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Triterpenoid saponins from corms of *Crocus sativus*: Localization, extraction and characterization

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ABSTRACT

A mixture of highly glycosilated triterpenoid saponins (CS5) isolated from the corm of *Crocus sativus* or saffron showed cytotoxic activity against HeLa tumoral cells. The main reverse phase HPLC fraction of this mixture (CS5-1) contains two new oleanane-type saponins, denominated **Azafrine 1 (1)** and **Azafrine 2 (2)**. The bidesmosidic saponins were respectively characterized as (1) 3-*O*-β-D-glucopyranosiduronic acid echinocystic acid 28-*O*-β-D-galactopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-α-D-rhamnopyranosyl-(1 → 2)-[4-*O*-di-α-L-rhamnopyranosyl-3,16-dihydroxy-10-oxo-hexadecanoyl]-α-D-fucopyranoside and (2) 3-*O*-β-D-galactopyranosiduronic acid echinocystic acid 28-*O*-β-D-galactopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 2)-[4-*O*-di-α-L-rhamnopyranosyl-3,16-dihydroxy-10-oxo-hexadecanoyl]-β-D-fucopyranoside. The surfactant properties of saponins, probably involved in the cytotoxic activity of CS5 and their exclusive localization in the external part or the corm, indicate their possible role as phytoprotectans. The similarity of their structural compositions to that of other triterpenoid saponins which are of special use in the pharmaceutical industry suggest a new application for *C. sativus* crops through the exploitation of corm for saponin extraction.

1. Introduction

Crocus sativus or saffron is cultivated for its dried stigmas, also known as saffron, because they are used to produce the world's most prized and expensive spice. While the use of this spice in cooking has continued to grow, the cultivated surface of this crop in Europe has registered a continuous decrease in the last decades (Negbi, 1999). Screening for phytopharmaceuticals with industrially interesting properties in other saffron organs could increase the economic value of the whole plant, which is currently limited to their stigmas (Khan and Ather, 2006). The corm could be the organ used for such screening because it is responsible for the vegetative reproduction of the plant and because it is present during all its biological cycle, almost one year, in contrast to the stigmas that are only present a few days per year. Although the composition of saffron corm has not been intensively analyzed, the first studies revealed the presence of glucose, amino acids and saponins (an oleanoic acid glycoside and a steroid). At a later point the presence of amines, starch, fatty acids and sterols was also detected (Loukis et al., 1983). Now the apocarotenoid responsible for the anticancer properties of the saffron stigmas, crocetin (Bathaie and Mousavi, 2010), has also been detected in newborn corms (Rubio-Moraga et al., 2010). The dormant corm also showed the presence of an HPLC fraction active against tumoral cell lines (CF), that provoked the activation of macrophages by release of nitric oxide (NO) under non-cytotoxic concentrations (Escribano et al., 1999a,b, 2000). In addition, this fraction was toxic for the root development of *Arabidopsis thaliana* (Fernandez et al., 2000). Initially, the bioactivity of CF from *C. sativus* corm was attributed to a glycoconjugate identified as a putative proteoglycan. This study shows that the glycoconjugate is composed of saponins.

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Saponins often appear as complex molecule mixtures consisting of carbohydrate moieties (glycones) attached to cholesterol-like moieties called aglycones or sapogenins (Haralampidis et al., 2002). In nature, the most abundant triterpenoid saponins are oleanane derivatives such as oleanolic acid, hederagenin, glycyrrhetinic acid, presenegenin, quillaic acid or echinocystic acid (Kasai et al., 1999). Although the natural role of saponins in plants is usually as a defense from attack by pathogens and pests such as phytoprotectants (Morrissey and Osbourn, 1999), they are also used for industrial and commercial applications and as a source of raw materials for the production of cosmetics and medicines, especially against cancer (Man et al., 2010) or adjuvants in vaccines (Sun et al., 2009) in the pharmaceutical industry. Furthermore they are used as food additives and as an ingredient in photographic emulsions, fire extinguishers, foaming agents and denatured alcohol (Waller and Yamazaki, 1996). All these properties are also important from an agronomical point of view because they provide and add value to plants that contain saponins, e.g. soy (Zhang and Popovich, 2009) and ginseng products (Jia and Zhao, 2009).

In this study we optimized the isolation and purification of the saponins from saffron corm and we demonstrated that they are localized exclusively in the external part of the corm. We also performed the isolation, purification and structural elucidation of the main two new saponins denominated **Azafrine 1** and **Azafrine 2**. The use of these saponins as phytopharmaceuticals or even as phytoprotectants, as is the case of other saponins with a similar composition, is a promising contribution to the research into new applications for the *C. sativus* crops.

2. Experimental

2.1. General

Optical rotations were measured at 20 °C on an Elmer 241 MC polarimeter (Waltham, MA, USA). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Voyager-DE instrument (PerSeptive Biosystems, Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width), using sinapinic acid as matrix. Positive-ion mode spectra were recorded. Electrospray ionization mass spectrometric analyses were performed using an LCQ DecaXP ion-trap instrument equipped with an electrospray ion source (Thermo Finningan, San Jose, CA). The samples were dissolved in the mobile phase, 2-propanol/water (1:1) to a concentration of approximately 10–30 μg/μL and then introduced via a syringe pump at a flow rate of 5 μL/min. The capillary temperature was set to 225 °C. ESI-mass spectra were acquired in both positive- and negative-ion modes by scanning over m/z 50–2000 with a spray voltage of 4.8 kV and varying capillary voltages of 31.5–46.0 V, including tandem MS^n using automated MS/MS of the selected parent ion (precursor).

NMR spectra were recorded on a DRX-500 spectrometer (Bruker, MA, USA) and on a SYSTEM 500 spectrometer (Varian, Palo Alto, CA) equipped with a 5 mm HCN cold probe (500 MHz for ^{1}H, 125 MHz for ^{13}C). Samples were dissolved in 600 μL of pyridine-d_5 and were analyzed at 300 K. NMR experiments included TOCSY (20–200 ms), ^{1}H-^{13}C HSQC, gHSQCTOCSY, HMBC, DEPT and ROESY (300 ms), using conventional pulse sequences. Chemical shifts (δ) are expressed in ppm relative to pyridine-d_5 (^{1}H, 7.19 ppm; ^{13}C, 123.15 ppm). Data were processed using ProSpect ND software. The methylation analysis (Ciucanu and Kerek, 1984), qualitative/quantitative analysis (Kamerling and Vliegenthart, 1989) and the absolute configuration determination (Gerwig et al., 1979) of the monosaccharides were carried out by gas-liquid chromatography–mass spectrometry (GC–MS) using a Fisons Instruments GC 8060/MD 800 system (Interscience) with a capillary column AT-1 (30 m × 0.25 mm, Alltech, Flemington, NJ). Isolations and purifications were carried out using an 1100 HPLC system (Hewlett Packard, Palo Alto, CA) connected on line with a photodiode array detector, with a dynamic range from ultraviolet to visible region (190–800 nm).

2.2. Cytotoxicity bioassays

The cervical epithelia carcinoma (HeLa) cell line was obtained from the American Type Culture Collection (Rockville, MD). The antitumor cytotoxicity assay was performed as previously described (Escribano et al., 1999b). All statistical analyses were performed with SPSS software. Statistical differences ($p < 0.05$) between mean values were determined by one-way ANOVA followed by Studentĭs test.

2.3. Plant material

The corms of August from *C. sativus* were collected from farmers in Tarazona de la Mancha (Albacete), and stored at −80 °C until use. A voucher specimen was deposited in the Biotechnology Division, Instituto de Desarrollo Regional, Castilla-La Mancha University, Albacete, Spain.

2.4. Extraction and isolation of CF

CF was isolated as previously described (Escribano et al., 1999b).

2.5. Extraction and isolation of CS5

The external part of 1 kg of corms from August was lyophilized and extracted 3 times with 50% aqueous isopropanol at room temperature. The extract was concentrated and the oily residue was re-dissolved in 30% aqueous MeOH and precipitated with acetone (1:1). After centrifugation, the pellet was re-dissolved three times in pure MeOH and precipitated with acetone (1:1) to obtain a crude mixture of saponins (Csap). Csap was dissolved in 50% aqueous acetonitrile and fractionated on a reversed-phase HPLC Cosmosil 5C_{18}-AR II column (4.6 × 250 mm, Hewlett Packard, Palo Alto, CA). The column was equilibrated with 36% acetonitrile (v/v), containing 0.05% trifluoroacetic acid (v/v), and eluted with the following acetonitrile gradient containing 0.05% trifluoroacetic acid (v/v): from 36 to 44% in 15 min, from 44 to 82% in 1 min, 82% for 5 min, from 82 to 36% in 1 min, and 5 min to re-equilibrate the column. The flow rate was 1.5 mL/min and the detection wavelength was 208 nm. CS5 was obtained from 10 to 15 min.

2.6. Extraction and isolation of *1* and *2*

CS5 was re-fractionated again on HPLC as previously described, in order to purify the main HPLC fraction, denominated fraction CS5-1. To isolate **1** and **2**, CS5-1 was separated with silica gel 60 F254 TLC plates (Merck) using $CHCl_3$-MeOH-H_2O (56:37:7).

Two bands were visualized by staining with 0.2% orcinol in 20% H_2SO_4 and by heating in an oven at 150 °C for 5 min. A semi-preparative TLC was conducted to isolate these two bands. Each band was scraped from the backing material and was extracted with $CHCl_3$–MeOH–H_2O (14:6:1) and filtered. The solvent was eliminated under N_2 and the TLC-fractions were re-purified by RP HPLC as described above to obtain **1** (12 mg) and **2** (17 mg), both with the same retention time (10.9 min).

2.7. Localization of saponins

The external and the internal part of 100 g of corms taken in August were lyophilized and extracted as CSsap. The fractiona-