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SCREENING OF ANTIOXIDANT ACTIVITY AND ANTIPROLIFERATIVE EFFECTS OF VARIOUS EXTRACTS OF SAFFRON PLANT (*CROCUS SATIVUS* L.)

Iliass Lahmass[1], Mouad Lahmass[1], Adil Laaziz[1], Ilham Touiss[2], Sabir Ouahhoud[3], Redouane Benabbas[3], Ennouamane Saalaoui[3]*

Address(es): Titul(s) Firstname Surname of the corresponding author

[1] Laboratory of Biotechnology, Environment, Agri-food and Health, Faculty of Science Dhar Mahraz, Sidi Mohamed Ben Abdallah University, P.B. 1796 Atlas Fez, Morocco.

[2] Laboratory of Life and Health Sciences, Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmacy, University Abdelmalek Essaadi, Tangier, Morocco.

[3] Laboratory of Biochemistry and Biotechnology, Faculty of Sciences, Mohammed First University, BP 717, Oujda, 60000, Morocco.

*Corresponding author: iliass.lahmass@gmail.com

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ABSTRACT

Saffron is a plant classified in the spice family. This plant and its by-products have been documented for their diverse array of biological effects, including anti-proliferative and antioxidant capacities.

This study aimed to assess the biological impact of saffron by-products by analyzing the total phenol content in methanolic and ethyl acetate extracts of five *Crocus sativus* by-products, specifically tepals, tunics, spaths, corms, stigmas, and leaves. Soxhlet extraction was employed to determine the biological activities of extracts from different parts of the plant. Antioxidant activity was assessed using a quantitative DPPH assay and a β-carotene linoleate model system. Additionally, the antiproliferative activity of our extracts was gauged through an MTT assay on human malignant cells, specifically U373 and A549.

Our findings demonstrated that various extracts from the Crocus plant exhibited substantial antioxidant activity, actively scavenging free radicals (Corms extracts showed highest activity 65%) and extracts of stigmas demonstrated an effective reduction of β-carotene oxidation 40%. Moreover, the plant extracts displayed a significant capacity to diminish cancer cell viability and impede the proliferation of U373 (Green leaves 10.6% of control growth) and A549 cells (36.8% was achieved with the tunics extract) in a concentration-dependent manner. In this study, the extracts manifested both antioxidant and antiproliferative activities. Consequently, further investigations are warranted to explore additional biological properties of this widely utilized spice.

Keywords: Antioxidant Activity, Antiproliferative Effects, Saffron, Waste Valorization

INTRODUCTION

Saffron, derived from the crocus flower, is obtained from the dried stigmas of *Crocus sativus* Linnaeus. Functioning as both a food flavoring and coloring agent, saffron holds a prominent position among the most widely used spices globally, enhancing culinary preparations. Renowned for its distinctive taste and aroma, saffron is also acknowledged as one of the most expensive spices employed in the food industry. *Crocus sativus* L., a member of the Iridaceae (Iris) family, is a prevalent plant extensively cultivated in various regions, including Iran, Spain, Greece, India, Azerbaijan, China, France, Egypt, Israel, Italy, Mexico, Morocco, and Turkey. Its widespread cultivation highlights the global significance and demand for saffron in diverse culinary traditions **(Pitsikas, 2016; Rios, Recio, Giner, & Manez, 1996)**.

Carotenoids found in saffron serve as potent biological antioxidants, shielding cells and tissues from the detrimental impacts of free radicals. Singlet oxygen, a crucial element in human health, is effectively influenced by these carotenoids. Saffron, renowned for its therapeutic properties, is particularly abundant in various carotenoids, including crocin and crocetin digentiobiose ester, which contribute to its distinctive color. Additionally, it contains glycoside (picrocrocin) and a volatile oil component, safranal, responsible for its unique aroma. The synergistic presence of these compounds enhances the overall health benefits and sensory attributes of saffron **(Fernández & Pandalai, 2004; Winterhalter & Straubinger, 2000)**.

Beyond its extensive use as a food additive, saffron holds a significant place in traditional Persian and Ayurvedic medicine. Recognized for its therapeutic properties, saffron is employed to address various health concerns, including throat problems, menstrual disorders, and issues related to aphrodisiac, adaptogenic, antispasmodic, carminative, and depressive conditions. This spice has established itself as a valuable component in traditional healing practices, providing a holistic approach to addressing a range of health-related issues **(Abu-Izneid *et al.*, 2022; Akhondzadeh, Fallah-Pour, Afkham, Jamshidi, & Khalighi-Cigaroudi, 2004)**.

Over the past century, numerous studies have substantiated the beneficial effects of saffron, showcasing its potential in addressing various health issues. Saffron has been shown to have therapeutic properties that can contribute to the treatment of coronary heart disease and hepatitis, while also promoting immunity **(Khazdair, Boskabady, Hosseini, Rezaee, & Tsatsakis, 2015)**. Additionally, saffron extract has demonstrated efficacy in managing conditions such as depression, anxiety disorders, and schizophrenia **(Marx *et al.*, 2019)**. Furthermore, its multifaceted benefits encompass potential anti-obesity effects (Mashmoul, Azlan, Khaza'ai, Yusof, & Noor, 2013), antioxidant properties **(Baba *et al.*, 2015; Lahmass *et al.*, 2018)**, anti-diabetic attributes **(Arasteh *et al.*, 2010; Elgazar, Rezq, & Bukhari, 2013; Iliass Lahmass *et al.*, 2017; Mohajeri, Mousavi, & Doustar, 2009)**, neuroprotective and memory-enhancing qualities **(Abe & Saito, 2000; Ghadrdoost *et al.*, 2011)**, antinociceptive and anti-inflammatory actions **(Poma, Fontecchio, Carlucci, & Chichiricco, 2012)**, as well as antitumor and anticarcinogenic activities **(Abdullaev & Espinosa-Aguirre, 2004; Bolhassani, Khavari, & Bathaie, 2014)**. Notably, saffron extract has shown significant inhibition of the growth of colorectal cancer cells **(Aung *et al.*, 2007; Bajbouj, Schulze-Luehrmann, Diermeier, Amin, & Schneider-Stock, 2012)**.

The saffron plant is recognized as a source of numerous bioactive compounds possessing cytotoxic, anti-tumoral, chemopreventive, and anti-mutagenic properties **(Mirhadi, Nassirli, & Malaekeh-Nikouei, 2020; Samarghandian, Borji, Farahmand, Afshari, & Davoodi, 2013).** Additionally, Saffron alone or combined with stamina exercise, increased hippocampus BDNF, serotonin, and increased NT-3 mRNA expression **(Akbari-Fakhrabadi *et al.*, 2021)**.

Previous research has not explored the effects of various parts of *Crocus sativus* and their extracts using the soxhlet method. Therefore, the objective of this study is to, for the first time, elucidate the antioxidant properties and antitumor activity of different extracts obtained through soxhlet extraction from corms, tepals, tunics, spaths, stigmas, and leaves of the *Crocus sativus* plant.

MATERIALS AND METHODS

Preparation of plant extract

The bulbs (corms) of saffron plants were sourced from Taliouine, located in South Morocco. The cultivation of these plants was carried out without the application of any chemical treatments in the Oujda region, situated in the East of Morocco. Collection of various parts of the plant was manually conducted between October

and November. Subsequently, individual plant elements were dried at room temperature in the absence of light.
Dried, milled *Crocus sativus* (100 g) was successively extracted with 500 mL each of hexane, ethyl acetate, methanol, and distilled water for 6–8 hours using a conventional Soxhlet apparatus (500 mL boiler). The resulting liquid extracts were filtered and evaporated to complete dryness at the conclusion of each extraction, employing a vacuum at 35 °C and a rota-vapor apparatus. Subsequently, the dried extracts were stored at 4 °C for subsequent studies. All experiments were conducted in triplicate to ensure reliability. The selection of these solvents was based on their differing polarities, facilitating the extraction of various types of compounds ranging from non-polar to polar.

DPPH radical scavenging activity assay

The method involves neutralizing DPPH free radicals using an antioxidant sample. The radical scavenging activity was determined following the technique reported by Lamien-Meda and collaborators with certain modifications **(Lamien-Meda *et al.*, 2008)**. Specifically, 100 µl of the sample was combined with 200 µl of freshly prepared DPPH (20 mg/l in methanol) and thoroughly mixed. The mixture was vigorously shaken and allowed to reach a steady-state at room temperature for 30 minutes. The reduction in DPPH coloration was quantified by measuring the absorbance at $\lambda = 517$ nm.
The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100.$$

Where A_0 is the absorbance of the control (without extract or positive control) and A_1 is the absorbance in the presence of the extract.

Antioxidant assay using β-carotene linoleate model system

The antioxidant activity of extracts was evaluated by the β-carotene-linoleate model system with some modification **(Tepe, Sokmen, Akpulat, & Sokmen, 2006)**. A solution of β-carotene was prepared by dissolving 2 mg of b-carotene in 10 ml of chloroform. 2 ml of this solution were pipetted into a 100 ml round bottom flask. After chloroform was removed under vacuum, 20 mg of purified linoleic acid, 200 mg of Tween 20 emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (2.5 ml) of this emulsion were transferred into different test tubes containing different extract concentrations and methanol. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The tubes were placed, at 50 °C, in a water bath, and measurement of absorbance was recorded after 2 hours; a blank, devoid of β-carotene, was prepared for background subtraction.
The same procedure was repeated with the synthetic antioxidant, ascorbic acid, as the positive control. Antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity} = \frac{\text{initial β-carotene content} - \text{β carotene content after 2 h of assay}}{\text{initial β-carotene content}} \times 100$$

Cell culture

In our study, the work was done on two types of human malignant cells: line U373 (human glioblastoma cell), line A549 (Lung Carcinoma Cells), obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% of antibiotic–antimycotic solution (containing 10 000 units/mL of penicillin base, 10 000 µg/mL of streptomycin base; Gibco). The cells were maintained under standard conditions of temperature (37 °C), humidity (95%), and carbon dioxide (5%).

Cell viability assay

The antiproliferative activity of our extracts was measured by MTT assay. In the exponential growth phase, cells were seeded in 96-well plates using 100 µL of cell suspension at a density of 20000 cells per well and incubated overnight. Then, the medium was replaced with a new culture medium (blank wells) or supplemented with increasing concentrations of the corresponding saffron extract. Cell viability was determined after 72 hours. In order to determine the number of viable cells, 100 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3 h; subsequently, the medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to lyse the cells and resuspend the formazan (the metabolic product of MTT). Quantities of formazan product directly related to the number of viable cells were measured at 570 nm using a Biorad microplate reader.

Qualitative determination

One hundred microliters (µL) of extract samples were injected into a High-Performance Liquid Chromatography (HPLC) system for the identification of chemical compounds in the saffron extract. A Waters Symmetry® C18 column (4.6 µm x 250 mm) was employed. The mobile phase consisted of a linear gradient of methanol (10–100%) in water (15% acetonitrile), and the flow rate was set at 1 ml/min. Elution was performed at room temperature with a maximum elution time of 60 minutes **(Caballero-Ortega, Pereda-Miranda, & Abdullaev, 2007)**. The sample volume used for each analysis was 20 µL of the test solution, and the experiments were conducted in triplicate for each sample.

Statistical analyses

All samples were treated and analyzed in triplicate. Means and standard deviations (SD) were calculated using Microsoft Office Excel.

RESULTS

DPPH scavenging activity

The results of the determination of the antioxidant activity of different extracts of the saffron plant using DPPH methods are presented in fig. 1.

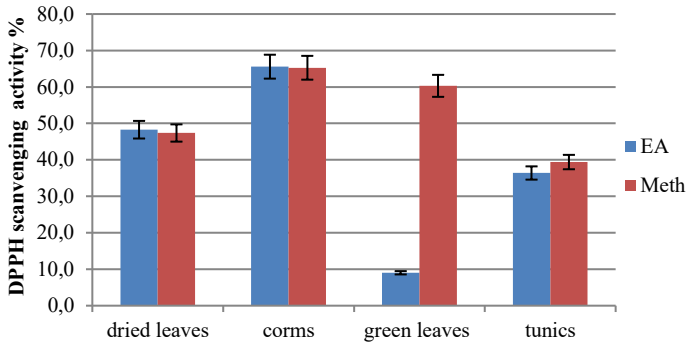

Figure 1 DPPH radical-scavenging activities of dried leaves, corm, green leaves and tunics extracts of saffron plant in ethyl acetate (EA) and methanol with a concentration of 500 µg/ml of each extract. Values are means ± SD of three determinations.

The corms extracts were more active than those of dried leaves, green leaves, and tunics. For both extracts, the methanolic and ethyl acetate extract exhibited almost the same activity except for green leaves methanolic extract, which revealed a strong antioxidant effect compared to that of ethyl acetate.

β-carotene assay

Data corresponding to incubations of β-carotene/linoleate with various concentrations of saffron extracts and ascorbic acid are presented in Table 1.

Table 1 Antioxidant activity of different parts of *Crocus sativus* (corms, tunics, stigmas and leaves) extracts at different concentration in a β-carotene/linoleate model system. Data are mean ± SD of three independent extracts.

Samples	concentration (µg/ml)	oxidized β carotene (%)		
control	-	96,485	±	0,41
tunics	100	37,012	±	3,14
	50	67,673	±	6,11
	20	86,754	±	6,58
stigmas	100	27,596	±	14,67
	50	82,731	±	2,31
	20	94,965	±	1,44
corms	100	40,318	±	5,14
	50	53,924	±	3,37
	20	85,971	±	6,42
leaves	100	85,062	±	2,26
	50	93,448	±	1,72
	20	98,454	±	0,97
ascorbic acid	100	23,94	±	6,13

The control, representing the percentage of initial β-carotene oxidized without the addition of extract or ascorbic acid, was 96.485%. The leaf extract did not exhibit

a significant reduction in this percentage within the tested range. In contrast, all other extracts (stigmas, corms, tunics) demonstrated an effective reduction of oxidation. The antioxidant activity of the extracts exhibited a concentration-dependent increase in all the samples.

Cytotoxicity of Crocus sativus extracts in U373 and A549 cells

To assess the antitumor activity of all extracts, a cytotoxicity MTT assay was conducted, and net growth inhibition was calculated in comparison to negative control growth. Among the tested extracts, green leaves displayed the highest activity (10.6% of control growth), followed by tunics extract (11.7%) and dried leaves (77.5%) for U373 (Fig. 2). No significant activity was observed with the corms extract (102.9%). The percentage of inhibition of cancer cells A549 (Fig. 3) was lower compared to that observed in U373. The maximum growth inhibition of 36.8% was achieved with the tunics extract (Fig. 1), while green leaves and dried leaves exhibited almost similar activity (49.5%; 53.6%, respectively). The corms extract displayed a lower activity (74.5%).

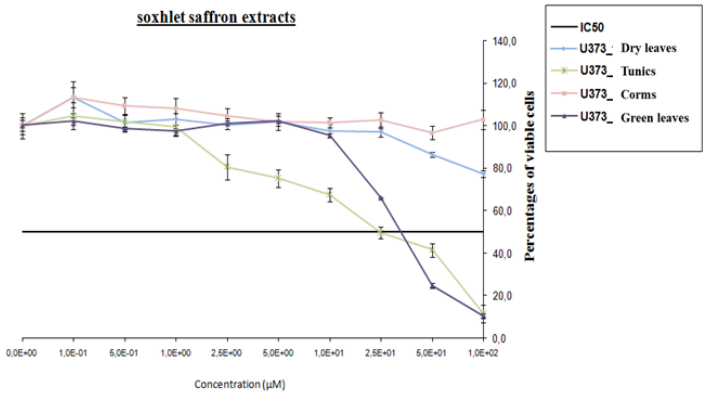

Figure 2 Inhibitory effect of different extracts from by-products (green leaves, corms, tunics and dry leaves) of *Crocus sativus* plant on tumoral human cells U373. Data are mean ± SEM of triplicate

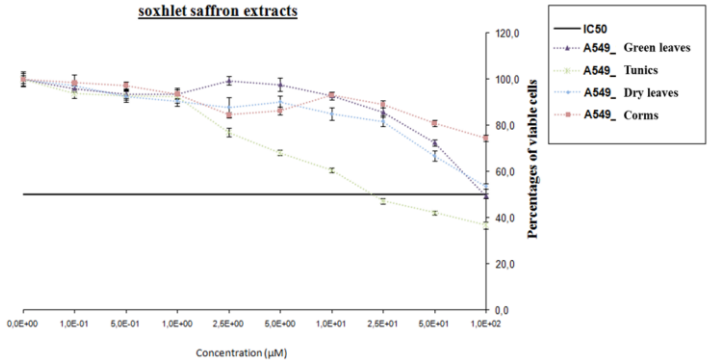

Figure 3 Inhibitory effect of different extracts from by-products (green leaves, corms, tunics and dry leaves) of *Crocus sativus* plant on tumoral human cells A549. Data are mean ± SEM of triplicate

Qualitative determination

One hundred microliters (µL) of extract samples were introduced into High-Performance Liquid Chromatography (HPLC) for the analysis of chemical compounds in saffron extract. Carotenoid compounds were identified by their retention times and quantified using the corresponding standard calibration curves (see Fig. 4). The HPLC chromatogram of the saffron extract revealed crocin and safranal as the predominant compounds in the extract.
Our analysis further identified various forms of crocins in the saffron samples under investigation.

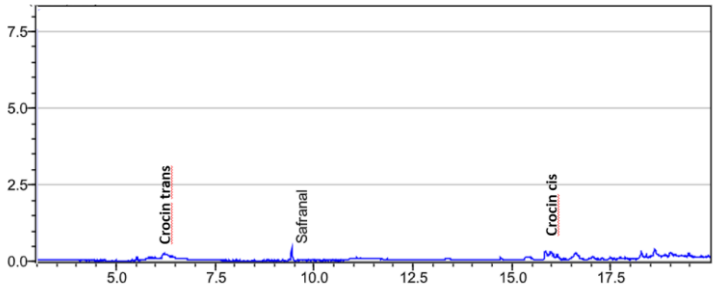

Figure 4 HPLC chromatograms of saffron extract reveal distinct peaks corresponding to various components present in the stigma. Qualitative determinations were conducted using a Waters Symmetry® C18 column, employing a linear gradient of methanol (10–100%) in water (with 15% acetonitrile), and a flow rate of 1 ml/min.

DISCUSSION

Our extract demonstrated the ability to reduce and decolorize the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) by donating hydrogen, leading to a yellow-colored product **(Blois, 1958)**, and DPPH is commonly employed as a substrate to assess the antioxidative activity of antioxidants **(Oyaizu, 1986)**. This method relies on the reduction of a methanolic DPPH solution in the presence of a hydrogen-donating antioxidant, resulting in the formation of the non-radical form DPPH-H through the reaction. This suggests that our extracts possess hydrogen-donating capabilities and function as antioxidants, particularly in the case of the two types of corm extracts and the methanolic extract of green leaves. Furthermore, these findings align with a study by Lahmass, who reported a significant radical scavenging capacity and robust antioxidant activity against free radicals **(I. Lahmass *et al*., 2017)**.
The coupled oxidation of β-carotene and linoleic acid in this model system leads to rapid discoloration of β-carotene in the absence of an antioxidant, resulting in the generation of free radicals. The linoleic acid-free radical, formed through the abstraction of a hydrogen atom from one of its diallelic methylene groups, initiates an attack on the highly unsaturated β-carotene molecules. This process leads to the oxidation and partial breakdown of β-carotene, causing a loss of orange coloration, which is monitored spectrophotometrically. Different antioxidants can impede the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system **(Jayaprakasha, Singh, & Sakariah, 2001)**.
In our study, the control showed that 96.48% of initial β-carotene was oxidized in the absence of an extract or ascorbic acid. Leaf extract did not significantly reduce this percentage within the tested range, whereas both corms and tunics extracts exhibited average oxidation-reduction. Stigmas demonstrated a higher ability to prevent β-carotene bleaching and approached the positive control. The oxidation of β-carotene incubated with tunics extract in the range of 20–100 µg/mL was 86.75–37%, corresponding to a decrease in oxidation between 10 and 61.6% compared to the control. For corms, β-carotene oxidation ranged from 94.95% to 40.31% at concentrations of 20–100 µg/mL, resulting in a decrease in oxidation between 58 and 10.9% relative to the control. Stigmas extract was also effective as an antioxidant, reducing oxidation to 27.596%, representing a 71.4% decrease compared to the control at a concentration of 100 µg/mL, exhibiting effectiveness similar to ascorbic acid in inhibiting oxidation.
As highlighted by Saklani and collaborators, the inhibition test of linoleic acid oxidation coupled with β-carotene appears to be a useful mimetic model of lipid peroxidation in biological membranes **(Saklani *et al*., 2017)**.
According to qualitative analysis, extracts from the Crocus plant exhibited a notable concentration of crocin, followed by safranal. Crocin, as the primary carotenoid in saffron, may play a significant role in conferring antioxidant properties. Safranal, a monoterpene aldehyde and the primary constituent of saffron plant essential oil, has also demonstrated antioxidant activity **(Abdullaev & Espinosa-Aguirre, 2004)**.
Furthermore, the main active compounds found in saffron have been shown to impede the growth of cancer cells and inhibit the formation of tumors. Furthermore, we evaluated the antiproliferative activity of by-products of *Crocus sativus* using the MTT assay on U373 and A549 cancer cells after treatment with increasing extract concentrations (0 to 100 µg/mL). Fig. 2 and 3 depict the viability, expressed as a percentage of the control, of cancer cells in the presence of different extract concentrations. The extracts demonstrated a significant dose-dependent effect on cell proliferation, with green leaves and tunics extract yielding the best results across all tested conditions and cell lines, leading to a significant reduction in cell viability. Corms and dry leaves exhibited less capacity to reduce cancer cell viability compared to other parts of the saffron plant. These results suggest that the antioxidant activities and antiproliferative effects of our extracts may be attributed to phenolic compounds and carotenoids, which appear to be the most active compounds from *Crocus sativus* against cancer cell proliferation.
Numerous studies have explored the chemopreventive potential of saffron stigma extracts against cancer. For instance, Samarghandian *et al*. investigated the antiproliferative effect of an aqueous saffron extract on lung cancer cells (A549). Their study suggested a dose and time-dependent reduction in A549 cell proliferation following saffron administration, attributed in part to the inhibition of cell proliferation and induction of apoptosis in cancer cells **(Samarghandian *et al*., 2013)**. Additionally, Ahmadnia *et al*. reported the in vitro inhibitory effect of aqueous saffron extract on the proliferation of human prostate cancer cells (PC3) in a dose-dependent manner **(Ahmadnia *et al*., 2021)**.
Ethanolic saffron extract demonstrated the potential to induce cytotoxic and apoptotic effects in carcinomic human cells (A549). The study revealed a concentration- and time-dependent reduction in cell viability and pro-apoptotic effects in the cancer cell line, suggesting its potential as a chemotherapeutic agent in lung cancer **(Samarghandian *et al*., 2013)**.

Saffron was found to significantly reduce the diethylnitrosamine (DEN)-induced increase in the number and incidence of hepatic dyschromatic nodules in rats, exhibiting a notable chemopreventive effect against liver cancer by modulating oxidative damage and suppressing the inflammatory response **(Noureini & Wink, 2012)**.

Moreover, crocin, a carotenoid from saffron, demonstrated significant neuroprotective effects against acrylamide-induced cytotoxicity in PC12 cells due to its antioxidant properties. Another study reported the enhancement of cytotoxic and apoptogenic properties of crocin in cancer cell lines, HeLa and MCF-7 **(Noureini & Wink, 2012)**. Overall, carotenoids and apocarotenoids are considered potential drugs in the regulation of cancer disease, exhibiting anticancer effects through various mechanisms, including the modulation of transcription factor activity and induction of apoptosis **(Cerdá-Bernad, Valero-Cases, Pastor, & Frutos, 2022)**.

In conclusion, this study represents the first report demonstrating the antioxidant and inhibitory effects of various extracts obtained from by-products of *Crocus sativus* using the soxhlet method extraction. Our findings suggest that these by-products constitute promising sources of bioactive compounds. The extracts exhibited notable antioxidant activity in several in vitro oxidative stress systems and demonstrated potential anticancer activity against carcinomic human cells. Specifically, our extracts displayed a robust inhibitory effect on the proliferation of A549 and U373 cells in vitro. Despite these promising results, further research on by-products of *Crocus sativus* L. is warranted to provide a more comprehensive understanding of the mechanisms underlying these bioactive properties and to facilitate the utilization of different extracts derived from saffron by-products.

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