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Abstract: Saffron is cultivated for production of the saffron spice. Nevertheless, a huge amount of saffron by-products including corms, tepals and leaves with little or no commercial value are generated during the processing of the spice. This biomass contains bioactive compounds whose exploitation can increase the profitability and sustainability of this traditional crop. A significant amount of polyphenols, mainly glycosides of kaempferol, luteolin and quercetin have been determined in tepals and leaves of saffron. Proliferation of Caco-2 cells was greatly inhibited by the tepal and leaf extracts (ED50 0.42 mg/ml), while the corm extract caused some signs of toxicity and completely abolished proliferation (ED50 0.05 mg/ml). To our knowledge, these are the first data reporting the inhibition of the proliferation of Caco-2 cells by extracts from tepals and leaves of saffron, and polyphenols could be responsible for this effect.

Highlights

1. Glycosides of kaempferol, luteolin and quercetin in tepals and leaves of saffron.
2. Proliferation of Caco-2 cells was greatly inhibited by tepal and leaf extracts.
3. Corm saffron extract caused toxicity and abolished proliferation of Caco-2 cells.
4. Saffron's corms, tepals and leaves are potential sources of anticancer molecules.

Polyphenol composition and *in vitro* antiproliferative effect of corm, tepal and leaf from *Crocus sativus* L. on human colon adenocarcinoma cells (Caco-2)

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Abstract

Saffron is cultivated for production of the saffron spice. Nevertheless, a huge amount of saffron by-products including corms, tepals and leaves with little or no commercial value are generated during the processing of the spice. This biomass contains bioactive compounds whose exploitation can increase the profitability and sustainability of this traditional crop. A significant amount of polyphenols, mainly glycosides of kaempferol, luteolin and quercetin have been determined in tepals and leaves of saffron. Proliferation of Caco-2 cells was greatly inhibited by the tepal and leaf extracts (ED_{50} 0.42 mg/ml), while the corm extract caused some signs of toxicity and completely abolished proliferation (ED_{50} 0.05 mg/ml). To our knowledge, these are the first data reporting the inhibition of the proliferation of Caco-2 cells by extracts from tepals and leaves of saffron, and polyphenols could be responsible for this effect.

Keywords: Bioactive compound; By-product; Cancer; Crocus sativus; Health-promoting; Iridaceae

1. Introduction

Saffron (*Crocus sativus* L.) is a triploid sterile plant belonging to the Iridaceae family (Gooldblatt et al., 2003). It is the most representative species of the genus *Crocus*, which comprises more than 88 species distributed across Central and Southern Europe, North Africa, and from Southwest Asia to Western China (Mathew, 1982; Petersen et al., 2008). *C. sativus* is traditionally cultivated for production of the saffron spice, which is derived from the dried stigma of the flowers and is considered the most expensive spice in the world. In addition, the use of saffron for medical benefit has played an important role in traditional medicine of different cultures on earth (Abdullaev, 1993). In modern pharmacy saffron has reputed to be useful in treatment of numerous human diseases including cancer (Abdullaev, 2002; Tarantilis et al., 1994).

Nevertheless, a huge amount of saffron by-products with little or no commercial value is generated during processing of the stigmas. Around 350 kg of tepals, 1,500 kg of leaves, and hundreds of corms too small for flowering and/or with physical or biological damages to be replanted are discarded in order to obtain only 1 kg of dry stigmas (Smolskaite et al., 2011). This biomass is however a potentially significant source of bioactive compounds whose exploitation would greatly increase the profitability and sustainability of saffron production (Santana-Méridas et al., 2012).

Numerous bioactive, health-promoting properties have been found in corms and tepals. Extracts from tepal have antidepressant (Moshiri et al., 2006), antinociceptive and anti-inflammatory properties (Hosseinzadeh & Younesi, 2002), as well as free radical scavenging and antityrosinase activities (Kubo & Kinst-Hori, 1999; Li et al., 2004). These extracts can also lower blood pressure and contractile response (Fatehi et al., 2003). Bioactive components in corm include proteoglycans showing cytolytic activity against tumoral (Escribano et al., 1999; 2000a) and plant (Fernández et al., 2000) cells, and triterpenic saponins with anticancer (Rubio-Moraga et al., 2011) and fungicidal (Rubio-Moraga et al., 2013) activities. Phenolic compounds with radical scavenging activity

(Esmaeili et al., 2011), and a mannan-binding lectin (Escribano et al., 2000b) have also been found in corm. Although saffron leaves have not received much attention as a source of bioactive components, the presence of a number of phenolic compounds that could be used as natural antioxidants has been described (Sánchez-Vioque et al., 2012; Williams et al., 1986).

Flavonoids have numerous biological activities, including effects on cancer-related biological pathways such as carcinogen bioactivation, cell cycle regulation, angiogenesis, oxidative stress, and inflammation (Le Marchand, 2002). Luteolin, kaempferol, quercetin, and apigenin are especially active polyphenols that show significant antiproliferative and cytotoxic effects against several cancer cell lines including Caco-2 cells (Manthey & Guthrie, 2002). The presence of these components in the corm, tepal, and leaf of saffron plants would support the use of these by-products as a source of health-promoting products inhibiting the proliferation of tumor cells (Richter et al., 1999; Seelinger et al., 2008).

This work is a continuation of previous investigations in which extracts of saffron corm, tepal, and leaf showed remarkable antioxidant and metal chelating activity, most likely due to their content in polyphenols (Sánchez-Vioque et al., 2012). The polyphenol content and composition of these extracts have now been determined. Because of reports of the antiproliferative effect of polyphenols on cancerous cells (Fresco et al., 2006; Huang et al., 2010; Megías et al., 2009; Middleton et al., 2000), the effect of the corm, tepal, and leaf extracts on the growth of a human cell line derived from a colon carcinoma has also been studied. Results support the health-promoting properties of the corm, tepal and leaf extracts because of their content in antioxidant phenolics, and their toxic/antiproliferative effect on Caco-2 cells.

2. Materials and methods

2.1. Plant material

Saffron plants were grown free of any chemical treatments in commercial areas of the region of Castilla-La Mancha (Spain). The different parts of the plant were collected according to the life cycle span of the crop as follows: tepals were picked manually in early October 2009, leaves were harvested in half april 2010, and the non-commercial corms (less than 20 mm in diameter) during July 2010. Corms, tepals, and leaves were dried at room temperature in the dark and ground (200 g) using an Ultra Centrifugal ZM 1000 mill (0.25 mm mesh) (Retsch, Haan, Germany).

2.2. Materials and reagents

Tissue culture media, fetal bovine serum, trypsin-EDTA solution, and non-essential amino acids for cell culture were from Invitrogen-Gibco (Barcelona, Spain). Trypan blue, Folin-Ciocalteu´s phenol reagent and the phenolic standards gallic acid, kaempferol-3-O-glycoside, quercetin-3,4-di-O-glycoside, apigenin-8-C-glycoside (vitexin) and luteolin-6-C-glycoside (iso-orientin) were from Fluka-Sigma–Aldrich® (St. Louis, MO, USA). Luteolin-8-C-glycoside (orientin) was purchased from AppliChem® GmbH (Darmstadt, Germany). The purity of these standards was in the 95% to 99.8% range. All other chemicals were of analytical grade.

2.3. Extraction of corm, tepal and leaf of saffron

Ground corms, tepals, and leaves (5 g) were extracted by stirring in 50 mL ethanol/water 70/30 (v/v) for 48h in the dark. The slurries were centrifuged at 5450 ×g for 30 min., the resulting supernatants were filtered (0.45 μm), and ethanol was removed using a rotary evaporator. Finally, extracts were freeze-dried and kept at −20ºC in sealed tubes.

2.4. Total polyphenol content

Total polyphenols were determined using the Folin-Ciocalteu method as described by Slinkard & Singleton (1977). Gallic acid was used as standard and total polyphenol content was expressed as gallic acid g equivalents.

2.5. Identification and quantification of polyphenols by LC-MS analysis

Corm, tepal and leaf extracts were analyzed using the LC-MS (ESI) technique in a Shimadzu LC/MS-2010A apparatus equipped with a LC-10ADvp binary pump, a DGU-14A degasser, a SIL-10ADvp autosampler, and a SPD-M10Avp Photo Diode Array Detector. Samples (10 mg/mL methanol) were injected in a 250 mm × 4.6 mm, 5 μm particle size Discovery HS-C18 column (Supelco, Bellefonte, PA, USA) at a flow rate of 0.4 mL/min. The following gradient of mobile phase A (0.1% formic acid in water at pH 2.5) and mobile phase B (methanol) was used for the separation of polyphenols: Initial 75% A, 25% B; 2 min 75% A, 25% B; 40 min 10% A, 90% B; 75 min 10% A, 90% B; 80 min 75% A, 25% B; 90 min 75% A, 25% B. The injection volume was 20 μl and UV detection was performed in the 200–700 nm range. Negative ion mass spectra were recorded in the 100–1000 m/z range. The interface conditions were as follows: capillary voltage -3.50 kV, CDL voltage −20.0 V, CDL temperature 250 ºC and detector voltage 1.48 kV. The nebulising gas flow rate was 1.5 L/min and the heat block temperature in the mass analyzer was 300ºC. The compounds present in the samples were tentatively identified according to their UV, mass spectra and retention times and compared with those of standards (when available), with data reported in the literature, and/or with positively identified compounds in other reference plant samples.

2.6. Cell culture and treatment

Caco-2 cells were kept at 5 % CO_2 in Dulbecco´s Modified Eagle Medium (1000 mg/ml glucose, 110 mg/ml pyruvate, and 580 mg/ml glutamine) supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids, 100 u/ml penicillin, and 100 μg/ml streptomycin. Fetal bovine serum was heat-inactivated at 56^{o}C for 30 minutes. Cells were subcultured once a week using trypsin-EDTA, and medium was renovated once in between passages. Trypan blue was used for routine cell counting.

Treatments were carried out under the same standard culture conditions. Cells were seeded in 96 well microplates (90 µl, $2x10^3$ cells/well) and the extracts were added (10 µl) to a final concentration of 0 to 1 mg extract/ml, 0.5 % (v/v) ethanol. Controls consisted of identical treatments except for addition of water or ethanol to a final concentration of 0.5 % (v/v) instead of the extracts. In addition to the cell viability assay as shown below, cells were routinely inspected under the phase-contrast microscope.

2.7. Cell proliferation using the neutral red viability assay

Cells in 96-well plates were incubated in fresh culture medium containing the vital stain neutral red (50 µg/ml) for 30 minutes. Cells were then washed using PBS, and the stain was extracted using acetic acid (75 µl, 1% v/v in ethanol 50% v/v). Absorbance was measured at 550 nm using a plate reader (Borenfreund & Puerner, 1985; Girón-Calle et al., 2010).

Data represent average and standard error of the mean (n=6). One way analysis of variance followed by Tukey test was carried out for pairwise multiple comparisons. The concentrations of extracts causing a 50% inhibition of cell proliferation (ED_{50}) were calculated by application of a four parameter logistic equation fit using the Sigmaplot 13.0 software package (Systat Software, San Jose, CA).

3. Results and Discussion

3.1. Extraction of corm, tepal and leaf of saffron and total polyphenol content of the extracts.

Extraction of bioactive components from the saffron by-products was carried out under mild conditions by stirring at room temperature in the dark in order to minimize degradation of polyphenols. In addition, this procedure is easy and inexpensive as compared to other methods of extraction that require application of heat, pressure, microwaves, or even ultrasounds, which would facilitate its implementation in an industrial setting.

Determination of polyphenols in the extracts using the Folin-Ciocalteau reagent showed that the leaf extract had the highest polyphenol content, followed by the extracts from tepals and corms (Table 1). There is a good correlation between the concentration of total polyphenols in the three extracts and the antioxidant and metal chelating activities that were previously reported for these extracts, namely inhibition of β-carotene bleaching, ferric reducing power, free radical scavenging, nitric oxide scavenging, and chelation of iron and copper (Sánchez-Vioque et al., 2012). Thus, all the antioxidant activities and the iron chelating activity followed the same pattern than the content in polyphenols: they were the highest in leaf, followed by tepal and finally corm extracts. Copper chelating activity was also the highest in leaves, although in this case, and unlike the content in polyphenols, the activity in corm was higher than in tepal (Sánchez-Vioque et al., 2012).

The polyphenol content in petals and leaves as referred to the original plant material (Table 1) were comparable to the total polyphenol content in other plant materials and seeds that are recognized as good sources of health-promoting polyphenols, e.g. grain legumes (Xu et al., 2007). The content in corm was a lot lower, about 20 times lower than in petals and leaves.

3.2. Polyphenol composition

Tables 2 and 3 show the polyphenol composition of the leaf and tepal extracts, respectively, as determined by HPLC-DAD-MS. The composition of the corm could not be determined because the concentration of polyphenols in the corm extract was too low for the HPLC-MS set up that was used. However, several phenolic acids, including caffeic, gentisic, p-coumaric, ferulic, syringic, and salicylic acids have been previously reported in corm (Esmaeili et al., 2011).

Glycosylated derivatives of luteolin ([M-H]-1 at m/z 447), and kaempferol ([M-H]-1 at m/z 787, 625 and 465) were the major tentatively identified polyphenols in leaves, representing 31% of total peak area (Fig.1, Table 2). Peak 13 (Rt 29.28 min.; 10.6% of total peak area) could be a phenolic

acid or an aglycone according to the low mass [M-H]-1 at m/z 261 and the UV λmax at 285 nm. Glycosides of quercetin ([M-H]-1 at m/z 463) and apigenin ([M-H]-1 at m/z 431) were also identified in leaf extract. This is consistent with a previous report describing the presence in saffron leaf of unidentified glycosides of kaempferol, quercetin, myricetin, and isorhamnetin, as well as flavones derivatives of tricin and apigenin (Williams et al., 1986).

Kaempferol glycosides were the major components found in tepals (Fig.1, Table 3). A kaempferol-di-glycoside ([M-H]-1 at m/z 609) probably containing sophorose as disaccharide [32] accounted for 53% of total peak area. Kaempferol-3-O-glycoside ([M-H]-1 at m/z 447, 3.1%), kaempferol-3-O-sophoroside-7-O-glucoside ([M-H]-1 at m/z 771, 2.9%), and quercetin-3,4-di-O-glycoside ([M-H]-1 at m/z 625, 2.9%) were found at lower concentrations. Previous reports showed the presence in saffron tepal of anthocyanins derived from delphinidin, petunidin and malvidin, as well as kaempferol, quercetin and isorhamnetin glycosides (Goupy et al., 2013; Nørbaek et al., 2002).

3.3. Effect of extracts on proliferation of Caco-2 cells.

Proliferation of Caco-2 cells under exposure to the corm, tepal, and leaf extracts was determined as an *in vitro* model of exposure of cancerous cells to bioactive components. Caco-2 cells were originally isolated from a human colon carcinoma, and when grown in standard culture conditions have a transformed, cancerous phenotype as long as they are not allowed to form a confluent monolayer (Artursson et al., 2001). They have been extensively used as a model of exposure to components in the diet and drugs (Girón-Calle et al., 2004; 2010).

As shown in Fig. 2, the corm extract completely inhibited proliferation after incubation for just one day, and kept inhibiting proliferation from there on. Observation of the cells treated with the corm extract under the microscope revealed that the cells attached over the dish upon seeding, but did not spread over the substrate as control cells did. Blebs and cellular debris, clear signs of

cytotoxicity, were observed in the following days, leading eventually to detachment of cells and the appearance of necrotic bodies. Cells exposed to extracts from tepal and leaf at the same concentration, 0.5 mg/ml, had a healthy appearance under the microscope throughout the seven days that lasted the incubation. Nevertheless, their growth was inhibited at all the time points, reaching a maximum inhibition of around 90 % by day 7 as compared to control cells.

An additional experiment was carried out in order to calculate the ED_{50} of the inhibition of proliferation by the extracts. Cells were treated with a range of concentrations of extracts from 0 to 1 mg/ml for 4 days (Fig. 3). A four parameter sigmoidal fit yielded ED_{50} values of 0.054, 0.420, and 0.426 mg/ml for corm, tepal, and leaf extracts, respectively. Thus, the inhibitory effect of corm extracts is almost 10 times higher than the effect of tepal and leaf extracts. These results indicate that the corm extract has a toxic effect on Caco-2 cells, while the extracts from tepal and leaf inhibited proliferation without completely inhibiting growth or causing any morphological sign of toxicity. The potential presence of compounds such as saponins (Rubio-Moraga et al., 2011) and glycoconjugates (Escribano et al., 2000a) in the corm extract could in part explain the cytotoxicity observed on Caco-2 cells although the real nature of the component (-s) responsible for these effects remains to be elucidated.

Our findings are consistent with reports showing antiproliferative and/or toxic effects of polyphenols or polyphenol rich extracts on Caco-2 cells and other cell lines (Girón-Calle et al., 2004; Kumar & Pandey, 2013; Manthey & Guthrie, 2002; Megías et al., 2009; Richter et al., 1999; Seelinger et al., 2008; Szewczyk et al., 2014; Wenzel et al., 2000). More specifically, Lacikova et al. (2009) reported that flavonoid content is associated with antiproliferative effects of leaf extracts from *Staphylea colchica* Stev against A431 human skin carcinoma cells, and Gul et al. (2013) found that the antiproliferative properties of *Abrus precatorius* leaf extracts against human colon adenocarcinoma cells (Colo-205), human retinoblastoma cancer cells (Y79), human hepatocellular carcinoma cells (HepG2) and Leukemia cells (SupT1) might be due to the synergistic actions of

bioactive flavonoids present in them. Likewise, Jaramillo-Carmona et al. (2014) have proven that the combination of quercetin and kaempferol exhibits a high cytotoxicity on human colon cancer (HCT-116) cells supporting that mixtures of polyphenols exhibit greater efficacy in comparison with single polyphenols.

4. Conclusions

Our data expand the knowledge on the polyphenol composition of saffron tepal and leaf as specific glycoside derivatives have been identified. To our knowledge, these are the first data reported on the inhibition of the proliferation of Caco-2 cells by extracts from tepals and leaves of saffron but no conclusion can still be established about the possible involvement of polyphenols in such activity. A partial purification of these extracts in order to obtain polyphenol-enriched fractions, and their subsequent assay against Caco-2 cells could clarify this point. Additionally, the potential synergism or antagonism among polyphenols or between polyphenols and other compound present in the extracts could be checked by comparison of the antiproliferative effects of crude extracts, polyphenol-enriched fractions and standard polyphenols.

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Table 1. Polyphenol content (gallic acid gram equivalents per 100 g) in the by-products of saffron production.

	In extracts[a]	In the original material[b]
Leaves	7.8	1.5
Tepals	4.8	1.7
Corms	1.3	0.1

[a] In freeze-dried extracts.

[b] In leaf, tepal and corm (dry matter).

Table 2. Major polyphenols detected and tentatively identified by liquid chromatography-mass spectrometry in leaf of *C. sativus*.

Peak	Rt (min)	Peak area (%)	UV λ_{max} (nm)	[M-H] $^{-}$ (*m/z*)	Tentative identification	Reference
1	6.51	2.26	219, 270, 306	195	n.i.	-
2	6.72	8.15	230, 256	290	n.i.	-
3	8.29	1.39	220	191	n.i.	-
4	18.06	2.39	223, 272, 331	369, 386	n.i.	-
5	20.86	2.63	224, 272, 338	787	n.i.	-
6	21.38	15.07	230, 272, 338	787	Kaempferol-8-C-glyc-6,3-O-diglycoside	Smolskaite et al., 2011
7	21.72	1.05	224, 270, 349	465, 625	Kaempferol-8-C-glyc-6-O-glycoside	Smolskaite et al., 2011
8	22.24	3.17	226, 270, 350	463	Myricetin/Quercetin glycoside	Chua et al., 2011
9	25.48	16.59	228, 256, 269, 348	447	Luteolin-6-C-glycoside (iso-orientin) + Luteolin-8-C-glycoside (orientin)*	Standards
10	26.41	2.56	227, 284	483, 787	n.i.	-
11	27.43	19.44	231, 284, 332	359	n.i.	-
12	28.27	1.59	229, 271, 332	431	Apigenin-8-C-glycoside (vitexin)	Standards
13	29.28	10.58	231, 285, 329	261	n.i.	-

Rt: Retention time, n.i.: Not identified, * Coelution occurs

Table 3. Major polyphenols tentatively identified by liquid chromatography-mass spectrometry in tepal of *C. sativus*.

Peak	Rt (min)	Peak area (%)	UV λ_{max} (nm)	[M-H]$^-$ (*m/z*)	Tentative identification	Reference
1	6.71	14.90	237, 262	281	n.i.	
2	9.10	2.91	268	389	n.i.	
3	14.84	2.91	267, 347	771	Kaempferol-3-O-sophoroside-7-O-glycoside	Goupy et al., 2013
4	24.85	2.94	254	625	Quercetin-3,4-di-O-glycoside	Standards
5	27.11	53.01	265	609	Kaempferol-di-glycoside	Goupy et al., 2013
6	30.05	1.31	266, 348	463	n.i.	
7	32.38	3.09	265	447	Kaempferol-3-O-glycoside	Standards

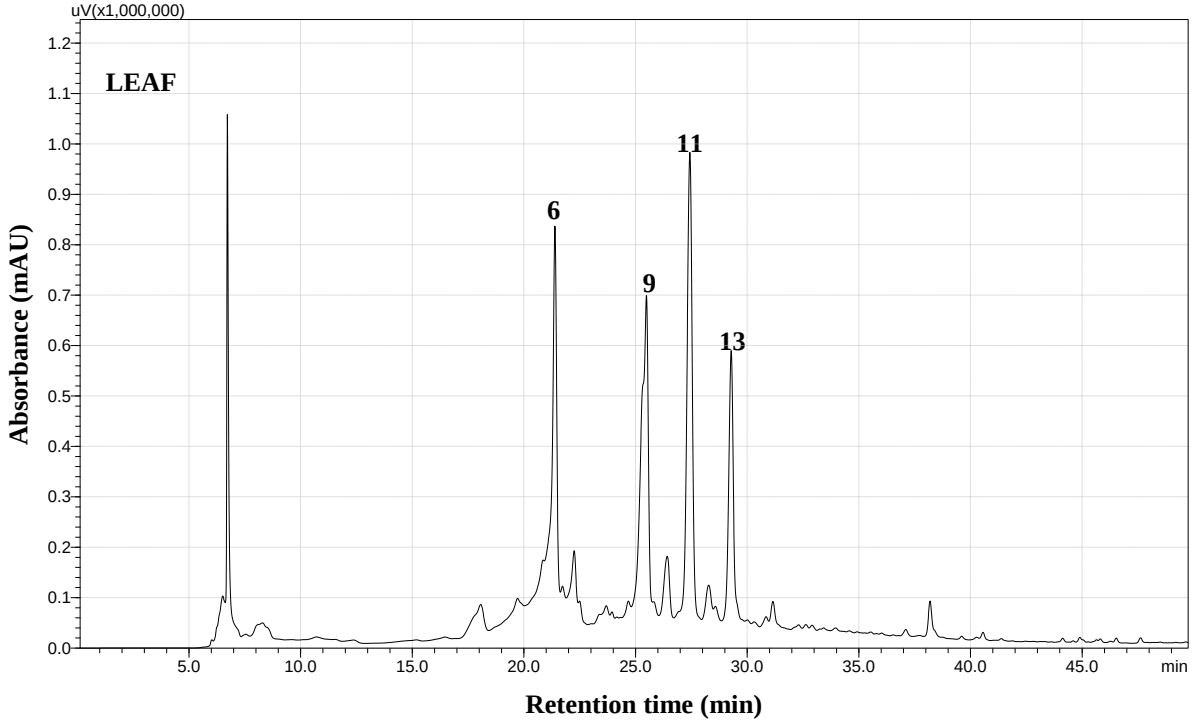
Rt: Retention time, n.i.: Not identified

Figure 1. Chromatograms at 280 nm of saffron leaf and tepal extracts analyzed by liquid chromatography-mass spectrometry showing the major peaks detected. Peak numbers as in Table 2 (leaf) and Table 3 (tepal).

Figure 2. Effect of corm, leaf, and tepal extracts on proliferation of Caco-2 cells. Cells were seeded in 96 well plates and grown in the presence of the extracts (0.5 mg/ml) for up to 7 days. Cell number was determined as uptake of the vital stain neutral red after 1, 2, 3, 4, and 7 days of treatment. Data are the mean of six replicates ± standard error.
* Indicates statistically significant differences as compared to controls for the same time period ($p<0.01$, ANOVA).

Figure 3. Effect of increasing concentrations of corm, leaf, and tepal extracts on proliferation of Caco-2 cells. Cells were seeded in 96 well plates and incubated in the presence of extracts (0 to 1 mg/ml) for 4 days. Cell number was determined as uptake of the vital stain neutral red. Data are the mean of six replicates ± standard error.

Figure 1.


uV(x1,000,000)
LEAF
6
9
11
13
Absorbance (mAU)
Retention time (min)
min

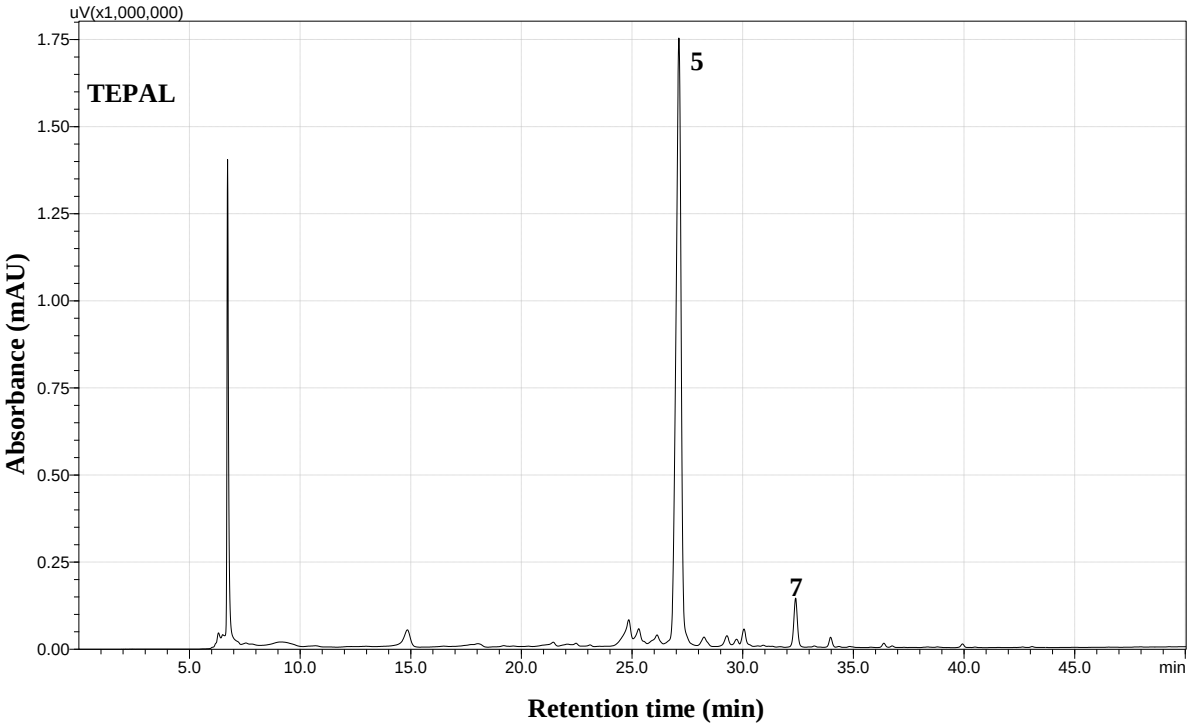

uV(x1,000,000)
TEPAL
5
7
Absorbance (mAU)
Retention time (min)
min

Figure 2.

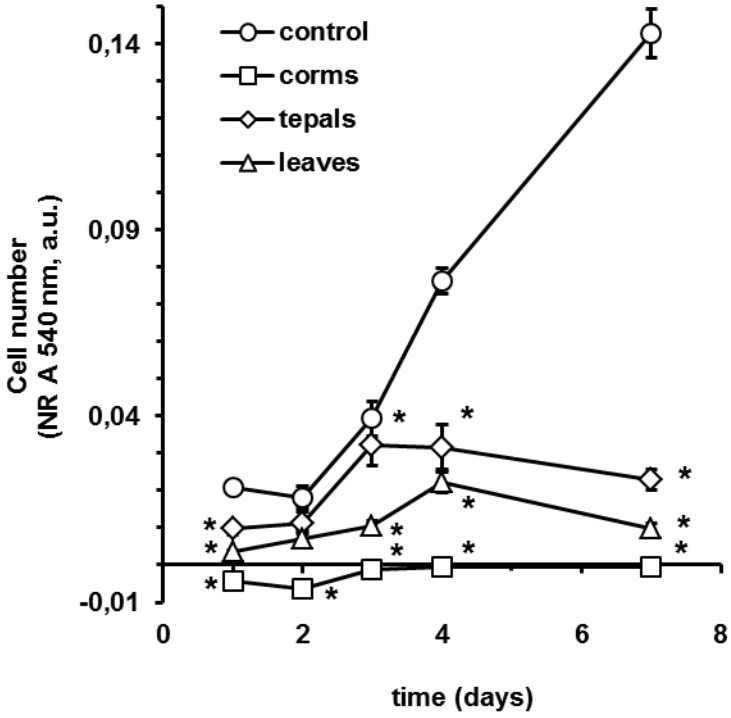
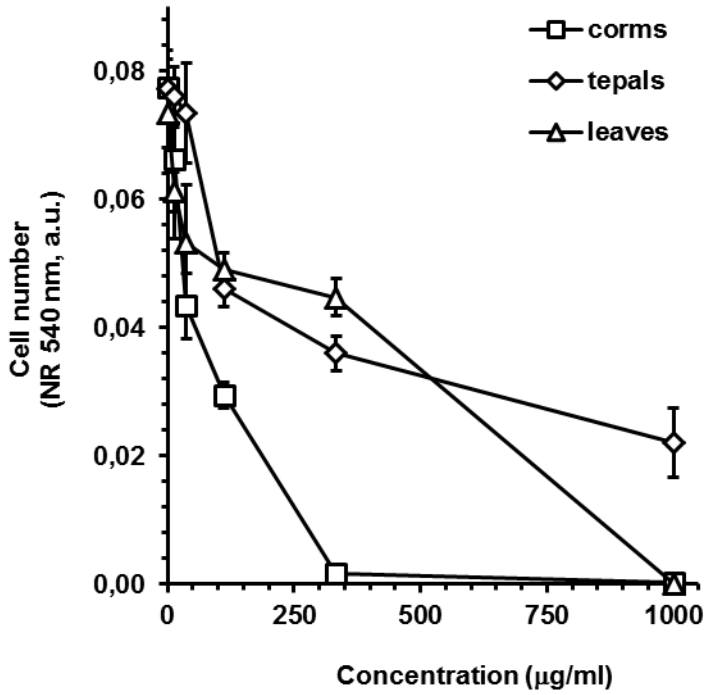

control
corms
tepals
leaves
Cell number
(NR A 540 nm, a.u.)
0,14
0,09
0,04
-0,01
0
2
4
6
8
time (days)

Figure 3.


corms
tepals
leaves
0,08
0,06
0,04
0,02
0,00
Cell number
(NR 540 nm, a.u.)
0
250
500
750
1000
Concentration (µg/ml)