Food Bioscience 57 (2024) 103501

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Evaluation of anti-cancer potential of saffron extracts against kidney and bladder cancer cells

Riccardo Vago [a,b], Francesco Trevisani [a], Pamela Vignolini [c,*], Chiara Vita [d], Francesco Fiorio [a], Margherita Campo [c], Francesca Ieri [e], Federico Di Marco [f], Andrea Salonia [a,b], Annalisa Romani [c,1], Arianna Bettiga [a]

[a] Urological Research Institute (URI), Division of Experimental Oncology, IRCCS San Raffaele Scientific Institute, Milan, Italy
[b] Università Vita-Salute San Raffaele, via Olgettina 58, Milan, Italy
[c] Laboratorio PHYTOLAB – DiSIA – University of Florence, via U. Schiff, 6, 50019, Florence, Italy
[d] PIN-QuMAP - Polo Universitario di Prato, piazza G. Ciardi, 25, 59100, Prato, Italy
[e] CNR, Via Madonna del Piano 10, 50019, Sesto Fiorentino, FI, Italy
[f] Bioinformatician Emerging Bacterial Pathogens Unit Div. of Immunology, Transplantation and Infectious Diseases- San Raffaele Scientific Institute, Milan, Italy

ARTICLE INFO

Keywords:
Saffron
Crocus sativus
Antioxidant compounds
Kidney cancer
Bladder cancer
Crocin
Safranal

ABSTRACT

Crocus Sativus L. (saffron) has been used for centuries as a medicinal plant in traditional medicine. Its main bioactive secondary metabolites, which include crocins, crocetin, picrocrocin and safranal, are known to possess significant antioxidant as well as anti-proliferative activities. With the aim to test the activity of saffron on kidney and bladder cancer cell lines, we tested the effect of extracts obtained from dried stigmas derived from different Italian regions (Lombardy and Tuscany) in two different years, on the cell viability. The treatment with saffron extracts showed a significant reduction of cell viability in most of our tumoral cellular systems, while the standards crocin and safranal alone at the same concentrations did not exerted any cytotoxicity. When the extracts and standards were mixed together, they exerted a milder inhibition of cell viability, indicating that the antitumor activity is likely a result of the synergy of the various compounds. In addition, in a circular economy vision, we recovered *Crocus Sativus* flowers and tested them on cancer cells, demonstrating a consistent reduction of cell viability, while kaempferol (main compound present in flower extracts) did not show compatible results. Our results extend the interests around saffron-derived bioactive molecules for their potential in therapeutics.

1. Introduction

In Italy, kidney and urinary tract cancers occupy the tenth place in terms of frequency among all cancer types with about 13,400 new cases in 2018, 8900 among men (4.5%) and 4500 among women (3%). Incidence increases with age and shows a peak during the eighth decade from birth, point at which the disease frequency is around one new case every 1000 men/year and about less than a half for women (Chow et al., 2010). Bladder cancer is one of the most frequent cancers in high-income countries. Statistics indicate a large incidence of bladder cancer in Italy when compared to other European and World countries, possibly attributed to differences in lifestyle habits and exposures, and given its relatively high prevalence (De Nunzio et al., 2020). Instead, renal cell carcinoma (RCC) affects over 400,000 individuals worldwide per year (Bray et al., 2018).

Most of the newly diagnosed bladder cancers (75%-80%) are classified as Non-Muscle Invasive tumors (NMIBC) and are treated by transurethral resection followed by intravesical instillation of drugs in high-risk patients (Babjuk et al., 2022) The Muscle Invasive Bladder Cancer (MIBC) account for the remaining 20%-25% of cases, which are characterized by poor prognosis and require radical surgical treatment or chemo-radiation with intensive surveillance (Witjes et al., 2021).

On the other hand, Renal Cell Carcinomas (RCCs) comprehend a heterogeneous group of tumors which derive from renal tubular

* Corresponding author.
E-mail addresses: vago.riccardo@hsr.it (R. Vago), trevisani.francesco1@gmail.com (F. Trevisani), pamela.vignolini@unifi.it (P. Vignolini), chiara.vita@pin.unifi.it (C. Vita), frank.fiorio045@gmail.com (F. Fiorio), margherita.campo@unifi.it (M. Campo), francesca.ieri@cnr.it (F. Ieri), dimarco.federico@hsr.it (F. Di Marco), salonia.andrea@hsr.it (A. Salonia), arianna.bettiga@gmail.com (A. Bettiga).
[1] Died, january 17, 2022.

https://doi.org/10.1016/j.fbio.2023.103501
Received 4 September 2023; Received in revised form 14 December 2023; Accepted 16 December 2023
Available online 24 December 2023

epithelial cells (Cinque, Capasso, et al., 2021; Cinque, Vago, Trevisani, 2021). In line with the World Health Organization, there are three most common histological subtypes of RCC, all differentiated by histological and molecular genetic modification: clear cell RCC (70%-80% of cases), papillary type 1 and 2 (10%-15%) and chromophobe (4%-5%) (Leibovich et al., 2010). The prognosis remains strictly connected to the aggressiveness at time of diagnosis for both tumors. In fact, if the 5-year disease-specific survival rate for a localized and low risk RCC (N0, M0 according to TNM classification) can be up to 90%, an incidental metastatic high risk (N2, M0/M1) RCC may be fatal rapidly, despite gold standard treatment such as surgery and oncological medical therapy (Cinque, Capasso, et al., 2021; Cinque et al., 2021). In case of bladder cancer, while survival rate for the NMIBC upon treatment is very high, the MIBC accounts only one third overall survival at 5 years from the diagnosis (Babjuk et al., 2022).

A correlation between the various cancer types and the components of saffron has been shown. Specifically, *Crocus sativus* L. (saffron) is a plant which belongs to the Iridaceae family and is known for being the most expensive spice in the world. This plant has been used for centuries as a medicinal plant in traditional Islamic and Arab medicine and, in the culinary scenario, it is appreciated for its ability to donate color, flavor and aroma to foods and beverages (Bagur et al., 2017). Its main bioactive secondary metabolites, which include crocins, crocetin, picrocrocin and safranal, are known to possess significant antioxidant activity, various *in vivo* and *in vitro* studies showed the pharmacological and biological effects of saffron, the antioxidant potential is considered to be the result of synergistic effect of all the bioactive components of saffron (Bukhari et al., 2018). The bioactive compounds of saffron have been found to play important role in the treatment of diseases via different mechanisms among which free radical scavenging activity being the most prominent (Bukhari et al., 2018). Crocins have been demonstrated to elicit reno-protective effects by acting against oxidative stress and inflammation in elderly rats (Samarghandian et al., 2016). While the details of how this is achieved are still unknown, their activity may rely on the antioxidant properties possessed by the crocin esters, which could influence the cell growth regulation, modulate genetic expression and the immune response. On the other hand, these compounds have been studied by the scientific community for other positive effects on the organism, demonstrating cardioprotective, neuroprotective, memory enhancing, antidepressant, anti-inflammatory, anxiolytic properties, as well as anti-proliferative activity (Ma et al., 2022). The literature on cancer prevention states that crocin and crocetin (molecules which belong to the carotenoid family) possess the ability to inhibit tumoral cell growth (Colapietro et al., 2019) and this led research towards the characterization of saffron and its components with the intent to reveal their therapeutic potential on different tumor types (Ma et al., 2022). *In vitro* and *in vivo* studies report that saffron exerts anti-tumoral effects by acting on numerous mechanisms (Christodoulou et al., 2015).

The production of saffron also generates a large amount of bio-residues: to produce 1 kg of stigmas, about 350 kg of tepals are obtained which are then thrown away (Vignolini et al., 2008). This biomass contains bioactive compounds, in particular kaempferol derivatives (Vignolini et al., 2008) the exploitation of which can increase the profitability and sustainability of this traditional production, in accordance with the principle of "Circular Economy".

Sánchez-Vioque et al. (2012), conducted *in vitro* studies on the antioxidant and metal chelating properties of tepals and leaves from saffron which have shown interesting results mainly in the inhibition of β-carotene oxidation, in the scavenging of NO radicals and in the chelating activity of Cu^{2+} (Sánchez-Vioque et al., 2012). Some researchers have reported data on the antiproliferative effects of polyphenols on cancer cells and the effect of tepals extracts on the growth of a human cell lines derived from colon cancer (Sánchez-Vioque et al., 2016), as well as their interesting application in cosmetic production (Belyagoubi-Benhammou et al., 2023). For all these reasons, it was decided to test also this matrix, in addition to the stigmas on kidney and bladder cancer cells. This study can further support an intriguing approach to innovatively improve the high-quality by-product fraction with the ultimate goal of optimizing and developing the production chain. There is scientific evidence in the literature linking saffron components to various types of cancer (Lambrianidou et al., 2020), such as breast cancer (Mir et al., 2020) and hepatocellular carcinoma (Amr Amin et al., 2011), but not much has been established yet on the properties of saffron components with respect to kidney and urinary tumors aside from some *in vitro* evidence of anti-tumoral activity on prostate cancer (D'Alessandro et al., 2013; Festuccia et al., 2014). For all these reasons this study aims at confirming on *in vitro* models for kidney and bladder cancers the anti-proliferative properties that saffron and its main compounds have demonstrated to possess by exposing cells to saffron extracts and pure crocins (Crc) and safranal (Sfr) standards. Here we tested the impact of saffron extracts derived from different Italian geographic areas and their main components as standard solutions on kidney and bladder cancer cellular models.

2. Materials and methods

2.1. Plant materials

Saffron samples were obtained from 2 geographic origins: Azienda Pura Crocus (Tuscany) and Azienda Cerchi nel Grano (Lombardia), from two different harvests made in 2017 and 2018. The collection of saffron flowers is handmade. The flowers are harvested in October in the early morning and the stigmas are separated from the other parts of the flower. Saffron samples were dried (40 °C) immediately after collection, kept in the dark and in closed jars and analyzed 30 days after drying.

2.2. Standards and solvents

Crocin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, Missouri, USA). Authentic standards of gallic acid and safranal for HPLC analysis was purchased from Sigma-Aldrich (St. Louis, USA), p-OH benzoic acid, kaempferol 3-glucoside and curcumin were purchased from Extrasynthèse S.A. (Genay, Lyon, France). All solvents were of HPLC grade purity (BDH Laboratory Supplies, United Kingdom).

2.3. Sample preparation

Saffron stigmas were subjected to aqueous extraction to simulate the extraction by specifications recommended by ISO/TS 3632-2:2003 and literature data (Shinwari & Rao, 2018), to recover the active principles present in the stigmas' samples.

The components from saffron stigmas (400 mg) were extracted with 5 mL H_2O, overnight and then filtered to eliminate plant residues.

Saffron flowers (from Tuscany) were dried at 40 °C then 300 mg of dried flowers were used for component extraction using 15 mL of 70% ethanol (pH 3.2 for HCOOH) over night and then filtered to eliminate plant residues, as reported by Vignolini et al. (2008).

After sample preparation, samples were diluted in cell culture medium considering their crocin concentrations.

2.4. Preparation of standards solutions

Crocin standard solution was prepared by dissolving crocin powder directly in the medium at different concentrations and subsequently filtered with a 0.22 μm filter. Safranal standard solution was prepared by mixing safranal and DMSO at different concentrations and subsequently by diluting the solution in cell culture medium at 1:1000.

2.5. HPLC/DAD analyses

Saffron extracts (stigmas and flowers) were analyzed by High Performance Liquid Chromatography/Diode Array Detector (HPLC/DAD) for the determination of saffron components. Analysis for polyphenols were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA), and were separated by using a 250 x 4.6 mm i.d. 5 μm Luna C18 column (Phenomenex, Torrance, CA, USA) operating at 25 °C. UV/Vis spectra were recorded in the 190-600 nm range and the chromatograms were acquired at 280, 308, 350 and 440 nm. The mobile phase was a two-steps linear solvent gradient system, starting from 90% H_2O (adjusted to pH 3.2 by HCOOH) up to 100% CH_3CN during a 40-min period, flow 0.8 mL min^{-1}.

The identity of polyphenols was ascertained using data from HPLC/DAD analyses by comparison and combination of their retention times and UV/Vis spectra with those of authentic standards and previously reported data (Vignolini et al., 2008). Quantification of individual compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geq 0.9998$) in the range 0-30 mg on the basis of authentic standards. In particular, crocin derivatives were determined at 440 nm using curcumin as reference compound. Flavonols were determined at 350 nm using kaempferol 3-glucoside as reference compound, picrocin was determined at 280 nm using p-OH benzoic acid as reference compound and safranal was determined at 308 nm using safranal as reference compound. In all cases, actual concentrations of the derivatives were calculated after applying, were possible, corrections for differences in molecular weight.

2.6. Cell culture

Human kidney cancer Caki-1 cells were purchased from the American Collection of Cell Cultures (ATCC, Manassas, VA, USA) and cultured in McCoy, 786-O (ATCC) in RPMI High glucose< RT-4 and RT-112 were purchased from the Cell Lines Service (CLS, Eppelheim, Germany) and cultured in RPMI and MCDK (a kind gift of Dr. Rampoldi's laboratory) in DMEM High glucose, supplemented with 10% (v/v), fetal bovine serum, 100 U/mL penicillin and100 μg/mL streptomycin at 37 °C with 5% CO2.

2.7. Cell treatment

Prior to each test, cells were seeded in growth medium in 96 well plates allowed to adhere for 24 h. For viability studies, seeded cells were incubated for 24 h, 48 h and 72 h in presence of the tested compounds or extracts. The tested concentrations for the standards and extracts (expressed as crocin content) were ranging from 50 μM to 5 mM.

2.8. MTT assay

Cells were seeded at an initial density of 5000 cells/well in 96-well plates in 100 μL and incubated for 24 h at 37 °C in humidified air containing CO_2 5%. The cells were then treated with different concentrations of standards and extracts by substituting the medium with the medium solutions containing the tested compounds. The control wells had DMSO in the medium at equal concentrations to those used for the tested compounds when needed.

Cell viability was measured after 24 h (MCDK) and 24 h, 48 h and 72 h (Caki-1, 786-O, RT-4 and RT-112) using 10 μL/well 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). The plates were incubated at 37 °C for 1 h. The medium was removed from the wells and the Formazan produced by the MTT reduction was solubilized in DMSO (100 μL/well). Absorbance was determined on a micro plate reader (Mithras LB 940 - Berthold) at 570 nm. The percentage of cell viability was calculated using the ratio AbsTEST/AbsCTRL.

2.9. Statistical analysis

Each experiment was performed in quadruplicate for every condition and repeated at least three times. Differences in viability percentages were assessed using Student's *t*-test followed by a Bonferroni post-hoc test ($p>0.05$). Results were expressed as mean ± SD and the analysis was performed by R-Studio environment for R version 3.6.3 using the package "Tidyverse".

3. Results

3.1. Characterization of the main bioactive components in stigmas' extracts

The aim of this study was to evaluate the anti-proliferative properties of saffron stigmas extracts and pure Crc and Sfr standards on *in vitro* models for kidney and bladder cancers, for this reason we first carried out a quali-quantitative analysis of our saffron samples. We analyzed four different stigmas samples of *Crocus sativus* derived from two different Italian geographic areas, Lombardy (L) and Tuscany (T), harvested in two consecutive years of production, 2017 and 2018. Procedures of harvesting and separation of flowers and removal of the stigmas, drying treatment and storage method have been standardized by the two farms. The extracts from dried stigmas were prepared and the content and identity of bioactive compounds was assessed by HPLC/DAD analysis by comparison and combination of UV spectra and retention times as previously reported (Masi et al., 2016; Vignolini et al., 2008).

In particular, crocins, safranal, picrocrocin and kaempferol derivatives (kaempferol 3-sophoroside glucoside and kaempferol 3-sophoroside) were identified as main components (Masi et al., 2016) and quantified (Table 1).

Climate, soil and geographic location can have a significant impact on increasing or decreasing the quantity and quality of plant performance (Farrokhi et al., 2021), even climate seasons affected directly the concentration of bioactive components and the antioxidant activity (Aires et al. 2011). Growing areas with higher air temperatures and no excessive rainfall during the flowering period resulted in higher yields of high-quality stigmas. In particular crocins were positively correlated with stigma yield and mean air temperature, but negatively with safranal (Cardone, Castronuovo, Perniola, Cicco, & Candido, 2020a).

The yield of stigma extracts was about 50%. In all extracts, crocins were the predominant component and does not drastically change among samples, while safranal, picocrocin and flavonoids showed great difference and variable ratios not only due to the geographical origin, but also to the year of production. Total Crocins and picrocrocin are higher in Tuscany samples with respect to the Lombardy ones, while safranal and kaempferol derivatives are higher in Lombardy samples. In particular, the major biologically active compounds are higher in 2018 both for Tuscany and Lombardy samples. The content of Crc were greater in the Lombardy extract from 2017 (L_2017), consisting of 66% of the overall compounds and lower in the Tuscany extract from 2017 (T_2017). Sfr content was under the detection level in Tuscany extract from 2018 (T_2018), and consisted of 0.4%, 5% and 21% among the overall compounds present in T_2017, Lombardy extract from 2018 (L_2018) and L_2017 respectively. Picocrocin was lower in the samples from L_ 2017 with lower content, 16%. Flavonoids content was higher in extract from Lombardy. These data allow us to have the information on the composition of the extracts needed for the interpretation of the results of the cells tests. In order to test the effects of the extracts on the cell viability in a comparable manner, normalization was performed according to the crocins content, as a compound known for its anti-tumoral properties (Table 1).

Table 1
Crocins, safranal, picrocrocin and flavonoids content in the extracts derived from dried stigmas of Tuscany and Lombardy 2017; 2018 *Crocus sativus*. Data shows the mean of three determinations (standard deviation < 5%). The relative content of the compounds was reported with respect to the crocins content: this ratio is shown in the table by using the %.

	Tuscany				Lombardy			
	2017		2018		2017		2018	
	μM	%	μM	%	μM	%	μM	%
Crocins	7.3	100	10	100	6.44	100	9.12	100
safranal	0.06	0.8	n.d	n.d	2	31	0.96	11
picrocrocin	5.8	69	6.67	79	1.6	25	5.57	61
flavonoids	0.74	10	0.9	9	1.66	26	1.88	20
TOTAL	**13.91**		**17.57**		**9.76**		**17.53**	

3.2. Effect of Tuscany and Lombardy saffron stigmas extracts on cell viability

Besides its typical spicy aromatic note which is much appreciated in the culinary world, Sfr has also been shown to possess cytotoxic potential towards certain cancer cells *in vitro* in the beginning of 1990 and research on this subject has increasingly continued during the past decade revealing Crc as the most promising anti-cancer compound in saffron (Abdullaev, 2002). To date, however, there has not been any recent report in literature on the effects that saffron exposition could have on urological cancers. We studied the *in vitro* cytotoxic effect of saffron aqueous stigma extracts and its single bioactive components on kidney and bladder cell line viability. According to an extensive literature review on toxicity of saffron and its constituents against normal and cancer cells (Milajerdi et al., 2016). Sfr can be considered as the most potent toxic constituent of saffron with selective toxicity even at low doses.

We investigated the cytotoxic effect of the four *Crocus sativus* stigma extracts L_2017, L_2018, T_2017 and T_2018 with different concentrations of aqueous extracts. For better comparison, we tested the samples at different concentrations normalized per Crc content as to have the same amount of this bioactive molecule and define more precisely the impact of the other components, which greatly vary among samples. Increasing doses of extracts ranging from 0.05 to 0.5 mM were assayed on RCC (Caki-1 and 786-O) and bladder cancer cells (RT4 and RT112) at 24, 48 and 72 h (Fig. 1 and Table 2). MDCK healthy renal cells were used as a control.

Tuscany extracts showed in all cases significant variation of the cell viability: T_2017 with 0.5 mM Crc resulted effective on 786-O at all time points; T_2018 with 0.5 mM Crc at 24 and 72 h (Fig. 2). Caki-1 viability was significantly reduced at 24 and 48 h in the presence of T_2017 with 0.5 mM Crc and at 24 h in the presence of T_2018 with 0.5 mM Crc. T_2017 with 0.5 mM Crc reduced the viability of RT-112 cells at all time points. Lombardy extracts were tested at 24 h on MCDK cells and 24, 48 and 72 h on the other cell lines and a significant decrease of the cell viability when exposed to L_2017 with 0.5 mM Crc at 24 h was observed. A substantial drop in viability was detected for 786-O, for L_2017 with 0.5 mM Crc and for L_2018 with 0.5 mM Crc. The inhibitory effect of L_2018 with 0.5 mM Crc was evident on Caki-1 at all time points, and for L_2017 with 0.5 mM Crc at 72h. Regarding bladder cancer cells, RT-112 showed a reduction of viability in the presence of both L_2017 and L_2018with 0.5 mM Crc. Analogously, RT-4 had significantly reduced viability in the presence of L_2017 with 0.5 mM Crc at all time points and at 72 h in the presence of L_2018 with 0.5 mM Crc.

In all cases, a reduction in viability was observed in presence of the extract at the greatest concentration adopted, normalized at 0.5 mM in Crc. At this concentration the Crc alone exerted milder inhibition of the viability. T_2017 had a greater impact on the cell viability with respect to T_2018. Considering the composition of the two extracts, this effect might be imputed to Sfr and picocrocin present in greater concentration in T_2017. Extract L_2017, which has the highest content of Sfr among the extracts, showed the greatest impact on viability, suggesting that Sfr combined with the other compounds could play a main role in the inhibition of cell viability.

Then, to better define the contribution of single bioactive molecules to the cytotoxic activity of the extracts, we tested the cytotoxic effect of different doses of safranal from 0.05 to 0.5 mM on 786-O, Caki-1 kidney and RT-112 bladder cells at 24, 48 and 72 h (Fig. 3A and B).

A dose-dependent, inhibitory effect on cellular viability exerted by the highest concentration of Sfr tested was observed only on 786-O, RT4 and RT112 cells, while Cack1 and MDCK did not show any reduction of cell viability at these doses. *In vitro* studies have shown that also Crc has inhibitory effects against a wide range of cancer cells (stronger than Sfr). For this reason, Crc is considered by the scientific community as the most promising anti-cancer compound in saffron (Lambrianidou et al., 2020) and, since Crc is the glycated form of saffron constituents, it seems

Table 2
Effect of saffron extract on the cell viability of 786-O, Caki 1 and MDCK kidney models and on RT4 and RT112 bladder cancer cells incubated for 24, 48 or 72 h with L_2017, L_2018, T_2017 or T_2018at 0.5 mM Crc. Reported values correspond to the mean of cell viability expressed as a percentage with standard error over three biological replicates. The percentage of cell viability was calculated using the ratio AbsTEST/AbsCTRL. *p<0.05, **p<0.01, ***p<0.001.

Extract at 0.5 mM Crc	Cell Line Viability expressed as a percentage (%)												
	786-O			Caki-1			RT112			RT4			MDCK
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h
L_2017	***	***	***	***	***	***	***	***	***	***	***	***	***
	17 ±0.4%	9.6 ±0.3%	7 ±1.0%	20 ±3.0%	9.2 ±0.3%	10 ±1.0%	19.2 ±0.4%	9 ±1.0%	7 ±1.0%	30 ±5.0%	17 ±5.0%	17 ±1.0%	8.8 ±1.1%
L_2018	***	***	***	**	**	***	***	***	***	**	***	***	80
	53 ±4.0%	29 ±4.0%	24 ±6.0%	74 ±7.0%	74 ±1.0%	76 ±4.0%	73 ±4.0%	41 ±2.0%	30 ±3.0%	71 ±5.0%	56 ±8.0%	61 ±6.0%	±5.0%
T_2017	***	***	***	***	***	**	***	***	***				
	70 ±1.0%	49 ±2.0%	41 ±2.0%	67 ±2.0%	64 ±2.0%	55 ±1.0%	47 ±7.0%	48 ±1.0%	18 ±1.0%				
T_2018	***	*	***	***	72	**	**	**	**				
	83 ±1.0%	75 ±2.0%	71 ±4.0%	73 ±2.0%		73 ±6.0%	78 ±7.0%	74 ±3.0%	70 ±1.0%				

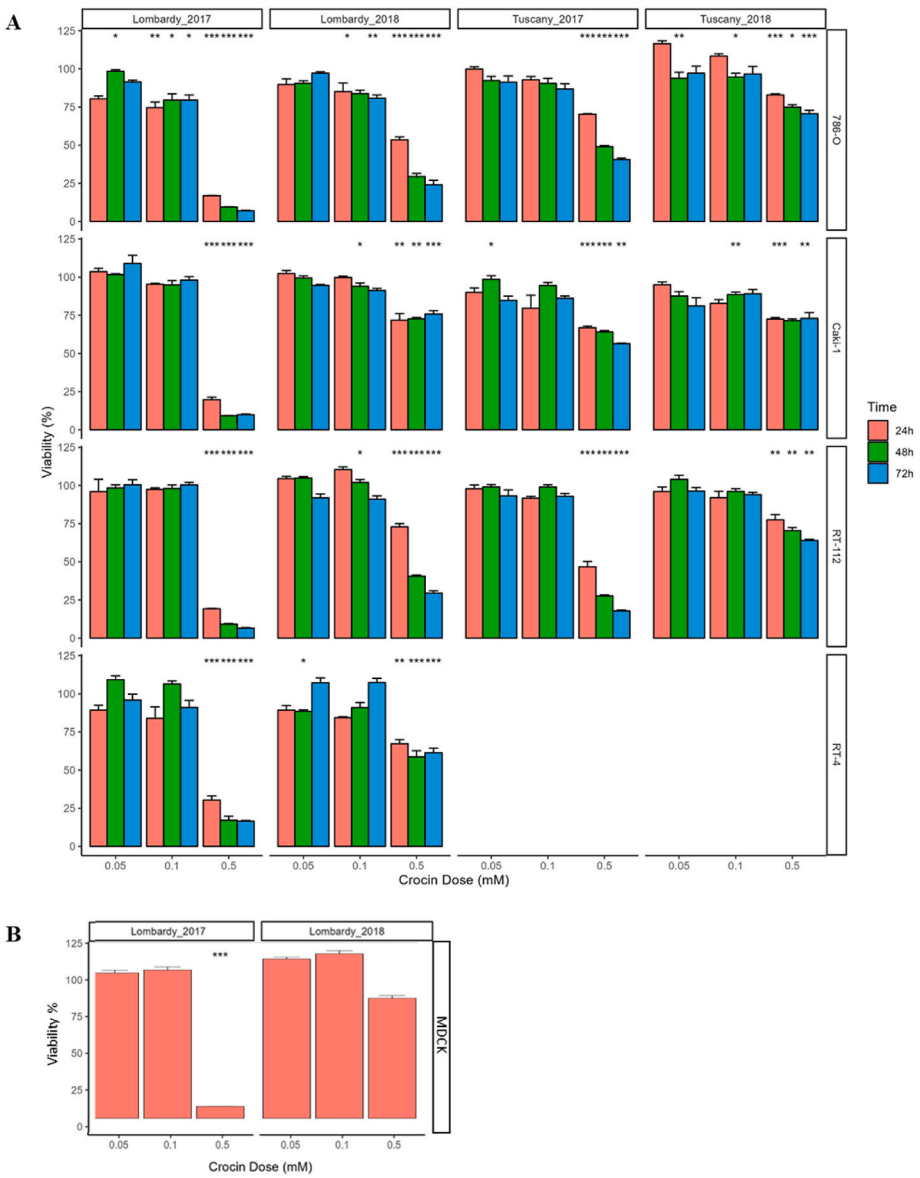

Fig. 1. Effect of saffron extract on the cell viability of 786-O (A), Caki 1 (A) and MDCK (B) kidney models and on RT4 and RT112 (A) bladder cancer cells incubated for 24, 48 or 72 h with L_2017, L_2018, T_2017 or T_2018. Reported values correspond to the mean of cell viability with standard error over three biological replicates. The percentage of cell viability was calculated using the ratio AbsTEST/AbsCTRL. *p<0.05, **p<0.01, ***p<0.001.

that sugars play a major role in strengthening its toxic effects. Cells were then incubated for 24, 48 and 72 h with increasing doses of Crc, ranging from 0.5 to 5 mM and its effect on cell viability was assessed (Fig. 3C and D).

Crc induced a dose-dependent, drastic reduction of cell viability in all the cell lines, which can be clearly detectable already after 24 h incubation and it is constant over time.

3.3. Evaluation of the impact of safranal supplementation in saffron stigmas extracts on RT112 bladder cancer cell viability

In order to further investigate the activity of Sfr in the context of saffron stigmas extracts, we compared the effect of T_2017 and T_2018 as such or after supplementation with 0.15 mM Sfr. Extracts were used as to have the same amount of Crc (0.5 mM). As reported in Table 1, T_2017 and T_2018 contain a minimal amount of Sfr or none at all, respectively. The effect of Sfr supplementation was assayed on RT-112 bladder cancer cells at 24, 48 and 72 h (Fig. 2).

Crc and Sfr or the combination of the two did not have any effect at the concentration used. The extracts confirmed to greatly reduce the cell viability and the L_2017 was the most active compared to the T_2017 and T_2018. The supplementation of 0.15 mM Sfr significantly improved the effect of both T_2017 and T_2018 at every time. Furthermore, the addition of Sfr to T_2018, which did not contain any detectable amount of it, produced an increase in the inhibitory effect reaching values similar to T_2017 at 24 and 48 h. T_2017+sfr resulted non-significantly different from L_2017 at 24 (30 ± 2%, $p>0.05$) and 48h (11 ± 1%, $p>0.05$), while at 72 h we noticed a slightly significant difference (7.2 ± 0.3%, $p<0.5$). The two solutions present the same content of Crc and Sfr but different content of picocrocin, which is greater in T_2017+sfr, and flavonoids, which is greater in L_2017. The effects on viability produced by incubation with the other solutions resulted significantly different from L_2017 at 24, 48 and 72 h. Comparing the effect of T_2017+sfr and T_2018+sfr, the former produced a greater inhibition of cellular viability despite presenting the same content of Crc and Sfr and a comparable content of flavonoids. It is possible to assign the different effects to the greater content of picocrocin or other non-detected compounds in T_2017+sfr.

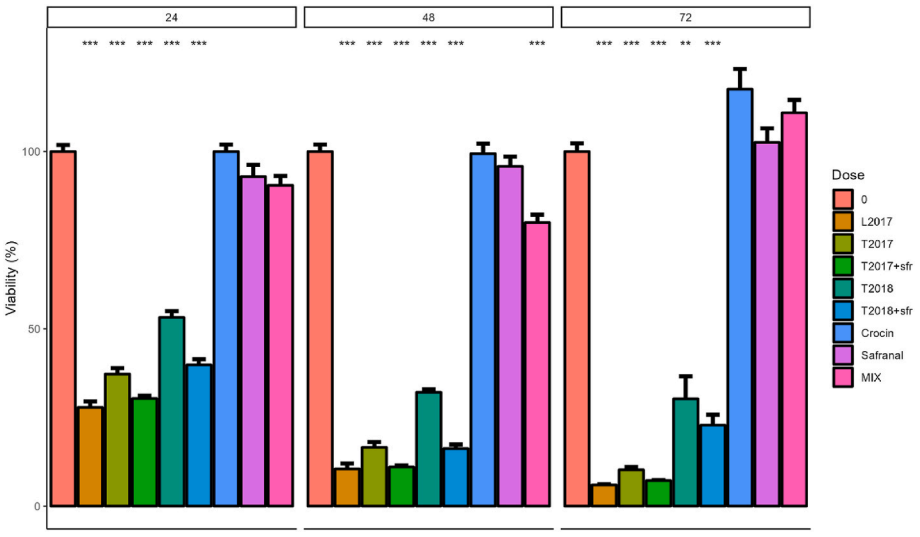

Fig. 2. Effect of saffron extracts supplemented with 0.15 mM safranal (+sfr) on RT112 bladder cancer cell viability incubated for 24, 48 or 72. Saffron extracts used contain the same amount of crocin (0.5 mM) as the standards, 0.5 mM crocin, 0.15 mM safranal and the combination of the two (mix) were used. Reported values correspond to the mean of cell viability with standard error over three biological replicates. The percentage of cell viability was calculated using the ratio AbsTEST/AbsCTRL.

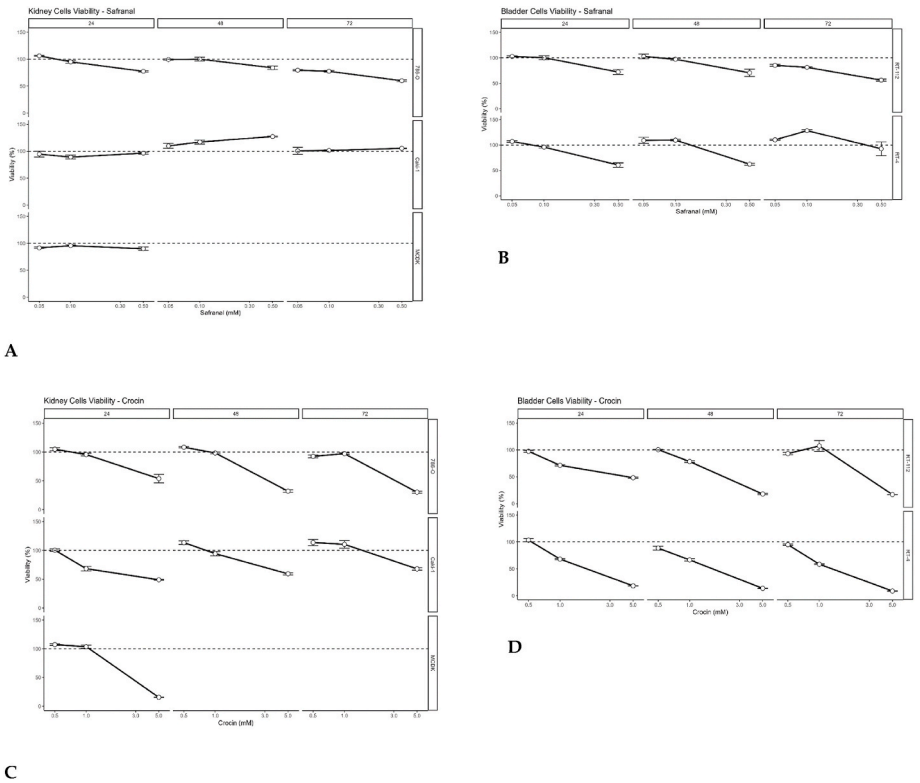

Fig. 3. Effect of safranal on the cell viability of 786-O, Caki 1 and MDCK (A) kidney cells and of RT4 and RT112 (B) bladder cancer cells incubated for 24, 48 or 72 h. Effect of crocin on the cell viability of 786-O, Caki 1 and MDCK (C) kidney cells and RT4 and RT112 (D) bladder cancer cells incubated for 24, 48 or 72. Reported values correspond to the mean of cell viability with standard error over three biological replicates. The percentage of cell viability was calculated using the ratio AbsTEST/AbsCTRL. The vehicle alone (DMSO 0.1%) employed for safranal and crocin solubilization was used as control.

3.4. Evaluation of the impact of saffron flowers extract on RT112 bladder cancer cell viability

Since petals and stamens represent the vast majority of the waste material, we investigated their potential on cancer cells. We recovered *Crocus sativus* flowers, processed them to extract bioactive compounds, which were assessed by HPLC/DAD analysis.

The quali-quantitative composition of the different classes of compounds in the extract was evaluated.

In particular we confirmed the presence of flavonols (kaempferol and quercetin derivatives), (86.2 mg/g dry sample) and crocins (23.56 mg/g dry sample); kaempferol derivatives are the main class in the extract and kaempferol 3-sophoroside (62.52 mg/g dry sample, 58% of total polyphenols) is the principal compound as previously reported (Vignolini

et al., 2008).

Subsequently, the activity of the flower extract was tested on RT112 bladder cancer cell line at 72 h with increasing doses of extract, ranging from 0.5 μM to 0.5 mM (Fig. 4). The effect of the main component in the extract, kaempferol 3-sophoroside, was also assessed alone with concentrations spanning from 0.09 μM to 90 μM.

The flower extract caused a dose-dependent, great decrease of RT112 cell viability, which was then nullified at the highest extract concentration (0.5 mM), while kaempferol 3-sophoroside alone did not exert any effect, even at 90 μM. Such concentration is slightly higher compared to that determining the IC50 for the flowers extract (150 μM), being the kaempferol 3-sophoroside the most abundant component in the extract. As a result of such observations, we would have expected this particular extract to be effective.

Overall, our data demonstrates that saffron is a good source of bioactive molecules, which are able to reduce the viability of cancer cells *in vitro*. Among the active components, Crc was the most effective molecule when alone, but it appears clear that the power of the extracts was represented by the mix of their constituents, which show a synergistic effect as a phytocomplex.

4. Discussion

Saffron has traditionally been used since ancient times in traditional medicine and its components have shown interesting bioactive properties (Cardone et al., 2020). Significant variations among saffron components were reported depending on different origins. Secondary metabolites, such as polyphenols, play important roles in plant growth, regulation, defense mechanisms, and structure of plants and their concentrations in the final product are influenced by environmental conditions (Teixeira et al., 2013). Growing areas with higher air temperatures and no excessive rainfall during the flowering period resulted in higher yields of high-quality stigmas. In particular crocins were positively correlated with stigma yield and mean air temperature, which, on the other hand, were negatively correlated with safranal (https://www.isprambiente.gov.it/files2019/pubblicazioni/stato-ambiente/SA_88_19_Indicatori_clima_annoXIV_2018.pdf). We have shown that the geographical area where saffron was cultivated determines radical changes in the total content and relative ratios among constituents. The geographical origin and their respective environmental conditions (altitude, rainfall's properties on soil, irrigation cycles, temperature, solar radiation time and humidity) were the only variables that could influence plant growth and development, exerting strong effects on the production of secondary metabolites (Parizad et al., 2019). The choice of the soil is very important in order to obtain a spice with high quality. While saffron adapts very well to a wide range of soil conditions, some soils with specific characteristics perform better than others. The typology of the soil also influenced the formation of tepals and floral stamens (by-products), which can be further exploited and used in other sectors, increasing agricultural income (Cardone et al., 2020). Recent data (D'Archivio et al., 2016; Masi et al., 2016) support the idea that the active compounds of saffron can be used as geographical markers of Italian saffron. Shayganfar et al. (2021) have shown that moderate salt stresses increase the production of flowers, stigmas and crocin derivatives. The drying process is also a very important step in saffron production and, given there is currently no standardized drying method, there is the need to investigate which process is the most effective in maintaining the bioactive principles in the final product. This might be hard to achieve, since drying time depends on the place, the temperature, the relative humidity and the quantity of material loaded. Drying conditions differ between countries and according to experience, available resources and climate for each region; all this leads to variations in the quality of the saffron (Cid-Pérez et al., 2021).

A relevant number of studies has demonstrated significant antitumor properties of saffron and its compounds on several cancer cell lines. Nevertheless, the specific mechanism of action has been not completely elucidated and the activity can be attributed to different processes such as inhibition of synthesis of DNA and RNA, inhibition or suppression of cancer cells proliferation, apoptosis, inhibition of metastasis and angiogenesis, and changes in the expression pattern of oncogenes or tumor-suppressive genes (Lambrianidou et al., 2020). In particular, Bax increment and Bcl-2 downregulation by disrupting the mitochondrial membrane potential and releasing the cytochrome *c* trigger caspase activation and apoptosis in multiple cancer cell models (Lambrianidou et al., 2020). Nevertheless, research is still ongoing to dissect the particular mechanism of action of each saffron component and their synergic effect as phytocomplex. Various analytical studies have revealed that saffron is characterized by a large number of potential biologically active compounds, but the most represented in terms of weight are crocins, a carotenoid responsible for saffron's special color. It is formed by esterification of crocetin (a dicarboxylic 20- carbon carotenoid, C20H24O4) with different glycosides which makes them soluble in water, unlike most carotenoids. Picrocrocin (monoterpene glycoside precursor of safranal and product of zeaxanthin degradation) is the second most abundant component of saffron dry matter (approximately 1%-13%) and it is the main molecule responsible for the bitter taste of saffron (Alonso et al., 2001). Safranal (C10H14O) which confers the saffron characteristic aroma, is a product of the natural deglycosylation of picrocrocin and it is present in low amount in fresh stigmas, but it is produced in high amount during storage by dehydration of its precursor picrocrocin (Melnyk et al., 2010). Our data indicate that crocins are active on all the cell lines tested and drastically influences these cells' viability. Safranal was not as effective as crocins, but its contribution (especially on bladder cancer cells) was clearly detectable both when used alone and when it was supplemented to the extracts to normalize its content. The synergic effect of the two, and probably more, components is likely to be ascribed by the presence of them as phytocomplexes. Depending on their chemical nature, they can be more or less soluble in different organic or inorganic solvents. Aqueous methanol,

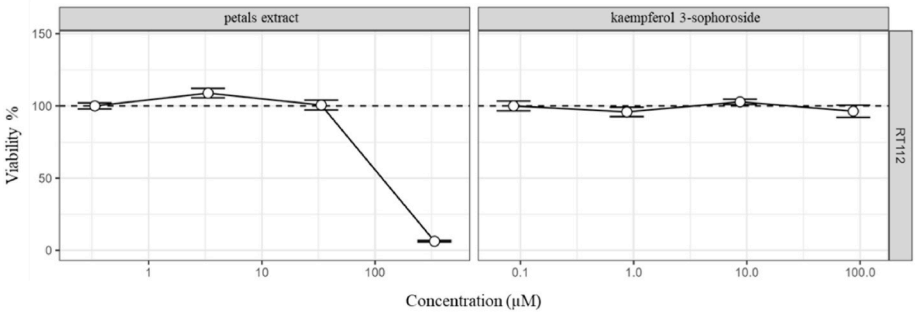

Fig. 4. Effect of flower extract and kaempferol 3-sophoroside on the cell viability of RT112 bladder cancer cells incubated for 72 h. Reported values correspond to the mean of cell viability with standard error over three biological replicates. The percentage of cell viability was calculated using the ratio AbsTEST/AbsCTRL.

ethanol or water are solvents commonly used for the extraction of many bioactive constituents and using a solvent with different polarity has significant effect on polyphenol content and antioxidant activity (Makhlouf et al., 2011). We used only water to extract the biologically active components from dried C. sativus stigmas to resemble as much as possible the natural environment by which we absorb the bioactive compounds contained in food when digesting. Already in 2008 Feizzadeh et al. sought to investigate saffron and its potential toxic effect on human bladder transitional cell carcinoma 5637 cell line confirming that saffron aqueous extract has inhibitory, dose dependent effects on the cell growth. We demonstrated that RT4 and RT112 bladder cancer cells from grade I NMIBC and grade II MIBC, respectively, were both sensitive to saffron stigmas extracts, indicating that the treatment can be beneficial in multiple stages of the tumor. The agri-food sector produces a large amount of waste products that can be used as a source of important biomolecules (e.g. polyphenols). *Crocus sativus* L. is cultivated for the production of stigmas, which constitute its valuable part and are used in various sectors: from food, to cosmetics, to pharmaceuticals. However, 90% of the harvest is represented by the flowers that are discarded. The high price of saffron and the new data on the activities of the tepals (Sánchez-Vioque et al., 2012, 2016) have led to a greater interest by the scientific community for this waste matrix, which can lead to the valorization of its use in various sectors. In this view, we investigated the potential impact of flowers extract on RT112 bladder cancer cell line, showing a dose-dependent drop of the cellular viability. The main component of the extract was kaempferol 3-sophoroside, which did not exert any effect when administered alone, indicating that the combinations of various components in the phytocomplex acts synergistically to affect the cell viability.

5. Conclusions

Bladder and kidney cancers represent together two of the most insidious types of neoplasms in the uro-oncological scenario, due to their molecular heterogeneity and frequent diagnosis delay. Several medical algorithms based on both surgery and oncological drugs regimen are changing over time with a significant improvement in the last decade in the overall survival (Trevisani et al., 2021). However, the aggressiveness of both bladder and renal cancers together with their drug resistance, remain a cumbersome issue for clinicians who continuously deserve new safe and tolerable medical therapies, especially in the advanced stages of the diseases. Therefore, there is an urgent need to create new medical compounds able to ameliorate patients' outcomes during therapy. In this perspective, the determination of therapeutic efficacy is one of the most crucial challenges in the development of an innovative drug. Even though our results represent only a starting point in tackling new possible biological, medical strategies able to decrease the cancer cell viability and proliferation over time (resulting therefore in a reduction of aggressiveness of both bladder and renal malignancies), we support the idea that these bioactive molecules could be a promising novel therapeutic approach.

In vivo tumor models are necessary to confirm the obtained results with the aim to evaluate various administration routes, including the intravesical instillations, which can be more focused and reduce the systemic side effects.

The valorization of biomass and the use of plant extracts are among the most innovative research topics. Saffron flowers contain important amounts of flavonoids and the results obtained from the flower extracts showed a dose-dependent decrease in cell viability, attributable to the synergistic activity of the present compounds. These results highlight the possibility to exploit flowers as a source of bioactive molecules with antitumoral activity. The exploitation of this waste matrix could increase the profitability and sustainability of this traditional production.

CRediT authorship contribution statement

Riccardo Vago: Writing – original draft, Supervision, Data curation, Conceptualization. **Francesco Trevisani:** Supervision. **Pamela Vignolini:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Chiara Vita:** Supervision. **Francesco Fiorio:** Data curation. **Margherita Campo:** Methodology, Data curation. **Francesca Ieri:** Methodology, Data curation. **Federico Di Marco:** Formal analysis, Data curation. **Andrea Salonia:** Resources. **Annalisa Romani:** Conceptualization. **Arianna Bettiga:** Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We express sincere thanks to Pura Crocus (Montalcino, SI) and Cerchi nel grano (Brescia) companies for the supply of saffron samples.

References

Abdullaev, F. I. (2002). Cancer chemopreventive and tumoricidal properties of saffron (*Crocus sativus* L.). *Experimental Biology and Medicine, 227*(1), 20–25.
Aires, A., Fernandes, C., Carvalho, R., Bennett, R. N., Saavedra, M. J., & Rosa, E. A. (2011). Seasonal effects on bioactive compounds and antioxidant capacity of six economically important brassica vegetables. *Molecules, 16*(8), 6816–6832.
Alonso, G. L., Salinas, M. R., Garijo, J., & Sánchez-Fernández, M. A. (2001). Composition of crocins and picrocrocin from Spanish saffron (*Crocus sativus* L.). *Journal of Food Quality, 24*(3), 219–233.
Amin, A., Hamza, A. A., Bajbouj, K., Ashraf, S. S., & Daoud, S. (2011). Saffron: A potential candidate for a novel anticancer drug against hepatocellular carcinoma. *Hepatology, 54*(3), 857–867.
Babjuk, M., Burger, M., Capoun, O., Cohen, D., Compérat, E. M., Escrig, J. L. D., … Sylvester, R. J. (2022). European Association of Urology guidelines on non–muscle-invasive bladder cancer (Ta, T1, and carcinoma *in situ*). *European Urology, 81*(1), 75–94.
Bagur, M. J., Alonso Salinas, G. L., Jiménez-Monreal, A. M., Chaouqi, S., Llorens, S., Martínez-Tomé, M., & Alonso, G. L. (2017). Saffron: An old medicinal plant and a potential novel functional food. *Molecules, 23*(1), 30.
Belyagoubi-Benhammou, N., Belyagoubi, L., Loukidi, B., et al. (2023). Bioactivity and applications of saffron floral bio-residues (tepals): A natural by-product for the food, pharmaceutical, and cosmetic industries [published online ahead of print. *Critical Reviews in Food Science and Nutrition, 13*, 1–15.
Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394–424.
Bukhari, S. I., Manzoor, M., & Dhar, M. K. (2018). A comprehensive review of the pharmacological potential of *Crocus sativus* and its bioactive apocarotenoids. *Biomedicine & Pharmacotherapy, 98*, 733–745.
Cardone, L., Castronuovo, D., Perniola, M., Cicco, N., & Candido, V. (2020). Saffron (*Crocus sativus* L.), the king of spices: An overview. *Scientia Horticulturae, 272*, Article 109560.
Chow, W. H., Dong, L. M., & Devesa, S. S. (2010). Epidemiology and risk factors for kidney cancer. *Nature Reviews Urology, 7*(5), 245–257.
Christodoulou, E., Kadoglou, N. P., Kostomitsopoulos, N., & Valsami, G. (2015). Saffron: A natural product with potential pharmaceutical applications. *Journal of Pharmacy and Pharmacology, 67*(12), 1634–1649.
Cid-Pérez, T. S., Nevárez-Moorillón, G. V., Ochoa-Velasco, C. E., Navarro-Cruz, A. R., Hernández-Carranza, P., & Avila-Sosa, R. (2021). The relation between drying conditions and the development of volatile compounds in saffron (*Crocus sativus*). *Molecules, 26*(22), 6954.
Cinque, A., Capasso, A., Vago, R., Lee, M. W., Floris, M., & Trevisani, F. (2021). The role of circulating biomarkers in the oncological management of metastatic renal cell carcinoma: Where do we stand now? *Biomedicines, 10*(1), 90.
Cinque, A., Vago, R., & Trevisani, F. (2021). Circulating RNA in kidney cancer: What we know and what we still suppose. *Genes, 12*(6), 835.

Colapietro, A., Mancini, A., D'Alessandro, A. M., & Festuccia, C. (2019). Crocetin and crocin from saffron in cancer chemotherapy and chemoprevention. *Anti-Cancer Agents in Medicinal Chemistry, 19*(1), 38–47.

D'Alessandro, A. M., Mancini, A., Lizzi, A. R., et al. (2013). *Crocus sativus* stigma extract and its major constituent crocin possess significant antiproliferative properties against human prostate cancer. *Nutrition and Cancer, 65*(6), 930–942.

D'Archivio, A. A., Giannitto, A., Maggi, M. A., & Ruggieri, F. (2016). Geographical classification of Italian saffron (*Crocus sativus* L.) based on chemical constituents determined by high-performance liquid-chromatography and by using linear discriminant analysis. *Food Chemistry, 212*, 110–116.

De Nunzio, C., Giannatempo, P., Passalacqua, R., Fiorini, E., Luccarini, I., & Brigido, A. (2020). Epidemiology and unmet needs of bladder cancer in Italy: A critical review. *Minerva Urologica e Nefrologica= The Italian Journal of Urology and Nephrology, 72*(1), 1–12.

Farrokhi, H., Asgharzadeh, A., & Samadi, M. K. (2021). Yield and qualitative and biochemical characteristics of saffron (*Crocus sativus* L.) cultivated in different soil, water, and climate conditions. *Italian Journal of Agrometeorology, 2*, 43–55.

Feizzadeh, B., Afshari, J. T., Rakhshandeh, H., Rahimi, A., Brook, A., & Doosti, H. (2008). Cytotoxic effect of saffron stigma aqueous extract on human transitional cell carcinoma and mouse fibroblast. *Urology Journal, 5*(3), 161–167.

Festuccia, C., Mancini, A., Gravina, G. L., et al. (2014). Antitumor effects of saffron-derived carotenoids in prostate cancer cell models. *BioMed Research International, 2014*, Article 135048.

International Organization for Standarization (ISO). (2003). *Saffron (Crocus sativus L.). Part 1 (specification) and Part 2 (test methods)*. Geneva, Switzerland: ISO. ISO 3632-1/2. Technical Specification.

Lambrianidou, A., Koutsougianni, F., Papapostolou, I., & Dimas, K. (2020). Recent advances on the anticancer properties of saffron (*Crocus sativus* L.) and its major constituents. *Molecules, 26*(1), 86.

Leibovich, B. C., Lohse, C. M., Crispen, P. L., Boorjian, S. A., Thompson, R. H., Blute, M. L., & Cheville, J. C. (2010). Histological subtype is an independent predictor of outcome for patients with renal cell carcinoma. *The Journal of Urology, 183*(4), 1309–1316.

Makhlouf, H., Saksouk, M., Habib, J., & Chahine, R. (2011). Determination of antioxidant activity of saffron taken from the flower of *Crocus sativus* grown in Lebanon. *African Journal of Biotechnology, 10*(41), 8093–8100.

Masi, E., Taiti, C., Heimler, D., Vignolini, P., Romani, A., & Mancuso, S. (2016). PTR-TOF-MS and HPLC analysis in the characterization of saffron (*Crocus sativus* L.) from Italy and Iran. *Food Chemistry, 192*, 75–81.

Ma, A., Zou, F., Zhang, R., & Zhao, X. (2022). The effects and underlying mechanisms of medicine and food homologous flowers on the prevention and treatment of related diseases. *Journal of Food Biochemistry, 46*(12), Article e14430.

Melnyk, J. P., Wang, S., & Marcone, M. F. (2010). Chemical and biological properties of the world's most expensive spice: Saffron. *Food Resesarch International, 43*(8), 1981–1989.

Milajerdi, A., Djafarian, K., & Hosseini, B. (2016). The toxicity of saffron (*Crocus sativus* L.) and its constituents against normal and cancer cells. *Journal of nutrition & intermediary metabolism, 3*, 23–32.

Mir, M. A., Ganai, S. A., Mansoor, S., et al. (2020). Isolation, purification and characterization of naturally derived Crocetin beta-d-glucosyl ester from *Crocus sativus* L. against breast cancer and its binding chemistry with ER-alpha/HDAC2. *Saudi Journal of Biological Sciences, 27*(3), 975–984.

Parizad, S., Dizadji, A., Habibi, M. K., Winter, S., Kalantari, S., Movi, S., … Moratalla-Lopez, N. (2019). The effects of geographical origin and virus infection on the saffron (*Crocus sativus* L.) quality. *Food Chemistry, 295*, 387–394.

Samarghandian, S., Azimi-Nezhad, M., Borji, A., & Farkhondeh, T. (2016). Effect of crocin on aged rat kidney through inhibition of oxidative stress and proinflammatory state. *Phytotheapy Research, 30*(8), 1345–1353.

Sánchez-Vioque, R., Rodríguez-Conde, M. F., Reina-Ureña, J. V., Escolano-Tercero, M. A., Herraiz-Peñalver, D., & Santana-Méridas, O. (2012). *In vitro* antioxidant and metal chelating properties of corm, tepal and leaf from saffron (*Crocus sativus* L.). *Industrial Crops and Products, 39*, 149–153.

Sánchez-Vioque, R., Santana-Méridas, O., Polissiou, M., Vioque, J., Astraka, K., Alaiz, M., Herraiz-Peñalver, D., Tarantilis, P. A., & Girón-Calle, J. (2016). Polyphenol composition and *in vitro* antiproliferative effect of corm, tepal and leaf from *Crocus sativus* L. on human colon adenocarcinoma cells (Caco-2). *Journal of Functional Foods, 24*, 18–25.

Shayganfar, A., Mohammadparast, B., Rostami, M., & Golfam, R. (2021). Salt stress causes a significant increase in anti-cancer crocins content of saffron stigma. *South African Journal of Botany, 143*, 61–68.

Shinwari, K. J., & Rao, P. S. (2018). Thermal-assisted high hydrostatic pressure extraction of nutraceuticals from saffron (*Crocus sativus*): Process optimization and cytotoxicity evaluation against cancer cells Innov. *Food science & emerging technologies, 48*, 296–303.

Teixeira, A., Eiras-Dias, J., Castellarin, S., & Geros, H. (2013). Berry phenolics of grapevine under challenging environments. *International Journal of Molecular Sciences, 14*, 18711–18739.

Trevisani, F., Di Marco, F., Raggi, D., Bettiga, A., Vago, R., Larcher, A., … Montorsi, F. (2021). Renal function outcomes in patients with muscle-invasive bladder cancer treated with neoadjuvant pembrolizumab and radical cystectomy in the PURE-01 study. *International Journal of Cancer, 149*(1), 186–190.

Vignolini, P., Heimler, D., Pinelli, P., Ieri, F., Sciullo, A., & Romani, A. (2008). Characterization of by-products of saffron (*Crocus Sativus* L.) production. *Natural Product Commununication, 3*(12), 1959–1962.

Witjes, J. A., Bruins, H. M., Cathomas, R., Compérat, E. M., Cowan, N. C., Gakis, G., Hernández, V., Linares Espinós, E., Lorch, A., Neuzillet, Y., Rouanne, M., Thalmann, G. N., Veskimäe, Ev Ribal, M. J., & van der Heijden, A. G. (2021). European association of urology guidelines on muscle-invasive and metastatic bladder cancer: Summary of the 2020 guidelines. *European Urology, 79*(1), 82–104.