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Chemical Composition of Saffron (*Crocus sativus* L.) from Four Countries

Heriberto Caballero-Ortega¹, Rogelio Pereda-Miranda², Leticia Riverón-Negrete¹, Juan M. Hernández¹, Mayra Medécigo-Ríos¹, Adriana Castillo-Villanueva¹ and Fikrat I. Abdullaev¹

¹Experimental Oncology Laboratory, National Institute of Pediatrics-SS, Torre de Investigación "Dr. Joaquín Cravioto", Av. IMAN # 1, 6° Piso Col. Insurgentes Cuicuilco, México D.F.

²Chemistry Faculty, National Autonomous University of Mexico Mexico

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Abstract

Saffron, the dried stigmas of *Crocus sativus* L., is widely used mainly as herbal medicine or food coloring, and as a flavoring agent. It is cultivated only in a few countries around the world. Comparative analytical and semi-preparative High Performance Liquid Chromatography (HPLC) studies using photodiode array analyses were performed on a Waters HPLC system for the separation of several ingredients from alcoholic extracts of four different saffron types. Ten saffron peaks were identified by comparison of their retention times with those of known reference compounds and quantified from samples of Azerbaijanian, Spanish, Indian and Iranian saffron as follows: picrocrocin, trimethyl hydroxy carboxaldehyde cyclohexene, kaempferol, cis/trans-crocins and safranal. 2-nitroaniline (Sigma Chemical Co.) was used as the internal standard. It was found that the total content of carotenoids in Azerbaijanian and Iranian saffron samples was higher in comparison to other samples. These HPLC analytical procedures are sensitive, reproducible and allow for higher scaling of the instrumental conditions for obtaining sufficient amounts (mg) of the different saffron components for further cytotoxic assessments. Spanish saffron metabolites were collected and tested for their cytotoxicity against human tumor cells. Our results confirm that glycosidic carotenoid-type metabolites are responsible for the antitumor effects demonstrated by saffron.

INTRODUCTION

Saffron is a food spice obtained from the flower stigmas of *Crocus sativus* L., a member of the Iridaceae family. It plays an important role in the culinary culture of different regions of the world, where it is used as a food additive, since it possesses powerful coloring and flavoring properties due to its glycosidic constituents (Abdullaev, 1993; Ríos et al., 1996; Abdullaev and Frenkel, 1999). However, recent studies have shown that saffron has anti-tumoral (inhibitory effect on malignant cell growth) and anti-carcinogenic (an inhibitory effect on the induction of cancer by chemical agents) activities in biological systems both in vivo and in vitro (Nair et al., 1991, 1995; Abdullaev and Frenkel, 1992a,b; Escribano et al., 1996; García-Olmo et al., 1999; Premkumar et al., 2001; Abdullaev et al., 2003). Currently, scientists have been able to isolate different saffron components and assess their cytotoxic effect on diverse types of tumoral cells (Abdullaev et al., 2002). The purpose of our study was to separate and compare the chemical composition of saffron extracts from four countries, in addition to testing the biological activity of each of the components isolated in colony formation assays.

MATERIAL AND METHODS

Saffron and Reagents

Pure saffron stigmas were obtained from local markets in four different countries: Azerbaijan, Spain, India and Iran, and were stored in the dark at 4°C until they were used. The methanol and acetonitrile used were purchased from Sigma Chemical Co. (St. Louis, MO). All of the reagents were of analytical degree. The water used was bi-distilled and purified using a Millipore system (Milli-Q).

Extraction Procedure

In order to achieve the separation of the saffron components from all samples, a methanolic extract was prepared using 20 mg of saffron stigmas from each country and grounded in 5 ml of methanol and 5 ml of bi-distilled water (2 mg/ml) for 2 hours at room temperature and in the dark while constantly being stirred. In order to separate the residual fraction of the extracts, the samples were centrifuged at 30,000 g for 20 minutes. They were filtered using Nylon Acrodisc Waters 13 (0.45 µm) membranes and stored at –20°C until they were used (Tarantilis et al., 1995).

Chromatographic Conditions

High performance liquid chromatography was used for the detection of the saffron components, in addition to a Waters equipment (Millipore Co., Milford, MA, U.S.A.) consisting of a solvent distribution system Model 600 E and a multiple UV wavelength photo-diode array detector (Model 996), linked to a computerized control system (Optiflex 466/LE, DELL). The uploading and processing of the chromatographic information was done using Millennium 2000 (Waters) software. The analytical chromatographic conditions used for the resolution of the extracts included a reverse phase Waters Spherisorb ODS2 column (4.6 x 250 mm internal diameter). The mobile phase used was a linear gradient of methanol in water from 10% to 100 percent containing 15 percent acetonitrile, different from the methods reported since they include 1% acetic acid in water, a maximum elution time of 60 minutes for each extract, a constant flow rate of 0.5 milliliters per minute in the mobile phase and an injection volume of 50 µl (Tarantilis et al., 1994; Tarantilis et al., 1995; Escribano et al., 1996; Lozano et al., 1999). 0.2 milligrams per milliliter of 2-nitroaniline was used as an internal standard. This compound was chosen for its separation and resolution from close peaks and for its detection at all assayed wavelengths (Lozano et al., 1999). Detection was performed simultaneously at 250, 310 and 440 nm. The main modification made to this method was the substitution with a solvent of similar polarity, but with a pH similar to neutral. When saffron components come into contact with an acid medium, their chemical structures change, a condition which constitutes a danger for maintaining the glycosidic nature of the majority of the saffron constituents intact and therefore, altering its biological activity. When the analytical method was standardized, the semipreparative method was developed. The solvent chosen was acetonitrile at the same concentration as that used in the analytical phase. The column used was a reverse phase Waters Spherisorb ODS2 (20 x 250 mm of internal diameter), at a flow rate of 9.45 ml/min and an injection volume of 500 µl (Abdullaev et al., 2002).

Colony Formation Assay

In order to determine which saffron components are responsible for its anti-tumoral activity, the effect of 10 isolated saffron fractions in HeLa cell colony formation assays was measured according to protocol (Abdullaev and Frenkel, 1992b):

- Seeding of HeLa cells (100,000 cells/dish)
- Incubation for 3 hours with each saffron extract (200 µl/ml) component
- Treatment with trypsin
- Seeding of 200 cells to start colony formation
- Incubation for 10 days

- Fixing and staining
- Counting of colonies. This assay was only carried out using the Spanish saffron sample.

RESULTS AND DISCUSSION

Ten fractions were identified from all of the extracts by comparing the retention times as previously described (Castelar et al., 1993; Tarantilis et al., 1995; Li et al., 1999; Lozano et al., 1999), as shown in Figure 1 and as follows: at 250 nanometers of wavelength, peaks number 1, 2 and 3 corresponded to picrocrocin, hydroxy-trimethyl-carboxaldehyde-cyclohexene (HTCC) and kaempferol, respectively. At 310 nanometers of wavelength, peak number 8 corresponded to safranal, and at 440 nanometers, peaks 4, 5, 6, 7, 9 and 10 corresponded to crocins 1, 2, 3, 4, 5 and 6, respectively. Using this methodology, we were able to obtain sufficient amounts (in milligrams) of each fraction to carry out the colony formation assays. Based on the chromatographic results, it can be speculated that different saffron samples do not differ in their chemical composition, but do vary in their metabolite concentrations.

Table 1 shows the different concentrations of the saffron components, expressed in milligrams per gram of total extract. It is clear that the Azerbaijanian and Iranian saffron samples have higher concentrations of these components. This could be partially due to the geographical locations where they were grown or to their degree of purity, since on occasions it is inevitable to spontaneously contaminate or induce leaf remains or flower petals in the mixture. Nonetheless, it is evident that the greater concentration of the components corresponded to diverse carotenoids in each sample.

In order to support the previous results obtained in our laboratory with reference to the cytotoxic activity that the total saffron extract possesses (Abdullaev and Frenkel, 1992a,b), each of the fractions obtained from the Spanish extract were tested on the growth of human HeLa tumor cells. Figure 2 shows that all of the fractions inhibit the formation of this cell type. It can also be appreciated that those fractions containing carotenoids develop a strong cellular inhibition, suggesting that this biological activity is provided by the compound activity of the saffron components.

These results are particularly important for the Azerbaijanian and Iranian extracts, since the carotenoids constitute a wide group of natural pigments responsible for the coloring and characteristic aroma of many vegetables, fruit and flowers, including saffron, but in addition it is known that thanks to its anti-oxidant potential, it can also be used as a cytotoxic and anti-tumoral agent (Abdullaev 1993; Nair et al., 1995; Escribano et al., 2000; Abdullaev et al., 2002). Saffron has been considered a new source of therapeutic agent for pharmaceutical use. Nonetheless and in spite of the high carotenoid concentration in saffron, these substances are very labile once purified. A new biotechnological methodology should be used to produce large quantities of these components for their use as chemopreventive agents.

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Tables

Table 1. Saffron compound concentrations

| Wavelength (nm) | Compound | Azerbaijan | India | Iran | Spain |
|-----------------|-------------|------------|-------|--------|-------|
| 250 | Picrocrocin | 26.93 | 3.92 | 32.45 | 10.25 |
| 250 | HTCC | 2.09 | 0.42 | 4.63 | 0.64 |
| 250 | Kaempferol | 1.14 | 0.29 | 0.95 | 0.26 |
| 440 | Crocin 1 | 48.75 | 6.76 | 45.99 | 11.95 |
| 440 | Crocin 2 | 37.34 | 3.67 | 28.27 | 6.57 |
| 440 | Crocin 3 | 2.70 | 0.23 | 3.03 | 0.80 |
| 440 | Crocin 4 | 2.33 | 0.35 | 2.21 | 0.49 |
| 310 | Safranal | 8.59 | 5.52 | 2.81 | 5.82 |
| 440 | Crocin 5 | 12.73 | 1.26 | 8.71 | 2.73 |
| 440 | Crocin 6 | 5.62 | 0.55 | 3.40 | 1.20 |
| | TOTAL | 148.22 | 22.97 | 132.45 | 40.71 |

The amounts are expressed in milligrams for each compound per gram of total extract.

Figures

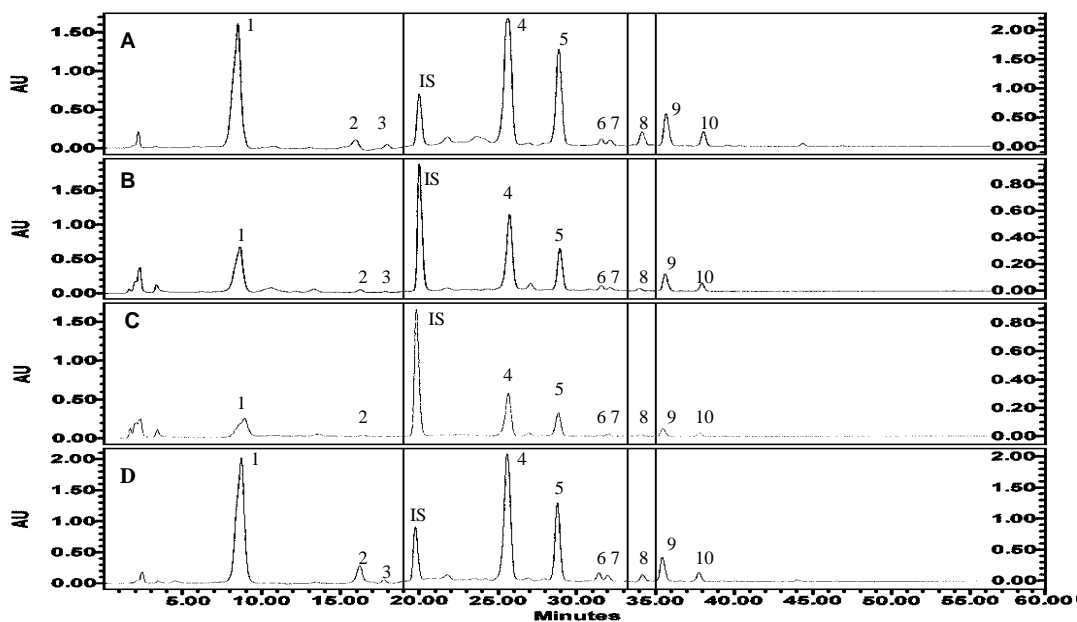


Fig. 1. Chromatograms of saffron extracts. A: Azerbaijanian saffron; B: Spanish saffron; C: Indian saffron; D: Iranian saffron. Saffron Components: Picrocrocin (1); Hydroxy-Trimethyl- Carboxaldehyde-Ciclohexene “HTCC” (2); Kaempferol (3); Crocin 1 (4); Crocin 2 (5); Crocin 3 (6); Crocin 4 (7); Safranal (8); Crocin 5 (9); Crocin 6 (10); Internal Standard (IS).

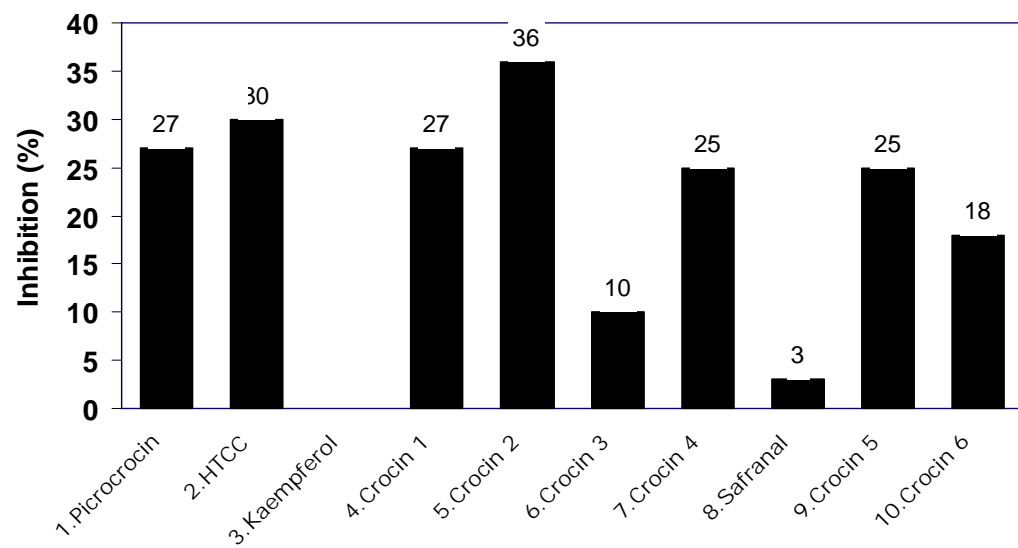


Fig. 2. Cytotoxic activities of Spanish saffron components *in vitro* on human HeLa tumor cells.