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Cytotoxic effect against 3T3 fibroblasts cells of saffron floral bio-residues extracts

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ABSTRACT

For every kilogram of saffron spice produced, about 63 kg of floral bio-residues (FB) (tepals, stamens and styles) are thrown away. Extracts of these bio-residues in water (W1), water:HCl (100:1, v/v) (W2), ethanol (E3), ethanol:HCl (100:1, v/v) (E4), dichloromethane (D5) and hexane (H6) were prepared. Their composition in flavonols and anthocyanins, and their effect on cell viability were determined. W1 was the richest in kaempferol 3-sophoroside (30.34 mg/g dry FB) and delphinidin 3,5-diglucoside (15.98 mg/g dry FB). The highest tested concentration (900 μg/ml) of W1, W2, E4, D5 and H6 did not significantly decrease the cell viability. Only E3 at that concentration caused a significant decrease of 38% in the cell viability. Therefore, all extracts studied are not cytotoxic at concentrations lower than 900 μg/ml, and W1 is proposed as the optimal for food applications due to its greater contribution of phenolic compounds.

1. Introduction

The flower of saffron (*Crocus sativus* L.) consists of six purple tepals, three yellow stamens and a long, white, filiform style ending in a stigma composed of three threads, which only represents 7.4% (w/w) of the flower weight (Serrano-Díaz et al., 2012). Saffron is cultivated for the stigma of its flowers which, after being dried, is the most valued spice. For every kg of spice produced, about 63 kg of floral bio-residues are generated, which so far are not exploited and are usually thrown away. Mechanization has been introduced into the saffron spice production process (Alonso, 2007). This causes an increase in production capacity, and a greater amount of floral bio-residues located in the producing companies which facilitates their possible use.

The interest in the valorization of the saffron floral bio-residues is increasing. Many studies have demonstrated their high phenolic content (Goupy, Vian, Chemat, & Caris-Veyrat, 2013; Montoro et al., 2012; Nørbæk, Brandt, Nielsen, Ørgaard, & Jacobsen, 2002; Serrano-Díaz et al., 2012; Termentzi & Kokkalou, 2008) and biomedical properties, such as antioxidant activity (Serrano-Díaz et al., 2012; Sánchez-Vioque et al., 2012), antityrosinase (Li, Lee, & Wu, 2004), antidepressant (Moshiri et al., 2006), antinociceptive and anti-inflammatory (Hosseinzadeh & Younesi, 2002), antifungal and cytotoxic against tumour cell lines (Zheng, Li, Ma, Han, & Qin, 2011) and arterial pressure reducer (Fatehi, Rashidabady, & Fatehi-Hassanabad, 2003). Goupy et al. (2013) and Nørbæk et al. (2002) have shown that these bio-residues are rich in K3-S and D3,5-diG, whose beneficial properties have been demonstrated (Kim, Ku, Lee, & Bae, 2012; Meiers et al., 2001).

Whole flowers and floral bio-residues have high ash, protein, crude fiber and available carbohydrate contents and are low in lipids. The insoluble/soluble dietary fiber ratio of floral bio-residues indicates they are balanced sources of dietary fiber (Serrano-Díaz, Sánchez, Martínez-Tomé, Winterhalter, & Alonso, 2013b). In the European Union, everything which is within a food dish must comply with the EC 258 (1997) concerning novel foods and novel food ingredients. This Regulation provides two alternatives to regulate

Abbreviations: TFA, trifluoroacetic acid; HCl, hydrochloric acid; TPC, total phenolic content; TAC, total anthocyanin content; K3-S, kaempferol 3-sophoroside; Q3-S, quercetin 3-sophoroside; K3-S-7G, kaempferol 3-sophoroside-7-glucoside; I3,4′-diG, isorhamnetin 3,4′-diglucoside; K3-R, kaempferol 3-rutinoside; K3-G, kaempferol 3-glucoside; K, kaempferol; D3,5-diG, delphinidin 3,5-diglucoside; P3,5-diG, petunidin 3,5-diglucoside; D3-G, delphinidin 3-glucoside; M3,5-diG, malvidin 3,5-diglucoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PBS, phosphate buffered saline.

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these products. The first pathway requires obtaining a novel food certificate after demonstrating that it has not adverse health effects. The second pathway requires demonstrating that it is a traditional food which was consumed before the entry into force of EC 258, (1997). In India, stamens are by-products commercialized under "Patti" saffron for food uses, styles are commercialized with the name of "Zarda" saffron and the tepals are sold for medicinal purposes (Indian Saffron & Walnut Industry, s.a., 2013), based on the aforementioned properties. However, written reference to the consumption of the saffron floral bio-residues as a whole has not been found so far, and toxicity studies to demonstrate the safety of consuming them have not been performed.

Therefore, the aim of this work was to determine the cytotoxic effect of extracts of saffron floral bio-residues in order to contribute to the demonstration of the safety of consumption of these products.

2. Materials and methods

2.1. Sample

The floral bio-residues from the saffron spice production were obtained from the company "Agrícola Técnica de Manipulación y Comercialización S.L." (Minaya, Spain) after the stigma separation from the flower during the 2010–2011 harvest. Floral bio-residues consisted of tepals, stamens and styles.

Fresh floral bio-residues were freeze-dried in a LyoAlfa 6–50 freeze-dryer (Telstar, Terrassa, Spain) for 48 h to constant weight (Serrano-Díaz, Sánchez, Alvarruiz, & Alonso, 2013a). They were crushed, sieved through a 500 μm mesh and then stored at −20 °C until their further analysis.

2.2. Extracts preparation

Freeze-dried saffron floral bio-residues were extracted with water (5 g in 150 ml) (W1), water:HCl (100:1, v/v) (5 g in 150 ml) (W2), ethanol (20 g in 200 ml) (E3), ethanol:HCl (100:1, v/v) (20 g in 200 ml) (E4), dichloromethane (40 g in 200 ml) (D5) and hexane (40 g in 200 ml) (H6). The six extracts were stirred for 1 h at 500 rpm and centrifugated at 3500 rpm. They were filtrated through a Buchner funnel (sintered disc of porosity grade 2) and concentrated under vacuum with a multivapor P-6 (Buchi, Flawil, Switzerland), dissolved in water and freeze-dried. Their yields were calculated.

2.3. Analysis of polyphenols

100 mg of W1, W2, E3 and E4 were re-dissolved in 10 ml of water:HCl (100:1, v/v) and 100 mg of D5 and H6 were re-dissolved in 10 ml of ethanol:HCl (100:1, v/v). Solutions were stirred for 1 h at 500 rpm, followed by centrifugation at 3500 rpm.

2.3.1. Total polyphenol and anthocyanin contents by UV–vis spectrophotometry

Re-dissolved extracts were diluted 1:100 with ultra-high-purity water and their absorbance at 280 nm was measured after 20 min using a Perkin-Elmer Lambda 25 spectrophotometer (Norwalk, CT, USA) with UV WinLab 2.85.04 software (Perkin-Elmer). TPC was quantified using a six-point calibration curve ($R^2 = 0.996$) of 1.0–20.0 mg/L gallic acid.

TAC of the re-dissolved extracts was determined in the same way as Serrano-Díaz et al. (2013a).

2.3.2. Flavonol and anthocyanin composition by HPLC-DAD

Re-dissolved extracts were filtered through a PTFE filter (0.45 μm) and analyzed by reverse-phase HPLC technique as described Serrano-Díaz et al. (2013a). Identification and quantification of flavonols and anthocyanins was done by HPLC-ESI-DAD-MS^n.

2.4. Cell viability

Non-tumourigenic 3T3 fibroblast cells Balb/c clon A31 cells were cultured as previously reported (Romero, Vilanova, & Sogorb, 2011).

Cell viability was assayed with the MTT test, which is based on the colorimetric determination of the formazan formed in the mitochondria from the MTT used as a substrate.

The W1, W2, E3 and E4 extracts were re-dissolved in water and D5 and H6 extracts were re-dissolved in ethanol The concentrations were 30 mg/ml (W1 and W2) and 40 mg/ml (E3, E4, D5 and H6). K3-S, K and D3,5-diG were dissolved in PBS at 8 mg/ml. The composition of PBS is 137 mM NaCl; 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.4). 3T3 fibroblast cells (200.000 cells/plate at the beginning of the exposure in 96 well plates) were exposed to dilutions of the six re-dissolved extracts and the three standard solutions for 3 days. Dilutions were prepared in cell culture medium at the beginning of each experiment as follows: 0.9, 9, 90 and 900 μg/ml for W1, W2, E3 and E4 extracts and 0.4, 4, 40 and 400 μg/ml for D5 and H6 extracts and K3-S, K and D3,5-diG standards.

Chemicals were removed and cells were incubated with 200 μl of MTT solution (1 mg/ml) for 3 h. MTT was removed and cells were washed with PBS. Finally, 200 μl dimethylsulfoxide were added to each well to lysate cells and to solubilize the formazan formed in the mitochondria. Formazan was determined in a Beckman Coulter AD340 microplate reader at 540 nm and by correcting the background absorbance with records at 690 nm. Each condition was assayed in 12 independent wells. Absence of cytotoxicity (100% viability) was attributed to the control cells. These control cells were cultured and grown in identical conditions to cells exposed to different extracts and standards dilutions but exposed only to solvents (in absence of extract or standards).The results were presented as a percentage of these controls.

2.5. Statistical analysis

One-way analysis of variance was performed on TPC, TAC, the results of the flavonol and anthocyanin quantification and the cytotoxicity. Mean values were compared by Duncan's test at $p < 0.05$ using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Yield of the extracts

The yields of the extracts in % (g of extract 100 g of dry floral bio-residues) were 41.1 for W1, 56.7 for W2, 9.7 for E3, 17.7 for E4, 2.3 for D5 and 1.5 for H6. These results showed that the yields obtained were related to the polarity of the solvents used in the extraction. The highest yields were found when more polar solvents (water) were used and the less polar solvent (hexane) showed the lowest yield. The extracts acidified with HCl also showed higher extracting capacity than the respective extracts without acidification. Saffron floral bio-residues are rich in sugars. (Serrano-Díaz et al., 2013b), and these compounds have low solubility in ethanol and very high solubility in water (Montañés, Olano, Ibáñez, & Fornari, 2007). This could be the reason why the aqueous extracts showed greater yield than the ethanolic extracts.

Table 1
Total phenolic and anthocyanin contents of different extracts of saffron floral bio-residues prepared with water (W1), water:HCl (100:1, v/v) (W2), ethanol (E3), ethanol:HCl (100:1, v/v) (E4), dichloromethane (D5) and hexane (H6).

Samples	Total polyphenol content (mg GA/g dw)[A,B]	Total anthocyanin content (mg D3,5-diG/g dw)[C,B]
Water (W1)	20.50 ± 1.16d	24.91 ± 1.19^{e}
Water:HCl (100:1, v/v) (W2)	13.57 ± 1.45^{c}	14.92 ± 0.57^{d}
Ethanol (E3)	4.60 ± 0.15^{b}	1.25 ± 0.06^{b}
Ethanol:HCl (100:1, v/v) (E4)	5.28 ± 0.70^{b}	3.67 ± 0.07^{c}
Dichloromethane (D5)	5.22 ± 0.98^{b}	0.27 ± 0.15^{a}
Hexane (H6)	2.20 ± 0.04^{a}	2.08 ± 0.16^{b}

Data expressed as mean ± standard deviation (n = 5). Different letter superscripts within a column indicate statistically significant statistical differences (p < 0.05) according to Duncan's multiple range test.
[A] Expressed in gallic acid equivalents (GA).
[B] dw: dry weight of the original sample.
[C] Expressed in delphinidin 3,5-diglucoside equivalents (D3,5-diG).

3.2. Total polyphenol and anthocyanin contents

Table 1 shows the TPC and TAC of the different extracts of saffron floral bio-residues. The highest TPC and TAC were observed in the aqueous extract without HCl (W1), followed by the aqueous extract with HCl (W2). Although W2 showed greater yield than W1 (3.1 Section), TPC and TAC were lower in W2; demonstrating that water:HCl (100:1, v/v) extracted greater amounts of other compounds besides polyphenols. The rest of the extracts showed much lower polyphenol contents than those of W1 and W2. The lowest TPC was observed in H6, while D5 showed the lowest TAC.

3.3. Phenolic composition

Aqueous extracts followed by ethanolic extracts were able to extract the most polar compounds. However, dichloromethane and hexane extracts were able to concentrate compounds already present in the aqueous and ethanolic extracts, but with a lower polarity.

Quantification of flavonols and anthocyanins of W1, W2, E3 and E4 is shown in Table 2. The flavonols found in higher concentrations were K3-S, K3-S-7-G and Q3-S. The highest concentrations of these compounds were found in W1, followed by E3. Lower concentrations were detected in W2 and E4 which were only different in the lower K3-S-7-G content of the E4. Therefore, these results show the negative effect of adding HCl to the solvent in a 100:1 (v/v) ratio. Besides, the ratio of flavonol concentrations is different in extracts prepared with HCl addition. K3-S has been described as the main flavonol in saffron floral bio-residues (Goupy et al., 2013). Extracts prepared with HCl in a 100:1 ratio (W2, E4) showed even lower K3-S concentrations than of other flavonols.

In all extracts, the other seven flavonols identified were minority (Table 2). The highest concentrations of K3,7-diG were observed in W1, E3 and E4; and those of K3,7,4′-triG and I3,4′-diG were found in W1. K3-R concentration was significantly higher in E4, and E3 presented the highest K3-G, K7-G and K contents.

Flavonols identified in this study were in accordance with those described by Montoro et al. (2012). Termentzi and Kokkalou (2008) showed higher flavonol contents in rutin equivalents per g of dry methanolic extract.

With respect to anthocyanins, the highest concentrations of D3,5-diG, P3,5-diG, D3-G and M3,5-diG were again presented in W1, while W2 showed the lowest concentrations of the five anthocyanins identified. Unlike W1, these results obtained for W2 were not in agreement with data by UV–vis (Table 1), since W2 showed greater TPC and TAC (Table 1) and the lowest concentrations of most flavonol and all the anthocyanins (Table 2). This could be because the HCl may produce some transformation of phenolic compounds, resulting in compounds which interfere at the reading wavelength used in UV–vis spectrophotometry. D3,5-diG, followed by P3,5-diG, and followed by D3-G were the major anthocyanins in W1 and E3. As was observed in the case of K3-S, anthocyanins ratios were also affected by the HCl addition to solvents. D3,5-diG has been described as the main anthocyanin (Nørbæk et al., 2002) however, in W2, the main one was D3-G. This could be because the acid hydrolyzed the glycosidic bond of D3,5-diG.

Table 2
Quantification of flavonols and anthocyanins of different extracts of saffron floral bio-residues prepared with water (W1), water:HCl (100:1, v/v) (W2), ethanol (E3) and ethanol:HCl (100:1, v/v) (E4) and re-dissolved in water:HCl (100:1, v/v).

	t_R	Water (W1)	Water:HCl(100:1, v/v/v) (W2)	Ethanol (E3)	Ethanol:HCl(100:1, v/v/v) (E4)
Flavonols					
Kaempferol 3-sophoroside-7-glucoside[A]	15.65 ± 0.01	5 601.79 ± 144.37^{d}	432.98 ± 28.99^{b}	1 373.38 ± 53.84^{c}	264.19 ± 38.64^{a}
Kaempferol 3,7-diglucoside[B]	19.28 ± 0.02	18.59 ± 0.28^{c}	3.38 ± 0.07^{a}	5.15 ± 0.03^{b}	5.48 ± 0.99^{b}
Kaempferol 3,7,4′-triglucoside[B]	19.74 ± 0.01	13.51 ± 0.32^{d}	0.37 ± 0.02^{a}	3.62 ± 0.15^{b}	8.75 ± 1.41^{c}
Quercetin 3-sophoroside[A]	21.72 ± 0.01	4 011.51 ± 349.77^{c}	95.56 ± 4.50^{a}	1 387.50 ± 58.06^{b}	65.00 ± 17.56^{a}
Isorhamnetin 3,4′-diglucoside[B]	22.55 ± 0.01	18.54 ± 0.46^{c}	1.42 ± 0.46^{a}	2.15 ± 0.03^{b}	n.d.
Kaempferol 3-sophoroside[A]	23.57 ± 0.01	30 341.60 ± 448.14^{c}	191.15 ± 3.40^{a}	26 070.14 ± 951.10^{b}	53.38 ± 6.33^{a}
Kaempferol 3-rutinoside[B]	25.32 ± 0.01	11.04 ± 0.26^{b}	0.85 ± 0.03^{a}	2.35 ± 0.13^{a}	51.49 ± 6.88^{c}
Kaempferol 3-glucoside[B]	27.35 ± 0.01	9.04 ± 0.31^{c}	0.57 ± 0.01^{a}	12.46 ± 0.23^{d}	8.41 ± 0.01^{b}
Kaempferol 7-glucoside[B]	27.52 ± 0.03	45.66 ± 1.13^{b}	0.91 ± 0.03^{a}	80.49 ± 2.72^{c}	2.92 ± 0.03^{a}
Kaempferol[B]	37.04 ± 0.01	8.53 ± 0.78^{b}	3.18 ± 1.19^{a}	45.70 ± 2.26^{c}	8.06 ± 2.28^{b}
Anthocyanins					
Delphinidin 3,5-diglucoside[C]	16.55 ± 0.01	15 984.94 ± 229.65^{d}	474.76 ± 11.46^{a}	2 319.24 ± 20.48^{c}	1 683.55 ± 200.98^{b}
Petunidin 3,5-diglucoside[C]	18.67 ± 0.01	3 967.68 ± 59.35^{d}	138.95 ± 14.06^{a}	1 163.11 ± 11.77^{c}	524.40 ± 62.65^{b}
Delphinidin 3-glucoside[C]	19.16 ± 0.01	1 185.97 ± 18.64^{c}	659.18 ± 17.92^{a}	616.16 ± 4.29^{a}	819.59 ± 96.15^{b}
Malvidin 3,5-diglucoside[C]	20.65 ± 0.01	386.18 ± 6.45^{c}	n.d.	166.34 ± 1.56^{b}	43.23 ± 5.09^{a}
Petunidin 3-glucoside[C]	21.51 ± 0.01	258.06 ± 4.84^{b}	178.90 ± 5.62^{a}	237.07 ± 13.31^{b}	276.65 ± 56.70^{b}

Data expressed as mean ± standard deviation (n = 5). Different superscript letters within a row indicate statistically significant differences (p < 0.05) according to Duncan's multiple range test.
n.d. – not detected.
[A] Expressed in equivalents of kaempferol 3-sophoroside (μg/g of saffron floral bio-residues on dry weight).
[B] Expressed in equivalents of kaempferol 3-glucoside (μg/g of saffron floral bio-residues on dry weight).
[C] Expressed in equivalents of delphinidin 3,5-diglucoside (μg/g of saffron floral bio-residues on dry weight).

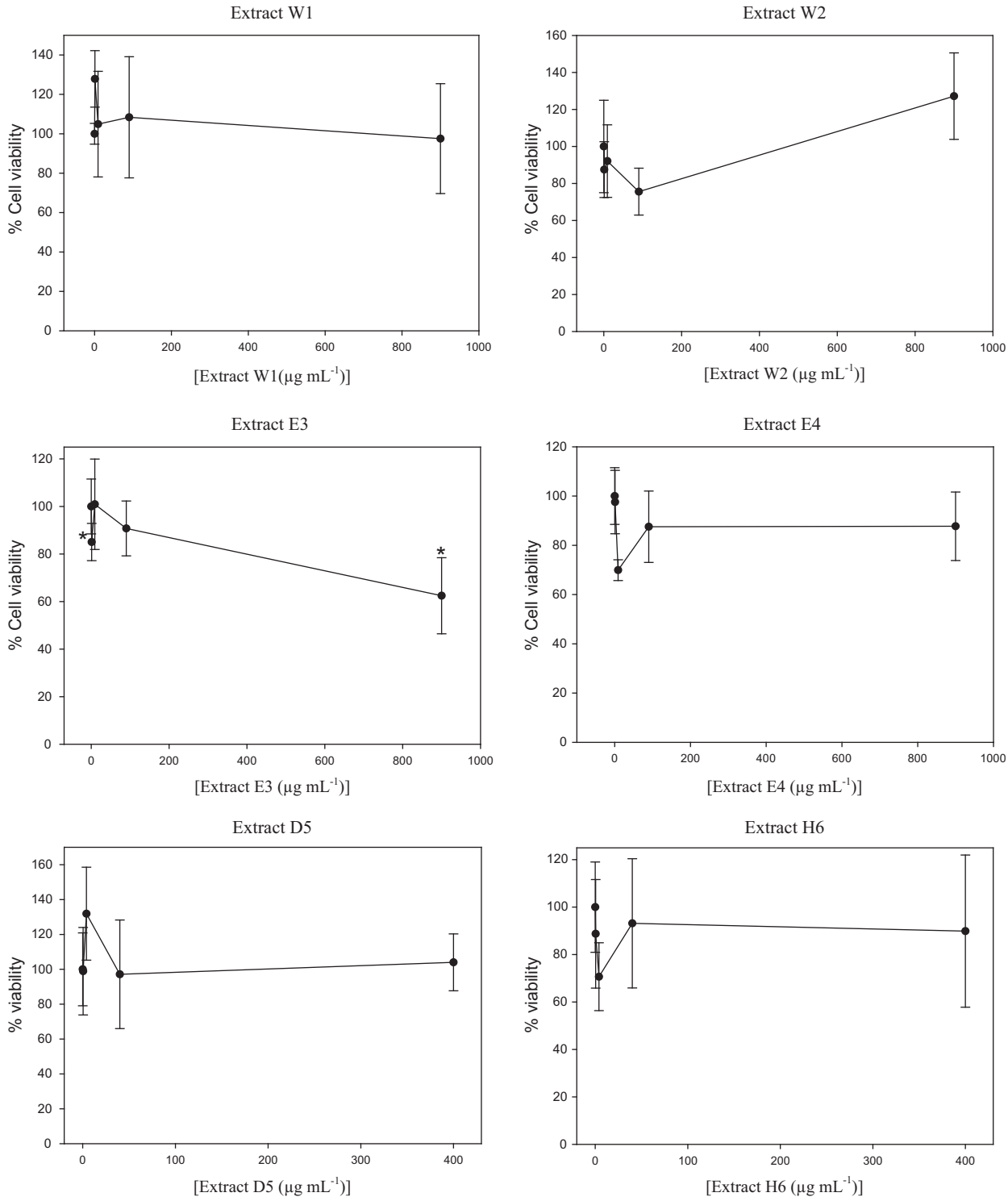

Fig. 1. Cytotoxicity induced after a 3-day exposure to different extracts of saffron floral bio-residues prepared with water (W1), water:HCl (100:1, v/v) (W2), ethanol (E3), ethanol:HCl (100:1, v/v) (E4), dichloromethane (D5) and hexane (H6) and redissolved in water (W1, W2, E3, E4) and ethanol (D5, H6). * Indicate which significant differences were found.

Regarding acidified ethanolic extract (E4), the D3-G and P3-G contents were superior to those of E3. This also may be due to hydrolysis of the glycosidic bonds of D3,5-diG and P3,5-diG, respectively.

Both anthocyanins and flavonols identified in this study were consistent with those described by Goupy et al. (2013) and Nørbæk et al. (2002). Goupy et al. (2013) detected contents of 22.74 mg for flavonols and 4.80 mg for anthocyanins per g of dry methanolic extract.

The chromatographic profiles identified in D5 and H6 were different to those found in the aqueous and ethanolic extracts. None of the five anthocyanins were detected in these two extracts. These results were not in agreement with those obtained by UV–vis spectrophotometry, since TACs were similar in E3 and H6, which could be due to the interferences of other compounds that also have absorbance at 520 nm. However, some flavonols (K in D5; and I3,4′-diG, K7-G and K in H6) were detected in these two

extracts at concentrations below 1 equivalent (μg/g) per g of saffron floral bio-residues dry weight and K3-S in H6 in concentrations up to 30 equivalents (μg/g) per g of saffron floral bio-residues dry weight. Termentzi and Kokkalou (2008) conducted a fractionation in different solvents of a methanolic extract of saffron tepals. As in this work, the only flavonoid identified in the dichloromethane fraction was K. Moreover, the composition of the dichloromethane fraction was completely different from other fractions (diethyl ether, ethyl acetate, butanol and water) since phenolic acids, isophorones and alkaloids were only detected in this fraction. Many compounds which were already present in aqueous and ethanolic extracts in amounts ranging from traces were concentrated in D5 and H6. Their spectrum was similar to that described for compounds belonging to the family of the chlorophylls.

3.4. Cell viability and cytotoxicity

The results of the analysis of extracts of different polarities are the first step towards the study of their toxicity. Fig. 1 displays alterations in viability of 3T3 fibroblast cells after 3 days of exposure to six extracts.

The viability of the cultures showed that the cytotoxicity induced after a 3-day exposure to extracts W1, W2, E4, D5 and H6 was very low. The highest tested concentration (900 μg/ml) did not cause a decrease in the cell viability. Only the E3 extract at this highest tested concentration caused a significant decrease (38%) in the cell viability.

The cytotoxicity induced after a 3-day exposure to the pure compounds, three of the main flavonols and anthocyanins (K3-S, K and D3,5-diG), was low as well. The highest tested concentration (400 μg/ml) of K did not cause any decrease in the viability of the cells, while the same concentrations of K3-S and D3,5-diG caused a significant decrease in the viability of 24% and 37%, respectively.

All the extracts prepared at the concentrations tested did not show cytotoxicity. The aqueous extract without acidification (W1) is proposed as the most suitable for food applications, since no cytotoxic effect at any concentration studied was observed and the K3-S and D3,5-diG contents were much higher than those of other extracts.

4. Conclusions

The cytotoxicity induced after a 3-day exposure to extracts W1, W2, E4, D5 and H6 is very low. All the extracts prepared are not cytotoxic at concentrations lower than 900 μg/ml. W1 is the richest extract in K3-S and D3,5-diG and, hence, it is proposed as the most suitable for food applications. The results presented in this paper are a first attemp to demonstrate the safety of the exposure to these floral bio-residues obtained in the saffron spice production.

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Erratum

Erratum to "Cytotoxic effect against 3T3 fibroblasts cells of saffron floral bio-residues extracts" [Food Chem. 147 (2013) 55–59]

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The publisher regrets that the reference Serrano-Díaz, J., Sánchez, A. M., Alvarruiz, A., & Alonso, G. L. (2013b) was incorrectly captured in the article's reference list. The correct reference appears below.

Serrano-Díaz J., Sánchez A.M., Martínez-Tomé M., Winterhalter P., & Alonso G.L. (2013b). A contribution to nutritional studies on Crocus sativus flowers and their value as food. Journal of Food Composition and Analysis, 31, 101–108.

Please note that the online version of this article has been corrected to this effect.

The publisher would like to apologise for any inconvenience caused.

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