Abstract. Among the different species of Crocus, only C. sativus has been extensively studied for the composition and the biological properties of its styles, since these constitute the well-known spice saffron, which is widely used in the Mediterranean, Indian and Chinese diet. With high performance liquid chromatography (HPLC) and UV/vis spectroscopy, the presence of hydrophilic carotenoids in the styles of three other Crocus taxa, endemic in Greece, C. boryi ssp. tournefortii, C. boryi ssp. boryi and C. niveus, is reported for the first time. Incubation of MCF-7 and MDA-MB-231 breast cancer cells for 48 h with different concentrations of all four Crocus style extracts showed a dose-dependent inhibitory effect on cell proliferation measured by the MTT assay. The antiproliferative effect was not related to the presence of estrogen receptors. Studies on the effect of trans-crocin-4 (the main carotenoid constituent of C. sativus styles, digentibiosyl-ester of crocetin), crocetin and safranal showed that the antiproliferative effect is attributed to the constituent crocins irrespective of the degree of glycosylation. These results show that the styles of the various Crocus taxa merit further investigation of their composition and mechanisms of action of their carotenoid constituents in order to establish if they could be used as chemopreventive or anticancer agents.

Breast cancer accounts for over one third of invasive carcinomas in women. Epidemiological studies have suggested that a high consumption of carotenoids may protect against the development of breast cancer (1, 2). Further in vitro and in vivo studies have suggested that many carotenoids, including non-vitamin A-precursors, such as lycopene, may be of use for cancer chemoprevention (3). The crocins are a family of glucosylated derivatives of crocetin (8,8’-diapocarotene-8,8’-dioic acid) which are found in the fruits of gardenia (Gardenia jasminoides Ellis) and in the styles (slender neck-like formation of the ovary, which at its apex forms the pollen-receptive surface of stigma) of Crocus sativus. In the species of the Crocus genus, the style starts from the underground ovary and is continuous up to the flower above the ground. Commercially the most important part of the style is the uppermost one (saffron), which is usually divided into branches and is coloured yellow, orange or red (4). Saffron as a spice is an important ingredient in the Mediterranean, Indian and Chinese diet and has been extensively used in folk remedies (5, 6). The genus Crocus consists of about 80 species worldwide, distributed from South-Western Europe, through Central Europe to Turkey and South-Western parts of Asia (4). Among these species, the cultivated C. sativus is the best known and studied. The composition and the biological properties of styles of other Crocus species have not been reported.

In vitro and in vivo experiments with the styles of C. sativus have shown their antiproliferative action towards specific cancer cell lines (HeLa, HL60, K562, P388, S-180 etc.) in a dose-dependent way, but the mechanism of action is not clarified yet (5, 7-14). Their effect on breast cancer cells has not been investigated, despite the fact that breast cancer is a common malignancy and a leading cause of death in women (15).

The aim of this study was to investigate the effect of extracts of the styles of C. sativus, C. boryi ssp. tournefortii, C. boryi ssp. boryi and C. niveus on breast cancer (MCF-7 and MDA-MB-231) cell proliferation. MCF-7 epithelial cells are estrogen-dependent (ER+: estrogen receptor positive) and of low invasiveness, and MDA-MB-231 cells are estrogen-independent (ER–) and of high invasiveness. In addition, the effect of the C. sativus constituents safranal (a monoterpene aldehyde), trans-crocin-4 (a di-gentibiosyl-ester of crocetin) and crocetin on breast cancer cell proliferation was also studied.
Materials and Methods

**Chemicals and materials.** All-trans retinoic acid and safranal were purchased from Sigma-Aldrich, Athens, Greece. The breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Middlesex, UK) and Eagle's minimal essential medium (EMEM) was from Biochrom KG Seromed®, Berlin, Germany. All other reagents were of analytical grade.

**Plant collection and extraction.** Crocus flowers were collected from regions of Southern Peloponnisos and the island of Serifos in the Cyclades in Greece during the autumn of 2004. *C. boryi* ssp. *boryi* was obtained from areas between Githion and Areopolis and *C. niveus* between Kardamyli and Kalamata, in Southern Peloponnisos while *C. boryi* ssp. *tournefortii* was collected from the area of St. Sostis, on Serifos. Styles were separated from the rest of the flowers and were dried at 37°C in a well-ventilated room. Styles of *C. sativus* were kindly supplied by the Cooperative of Krokos (Kozani, Greece).

The *C. sativus* style is divided into three branches with a length of 2.5-3.2 cm. In *Crocus boryi* ssp. *boryi* the style is divided in many slender orange or reddish branches, with a length of 0.8-1.0 cm. Style of *C. boryi* ssp *tournefortii* is also divided in many slender orange or reddish branches, with a length of 1.1-1.3 cm, while that of *C. niveus* is divided in three orange or bright red branches with a length of 1.3-1.4 cm. Specimens are deposited at the herbarium of the Department of Biology, University of Patras, Greece.

Dried styles of each *Crocus* taxon were subjected to extractions with methanol:water (50%, v/v) (3 ml / 50 mg) for 4 h (25°C) in the absence of light and with continuous stirring. The extracts were centrifuged, filtered through a 0.2-μm filter and evaporated to dryness in a Speed Vac system (Labconco Corp., Kansas City, Missouri, USA). The residues were stored at −20°C until further use. Samples were redissolved in methanol:water (50% v/v) before analysis.

**UV-Vis spectroscopy and HPLC analysis of *Crocus* styles extracts.** The spectra of the style extracts were recorded with a Biochrom 4060 (Pharmacia LKB) instrument. The extracts were analyzed with high performance liquid chromatography (HPLC) (Mod.10 AKTA, Amersham Biosciences, Piscataway, NJ, USA) on a Supelcosil C18 (5 μm, 25 cm x 4.6 mm, Sigma-Aldrich) column. Elution was performed with 20% v/v methanol containing 1% v/v acetic acid for 2 min, a gradient of methanol (20-70%) in the presence of acetic acid 1% for 50 min, with a gradient of methanol (70-100%) in the presence of acetic acid 1% for 5 min and 100% methanol containing 1% acetic acid for 1 min with a flow rate 0.5 mL/min. Detection wavelength was 440 nm.

**Isolation and identification of trans-crocin-4.** trans-Crocin-4 was isolated from the crude extract by semi-preparative HPLC on a Supelcosil C18 (5 μm, 25 cm x 8 mm) column. The mobile phase was a linear gradient from 20-70% methanol in water in 70 min, while the solvent flow rate was 1.5 ml/min and the sample injection volume was 50-100 μl. The peak eluted at 39.3±0.5 min, was collected and purified (>98%) after re-chromatography on an analytical column. The identity of the pure crocin constituent was studied by analytical HPLC using the method of Tarantilis et al. (16), electrospray ionization-mass spectrometry (ESI-MS) on a Micromass-Platform LC instrument (Micromass, Manchester, UK) and UV/Vis spectroscopy.

**Preparation of crocetin.** Crocetin was prepared by saponification of the *C. sativus* style extract as previously described (17). In brief, the extraction of *C. sativus* styles was hydrolysed with 10% sodium hydroxide aqueous solution at 65°C for 3.5h. The solution was acidified with phosphoric acid and the yielded precipitate was washed twice with water and then with methanol. The crocetin was crystallised with dimethylformamide and dried under vacuum. The crocetin was identified by thin layer chromatography (TLC) in acetone/dichloromethane 6:1, analytical HPLC (16) and UV/Vis spectroscopy.

**Cell culture and proliferation assays.** Cells were cultured as monolayers at 37°C in a humidified atmosphere of 5% (v/v) CO₂ and 95% air and in EMEM supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 100 μg/mL of insulin and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamycin and 2.5 μg/mL amphotericin B).

For cell viability assays, cells were seeded at an initial density of 5,000 cells/well in 24-well tissue culture plates, and incubated in medium with or without extracts or safron constituents at various concentrations for 48 h. At the end of treatment time, cells were washed and 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) in phosphate-buffered saline (PBS) was added to each microwell at a 1: 10 dilution for 4h (18). Cells were then washed with PBS and diluted in dimethyl sulphoxide. The measurement of the absorbance at 490 nm was performed in a Molecular Devices (Sunnyvale, CA, USA) E-max photometer. Each experiment was performed in triplicate and was repeated at least three times.

Statistically significant differences were evaluated by Student’s t-test using the Microsoft Excel statistical package. The acceptable level of significance was set at *p*<0.05.

**Results**

**HPLC analysis of extracts of styles of the various *Crocus* taxa.** The *C. sativus* styles fingerprint obtained by HPLC was in complete agreement with previous reports (7, 19). Eight peaks absorbing at 440 nm could be identified: trans-crocin-4 (the major carotenoid compound), trans-crocin-3, trans-crocin-2, cis-crocin-5, cis-crocin-4, trans-crocin-2, cis-crocin-5 and cis-crocin-1 (Figure 1D).

UV-Vis spectroscopy of all *Crocus* style extracts showed the presence of a double peak at 440 nm, which is characteristic of trans-carotenoids (Figure 1). HPLC analysis and monitoring at 440 nm showed that the carotenoids in *C. sativus* styles (Figure 1D) elute later than those of the other *Crocus* taxa (Figures 1A, B and C) which all have a similar HPLC elution pattern. As shown in Figure 1D, the spectrum of the *C. sativus* style extract also shows an absorption band at 250-260 nm (indicative of the glycosyl esters bonds of both cis-and trans-carotenoids and picrocrocin) and at 330 nm (characteristic of cis-carotenoids), as previously described (16). The same spectral absorption bands were also observed in the *C. boryi* ssp. *tournefortii* style extract (Figure 1A). Absorption bands at 250-260 nm and 350-360 nm were
observed in the *C. niveus* and *C. boryi* ssp. *boryi* extracts. In the endemic *Crocus* taxa the ratio of absorptivity at 250-260 nm to that at 440 nm is higher than that in *C. sativus*. This finding together with the earlier elution time of carotenoid constituents in the three endemic taxa suggests a higher degree of glycosylation of the carotenoids or a shorter carotenoid chain in the Greek endemic taxa.

Isolation of trans-crocin-4 and preparation of crocetin. By analytical HPLC, the isolated compound eluted at 34 min as one peak, the purity of which was calculated as 98%. The UV-vis spectrum of the isolated compound showed two absorption bands; one at 256 nm corresponding to glucosyl ester bonds of crocins and a double peak between 400 and 500 nm (maximum at 440 nm) characteristic of all trans-glycosidic carotenoids. The mass spectrum of trans-crocin-4 displayed an ion at m/z 999 [trans-crocin-4+Na⁺] and an additional signal at m/z 511.46 [trans-crocin-4+2Na⁺] /2.

After purification of the crocetin, TLC showed one compound with the Rf being 0.45. HPLC analysis showed the presence of one peak eluted at 55 min and disappearance of the peaks of the other crocins, whereas UV/vis spectroscopy showed one double peak between 400 and 500 nm (maximum at 440 nm) and a shoulder at 400 nm, as expected for crocetin.
Effect on breast cancer cell proliferation. With the exception of *C. niveus*, the *Crocus* style extracts significantly inhibited the proliferation of the ER⁺ MCF-7 and ER⁻ MDA-MB-231 breast cancer cells in a dose-dependent way (Figure 2), showing that the action of the constituents is not related to the presence of estrogen receptors. The inhibitory effect of *C. boryi* ssp. *tournefortii* and *C. niveus* on MDA-MB-231 (ER⁻) (IC₅₀ values <100 µg/mL) was statistically significantly higher than that on MCF-7 cells (ER⁺) (IC₅₀ values≈1000 µg/mL), suggesting selective action against this cell line. It is remarkable that *C. boryi* ssp. *boryi* had a high antiproliferative effect on both cell lines (IC₅₀ values<100 µg/mL).

The *C. sativus* style extract also inhibited the proliferation of breast cancer cells. The IC₅₀ values for *C. sativus* were 350 and 500 µg/mL for MCF-7 and MDA-MB-231, respectively. In order to identify the constituents, which mediate this action, the effects of *trans*-crocin-4 (the main *C. sativus* carotenoid constituent), crocetin and safranal on breast cancer cell proliferation were investigated. Safranal significantly (*p≤0.05*) inhibited the proliferation of MDA-MB-231 at concentrations higher than 125 µM and the proliferation of MCF-7 at concentrations higher than 500 µM. Similarly, *trans*-crocin-4 significantly (*p≤0.05*) inhibited the proliferation of MDA-MB-231 and MCF-7 at concentrations >200 µM (Figure 3A and B).

*Crocetin* inhibited the proliferation of MDA-MB-231 cancer cells in a similar way to *trans*-crocin 4, but with MCF-7 cells the inhibitory effect was significant even at low concentrations (25 µM). Crocetin (no sugars) was selected since crocins in the extract are numerous and vary in glycosylation.

All-*trans* retinoic acid (ATRA), a major representative of the retinoid family, has been suggested to inhibit the growth of leukemia, human colon and lung cancer cells (21-23). In our experimental protocol, ATRA at the concentrations of 10, 50 and 100 µM did not affect the proliferation of breast cancer cells after 48 h incubation. Previous studies on the effect of ATRA on breast cancer cell lines (MCF-7 and MDA-MB-231) have shown a significant antiproliferative action after three days of incubation (24, 25).
Discussion

Our results have shown that saffron extract and its constituents, i.e., trans-crocin-4, crocetin and safranal, significantly inhibit the proliferation of MCF-7 and MDA-MB-231 cells. The IC\textsubscript{50} values of the extract are lower than that reported for HeLa cells (2.3 mg/mL) (8). Both studies have shown that this inhibitory activity is mostly due to crocins. This is in accordance with other studies which have shown cytotoxic activity of dimethyl-crocetin, crocetin, and crocin in human chronic myelogenous leukemia K562 and promyelocytic leukemia HL-60 cells with 50% inhibition reached at concentrations of 1.2, 5.0 and 6.6 \textmu M (7). The cytotoxic activity of crocin has also been demonstrated with rat adenocarcinoma DHD/K12-PROb cells and human colon adenocarcinoma HT-29 cells with ID\textsubscript{50} (50% inhibitory dose) values of 0.4 and 1.0 mM, respectively (9), and with HeLa cells (ID\textsubscript{50}=3 mM). Other studies utilizing the method of colony formation as a measure of cell viability have also demonstrated that the IC\textsubscript{50} of saffron extract ranged from 100 to 250 \textmu g/mL depending on the type of malignant cells, but had no significant effect on normal mouse spleen cells (26-28).

The results showed that the action of crocins does not depend on the glycosylation of crocetin, but is more likely to be caused by the polyene backbone of the carotenoids. The fact that crocetin had a stronger antiproliferative action than trans-crocin-4 (an ester of crocetin) on the MCF-7 cells, leads to the conclusion that crocetin may suppress MCF-7 cell proliferation via an extra mechanism which does not exist in the MDA-MB-231 cells. Jagadeeswaran et al. have observed that an inhibition of proliferation of rhabdomyosarcoma cells was induced by crocetin, whereas Tarantilis et al. have reported that crocetin displayed a strong antiproliferative action on HL-60 and K562 cells (IC\textsubscript{50}=2 \textmu M) (7, 29, 30). Previous studies have shown that orally ingested crocins could not act as bioactive molecules by themselves in vivo except in the gastrointestinal tract since they are hydrolyzed to crocetin before being incorporated into the blood circulation (17). Therefore, the fact that crocetin is active is of great importance. On the other hand, Escribano et al. (8) have reported that crocetin had no cytotoxic effect in HeLa cells. The reported discrepancy could be due to the differences in cell lines and culture conditions used and different mechanisms of action. Abdullaev et al. (12) have shown that crocin had a dose-dependent inhibitory effect on the DNA, RNA and protein synthesis of different human malignant cells (HeLa, A-204, HepG-2, CCD), but no significant effect on colony formation.

The low level of safranal in the extract (<0.01% of extract) (20) and its lack of effect on breast cancer cell proliferation at concentrations lower than 125 \textmu M (18.78 \textmu g/mL), although the extract significantly inhibited cell proliferation at a concentration of 100 \textmu g/mL, show that the antiproliferative effect of the extract is not attributed to safranal, but mainly to the crocin constituents irrespective of the glycosylation degree (~20% of extract dry weight).

The exact mechanisms underlying this effect are largely unknown. Earlier studies on the effect of saffron constituents on other cancer cells have associated their antiproliferative effect with the inhibitory effect on cellular DNA and RNA synthesis and interaction with topoisomerase II, an enzyme involved in cellular DNA-protein interaction (12, 13). A third suggested mechanism is the metabolic conversion of naturally occurring carotenoids to retinoids, even though it has been reported that this is not a prerequisite for anticancer activity (10, 11). Escribano et al. (8) have described various changes in the morphology of treated cells suggestive of apoptosis, while another proposed mechanism is the inhibitory effect of these compounds on free radical chain reactions (5, 10). The possibility of induction of gap junctional communication by carotenoids has also been proposed (11, 14). Since the inhibitory effect of ATRA was not significant in the present experiment protocol, it may be suggested that the antiproliferative effect of the carotenoids derived from C. sativus styles is not due to conversion to retinoids. C. boryi ssp. tournefortii and C. boryi ssp. boryi are endemic in Greece while C. niveus is endemic only in Peloponnisisos (31). Screening of the crude extracts from their styles showed that the plants are rich sources of polar carotenoids. This is the first report on the composition of styles of other Crocus plants besides C. sativus although structural identification was not performed. In our experiments the highest antiproliferative action was caused by C. boryi ssp. boryi. With the MDA-MB-231 cells, extracts of all endemic Crocus plants displayed higher inhibitory effect than C. sativus. The high antiproliferative action of the extracts of the styles of the endemic Crocus plants, especially of C. boryi ssp. boryi, shows that further studies on their composition and biological properties, such as toxicity and bioavailability, are necessary in order to evaluate their anticancer potential.

Saffron toxicity has been studied by many researchers. Nair et al. (32) have reported that the toxicity levels are very low; the lethal dose (LD\textsubscript{50}) required to kill 50% of mice after receiving saffron extract, was very high (>600 mg/kg body weight). Abdullaev et al. (10) have also shown that the oral administration of the saffron extract at concentrations from 0 to 5 g/kg was non-toxic in mice. In combination with the fact that crocins (water soluble carotenoids) are easily absorbed (17, 33), we conclude that Crocus style extracts and their constituents should be further studied in vitro and in vivo in order to establish if they could be of value as chemopreventive or anticancer agents.
References


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