stigma extracts (250, 500 and 1000 µg/ml) for 24, 48, and 72 hours. Cell growth was determined by MTT based kit (Sigma). This product was designed for the spectrophotometric measurement of cell proliferation rate as a function of mitochondrial activity in living cells. On the other hand, the proliferation was also analyzed by counting cells with a Neubauer modified chamber, after Trypan Blue staining (1%, w/v) for cytotoxicity evaluation. Cell morphology was observed by optical microscope (20×) (Nikon, TE2000-PFS).

2.9. Statistical Analysis

Each experiment was repeated at least three times. Analysis of variance was conducted using one-way ANOVA test with SPSS (ver.19 ita) for Microsoft and means were compared by Duncan tests.

3. Results

3.1. Saffron Quality Determination

Stigmas of Civitaretenga *C. sativus* were dehydrated, powdered and subjected to aqueous extraction. The drying process was conducted at different times (2, 8 and 16 hours). To evaluate saffron taste, aroma and color, the extract was analyzed according to ISO/TS 3632 method. Saffron $E_{1\text{cm}}^{1\%}$ values, obtained by spectrophotometric analysis at different wavelengths, were reported in **Table 1**: all values remained inside ISO/TS 3632 standard ranges, except picrocrocin level after 2h of desiccation. 8 and 16 h dehydration processes decreased picrocrocin amount, respectively of 20% and 37.3%, and increased safranal content, of 7.4% and 22.2%, with respect to 2 h procedure. No considerable variation was observed in crocin amount.

3.2. Secondary Metabolite Analysis

Aqueous or methanolic extraction was performed on *C. sativus* stigmas. Total phenolic content was measured in both extracts: methanolic solution presented 53.52 ± 1.75 µg CAE/mg DW of total polyphenols with respect to the aqueous one that was only 31.46 ± 1.05 µg CAE/mg DW. HPLC-DAD analysis permitted crocin identification in saffron aqueous extract (**Figures 1 and 2**): *trans*-4-GG, *trans*-3-Gg, *trans*-2-gg, *cis*-4-GG, *cis*-3-Gg and *trans*-2-G isomers were detected, at specific retention times (tR, min), and quantified (Q, mg/g) with respect to authentic standards, as reported in **Table 2**. *Trans*-4-GG and *trans*-3-Gg molecules were the most abundant identified crocins. The chromatographic method was also applied to reveal the presence, or the absence, of principal saffron flavonoids (kämpferol, quercetin, genistein and myricetin) and phenolic acids (cumaric, caffeic, chlorogenic and gallic acid) in the sample (**Table 3**).

Table 1. Civitaretenga saffron $E_{1\text{cm}}^{1\%}$ values at 257, 330 and 440 nm for different spice dehydration times. In the lower part are also reported ISO 3632 standard $E_{1\text{cm}}^{1\%}$ value ranges.

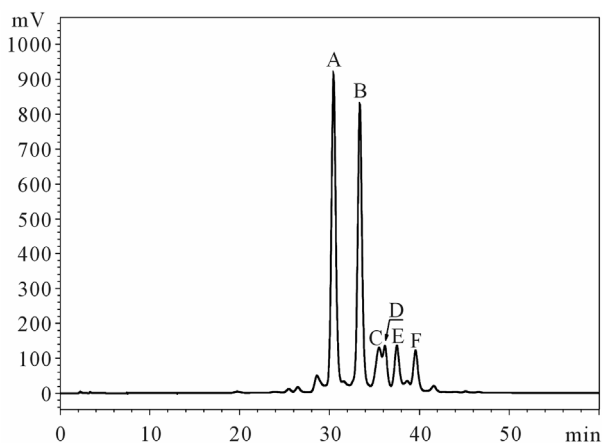
Saffron quality evaluation				
Saffron dehydration time (h)	$E_{1\text{cm}}^{1\%}$ 257	$E_{1\text{cm}}^{1\%}$ 330	$E_{1\text{cm}}^{1\%}$ 440	
2 h	75 ± 0.02	27 ± 0.02	94 ± 0.07	
8 h	60 ± 0.01	29 ± 0.01	95 ± 0.004	
16 h	47 ± 0.02	33 ± 0.01	92 ± 0.03	
ISO 3632 standard range	30 < X < 70	20 < X < 50	80 < X < 190	

Table 2. Crocin retention times (tR, min) and quantity (Q, mg/g) detected in Civitaretenga saffron by HPLC-DAD analysis.

Crocin identification		
Crocin	tR (min)	Q (mg/g)
<i>trans</i> -4-GG	30.51 ± 0.4	45.0 ± 0.2
<i>trans</i> -3-Gg	33.27 ± 0.6	39.2 ± 0.1
<i>trans</i> -2-gg	35.28 ± 0.6	4.5 ± 0.5
<i>cis</i> -4-GG	36.16 ± 0.7	3.9 ± 0.5
<i>cis</i> -3-Gg	37.54 ± 0.4	4.2 ± 0.4
<i>trans</i> -2-G	39.67 ± 0.5	3.2 ± 0.1

Table 3. Specific flavonoid and phenolic acid presence (+) or absence (–) in Civitaretenga saffron and relative retention times (tR, min) in HPLC column.

Secondary metabolite survey		
Molecules	Presence/absence (+/–)	tR (min)
Flavonoids		
Kampferol	+	20.0 ± 0.2
Quercetin	+	14.4 ± 0.1
Genistein	–	-----
Myricetin	+	9.9 ± 0.4
Phenolic acids		
Cumaric acid	–	-----
Caffeic acid	+	11.18 ± 0.6
Chlorogenic acid	+	10.59 ± 0.2
Gallic acid	+	8.71 ± 0.3

**Figure 1. HPLC-DAD chromatogram representing saffron extract crocins: *trans*-4-GG (A), *trans*-3-Gg (B), *trans*-2-gg (C), *cis*-4-GG (D), *cis*-3-Gg (E) and *trans*-2-G (F).**

3.3. Spice Antioxidant and Antiproliferative Activity

Civitaretenga *C. sativus* antioxidant properties were estimated by antiradical molecular assays on its stigma aqueous extract. DPPH test showed a sample IC₅₀ value of 3.76 mg DW whilst FRAP assay indicated that one gram of dried sample presented the same antioxidant power of 2.53 ± 0.15 mM FeSO₄. Saffron antiproliferative effects were tested on highly metastatic murine B16-F10 melanoma cells for 24, 48 and 72 hours after treatment with 250, 500 and 1000 µg/ml of spice extract. As reported in the **Figure 3**, MTT assay showed cell growth reduction at all treatments: in particular, with respect to the control, proliferation decreased of 40.7%, 63.9% and 73.6% respectively after 250, 500 and 1000 µg/ml of treatment at 72 h. Treatment cytotoxic properties were analyzed by Trypan Blue solution capacity to stain dead cells: cell mortality was always very reduced with respect to the control (max < 8%) (**Table 4**). Cell morphological observations were also performed by an optical microscope (**Figure 4**): 1000 µg/ml treatment for 72 h induced the development of cell dendritic evaginations, with respect to the control cells.

4. Discussion

Saffron derives from *C. sativus* stigmas. This spice increased its human applications and commercial value along the time. As Italy, along with Iran, Spain, India, Greece, Azerbaijan and Morocco, is one of the principal world saffron producers [33], this work focused on the determination of Civitaretenga saffron biochemical, organoleptic and nutraceutical properties. Not all saffron extracts can be considered equal because various geographical conditions, genetic factors and temperatures differently can modulate plant growth and secondary metabolite synthesis. Food quality, generally, depends on its chemical composition and preparing techniques. In saffron production, humidity percentage (about 12%) is very important to assure spice conservation and excellence: water content, in fact, regulates microbiological and enzymatic activities reducing spice preciousness [10].

Table 4. Murine B16-F10 melanoma cell toxicity after 24, 48 and 72 hours of treatment with 250, 500 and 1000 µg/ml of Civitaretenga saffron extract.

Treatment hours	Spice cytotoxicity percentages			
	Control	250 µg/ml	500 µg/ml	1000 µg/ml
24 h	6.3%	7.1%	11.7%	13.9%
48 h	7.1%	6.5%	13.5%	12.8%
72 h	6.4%	10.4%	12.8%	14.2%

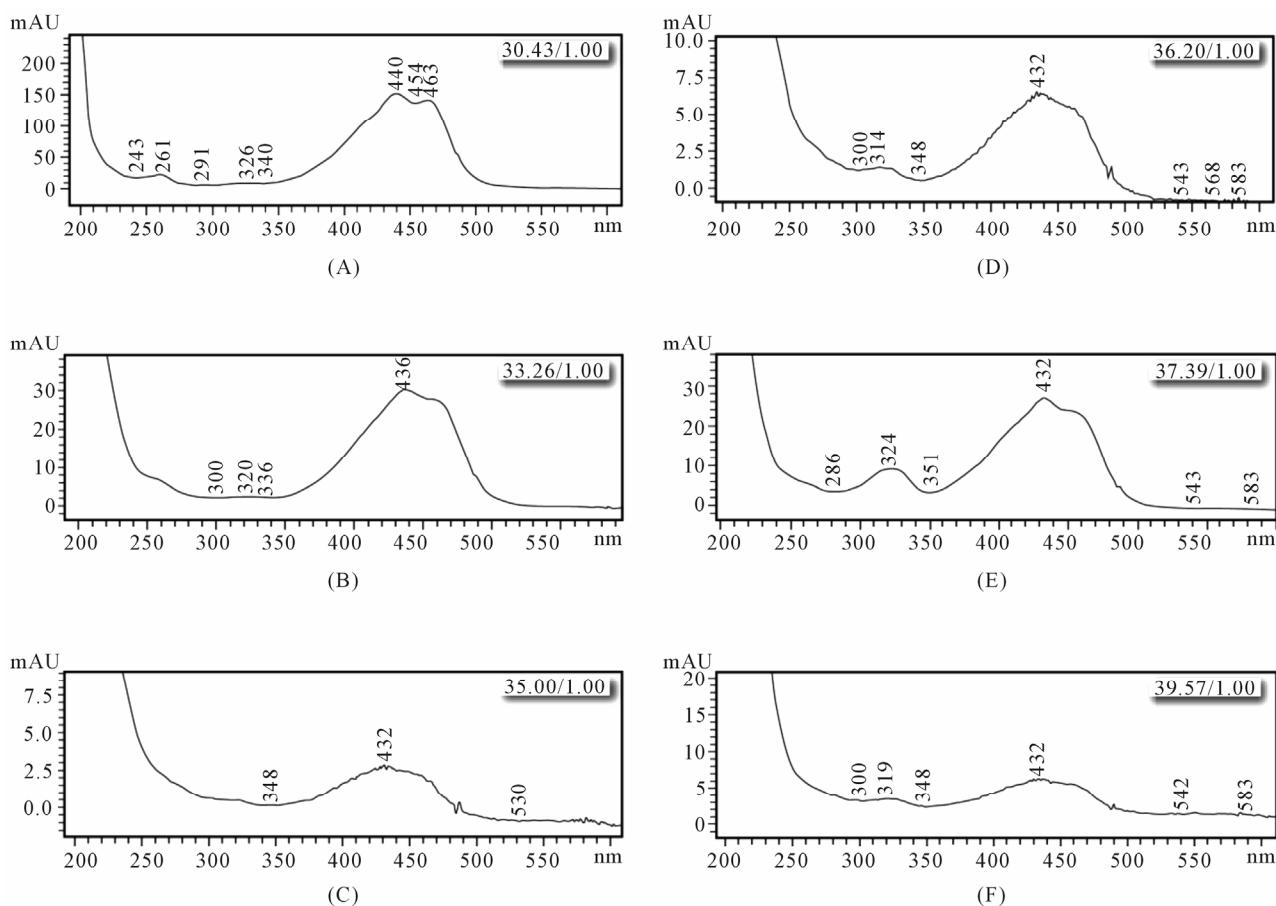


Figure 2. DAD crocin absorbance spectra: *trans*-4-GG (A); *trans*-3-Gg (B); *trans*-2-gg (C); *cis*-4-GG (D); *cis*-3-Gg (E) and *trans*-2-G (F).

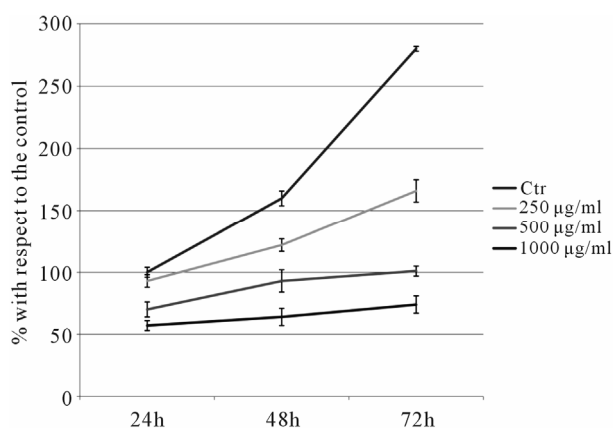


Figure 3. Murine B16-F10 melanoma cell growth determination by MTT assay at 24, 48 and 72 h after treatment with 250, 500 and 1000 µg/ml of saffron extract.

Civitarenga saffron quality was established according to international standardization [20] (Table 1). This study observed that desiccation procedure affected spice quality: long dehydration times (8 and 16 h) highly reduced saffron taste (picrocrocin level) and weakly increased spice aroma (safranal content), with respect to

short desiccation processes (2 h), but, interestingly, they didn't significantly modify its color (crocin amount). These results could be explained because safranal molecule comes from picrocrocin degradation, following extensive drying periods [34]. On the other hand, we concluded that spice color modulation and crocin stability, correlated to high temperature variations [35], would not be associated to different desiccation times. Secondary metabolite production in plants can vary from 6.8 to 32.1 µg CAE/mg DW [36]. Total polyphenolic content found in Civitarenga stigmas was very high: aqueous extract phenol quantity resulted within literature reported ranges whilst methanolic solution showed a 1.7-fold increased concentration, surely, due to the organic solvent capacity to capture less polar molecules, as flavonoids and phenolic acids. Oxidative reactions are essential processes in cell metabolism; however, they induce cell structural damages, apoptosis and several pathologies (cancer, atherosclerosis, diabetes, etc.), stimulating reactive oxygen species (ROS) synthesis. A lot of plant food compounds were recognized as antiradical substance sable to prevent and rescue cell oxidative stress and several pathologies [37-39]. To give more information about saf-

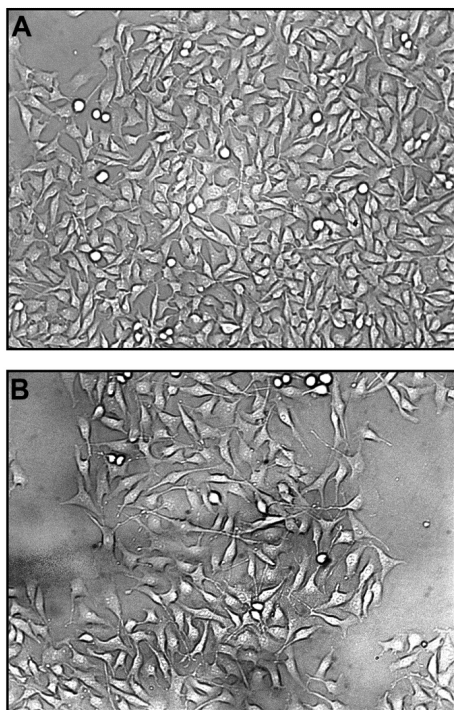


Figure 4. Morphological evidences of differentiation were identified in treated (1000 µg/ml) B16-F10 melanoma cells (B), with respect to the control ones (A) (20× images).

from antioxidant elements with respect to human diet, even if saffron methanolic extract had shown a great phenolic content, we decided to only analyze the exact secondary metabolite composition of aqueous solution, because of its major cell bioavailability and absorption capacity. Crocin levels (**Figure 1** and **Table 2**) were quite elevated with respect to the average values studied in other scientific reports [27]. Saffron secondary metabolite profile was characterized by the presence of different flavonoids (kempferol, quercetin and myricetin) and phenolic acids (caffeic, chlorogenic and gallic acid); nevertheless, HPLC-DAD analysis didn't detect any genistein or cumaric acid traces (**Table 3**). These results suggested us to investigate spice antioxidant properties. DPPH and FRAP assays revealed a good saffron anti-radical effect, though not superior to literature data [40]. Different research groups published saffron antineoplastic effects [41]. Obtained results (**Figure 3**) demonstrated that Civitaretenga stigma solution showed a great anti-proliferative effect, on murine B16-F10 melanoma cell line, but also a very low cytotoxicity (**Table 4**). Cell proliferation reduction could be associated with *C. sativus* extract differentiating properties. This hypothesis was, preliminary, supported by cell shape morphological evidences (**Figure 4**): with respect to the control, typical dendrite-like structures [42] were observed in cells, in particular, after 72 h of 1000 µg/ml treatment. All these data support the idea that Civitaretenga *C. sativus* saffron

is a spice of elevated quality and that it contains highly reducing and chemopreventive agents.

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