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Further Examination of Antiradical Properties of *Crocus sativus* Stigmas Extract Rich in Crocins

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Data for antiradical properties of saffron extract and its bioactive constituents (crocins, crocetin) are limited and poorly discussed in comparison with those of extracts containing potent scavengers. Further examination was sought using the Folin−Ciocalteu (F−C) reagent and various free radical species produced in cell-free or cell model systems. Oregano and turmeric methanol extracts, rich in well established scavengers, and also crocetin, rosmarinic acid, and curcumin, representing the major types of constituents in the three studied extracts, were used as "reference". On the same weight basis, saffron extract activity was found to be rather negligible in all cell-free systems with regard to that found for reference ones. On the contrary, in the human monocyte system, saffron extracts or free crocetin were found to reduce ROS production as effectively as the phenolic antioxidants. Our findings point out that saffron extracts exhibit a remarkable intracellular antioxidant activity that cannot be revealed using assays repeatedly applied to the evaluation of phenolic-type antioxidants.

KEYWORDS: Saffron; *Crocus sativus* L.; crocins; crocetin; oregano; turmeric; reactive oxygen species (ROS); antiradical properties; human monocytes; DPPH•; ORAC; lecithin liposomes

INTRODUCTION

Since ancient times, saffron, a spice comprised of the dried red stigmas of *Crocus sativus* L., has been highly valued for its distinct flavor and for the bright yellow hews it imparts to food preparations. The latter are owed to the presence of some unique water-soluble carotenoid metabolites, which are a group of crocetin (8,8′-diapocarotene-8,8′-dioic acid) glycosides, the so-called crocins.

During the last three decades, saffron carotenoids have attracted the interest of several researchers in the fields of pharmacology, biology, and medicine. The aim of these studies was either to give scientific evidence for the numerous therapeutic properties of saffron, known from ancient Greek, Persian, Roman, Ayurverdic, and Chinese remedies, or to search for alternative drugs among bioactive phytochemicals. The activities assigned up to now to crude saffron extracts—or to its major bioactive constituents—are diverse, i.e. antitumor/anticancer activities, antiatherosclerotic activities, and effects against ethanol-induced memory impairments (*1−3*). Recent findings about the metabolism and pharmakokinetics of saffron carotenoids point out that free crocetin, formed upon hydrolysis of crocins prior to or in the course of intestinal absorption, is the bioactive molecule (*4, 5*). In addition, it is postulated that, when absorbed into blood plasma, crocins and crocetin modulate intracellular oxidative stress by activation of antioxidant enzymes (*6, 7*) or by direct scavenging of reactive oxygen species (e.g. ref *8*). On the other hand, evidence on the antiradical activity of saffron extracts, crocins, or crocetin is limited (*9−11*) and inconclusive, as data are scarcely discussed in comparison to those for extracts or compounds of known antioxidant potency.

In the present study, the reactivity of methanol saffron extract was investigated toward the Folin−Ciocalteu (F−C) reagent and various free radical species produced in cell-free ($ABTS^{•+}$, DPPH•, peroxyl) or cell model systems ($O_2^{•-}$). The observed activity was evaluated with reference to those of oregano and turmeric extracts known to be good sources of chain-breaking antioxidants. Oregano extracts are rich sources of rosmarinic acid and flavonoids (*12*), whereas turmeric ones contain high amounts of curcuminoids (*13*). The major types of constituents in the three studied extracts, i.e. crocetin, rosmarinic acid, and curcumin (**Figure 1**), were also included in the study to get structure−activity information. Taking into account the general interest in functional foods and dietary supplements, substantiation of the actual saffron extract activity is a prerequisite in industrial applications and justifies the aim of our study.

MATERIALS AND METHODS

Samples and Standards. Dried stigmas of *Crocus sativus* L. were kindly donated by the Cooperative of Saffron Producers (Krokos

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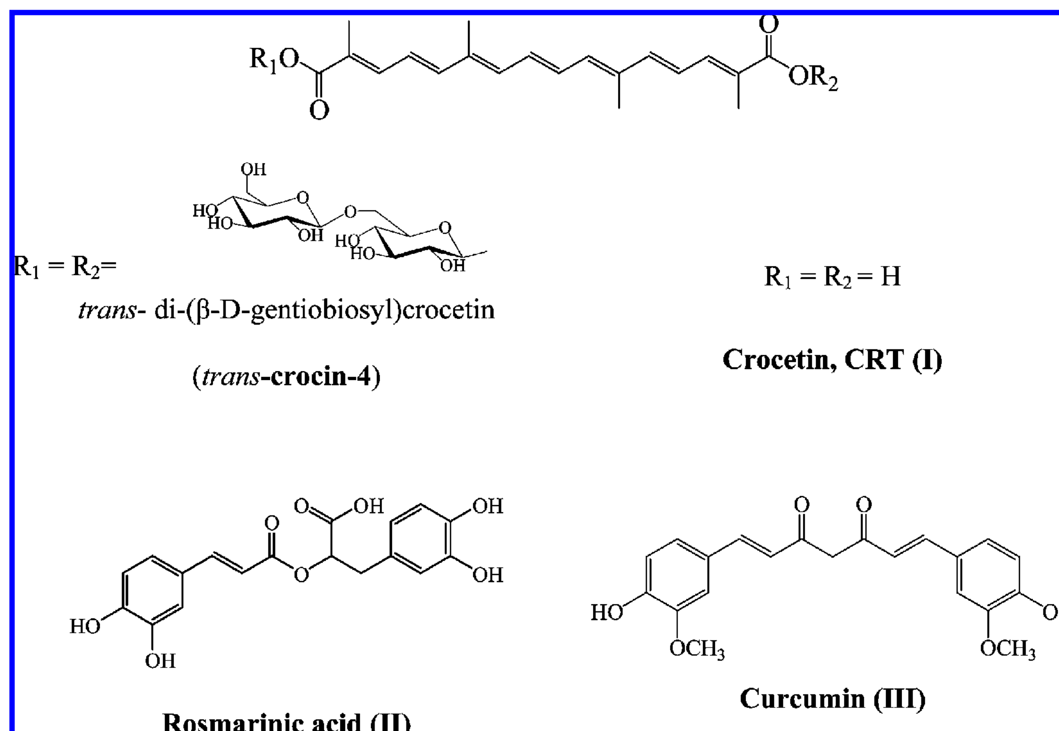

Figure 1. Structural characteristics of the major types of saffron (I), oregano (II), and turmeric (III) active constituents.

Kozanis, Greece). Dried material from botanically characterized *Origanum vulgare* L. ssp. hirtum was a gift from the Botany Department of the Mediterranean Agronomic Institute of Chania (Crete, Greece). *Curcuma longa* L. rhizomes were obtained from the local market. Refined olive oil was kindly donated by ELAIS S. A. (Piraeus, Greece) and purified as in ref *14*. Rosmarinic acid (97%) was purchased from Fluka Chemie (Buchs, Switzerland). Curcumin (97%) was from Roth Karlsruhe (Germany). Trolox (97%) was from Aldrich Chemical Co. (Steinheim, Germany). Human insulin was from Novo Nordisk A/S (Denmark).

Solvents and Reagents. All chemicals from various suppliers were of the highest purity needed for each assay: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) diammonium salt, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•), dihydroethidium (HE), and D-phosphatidylcholine (Lecithin, ~40%) from soybean were from Sigma Chemical Co. (St. Louis, MO). Folin−Ciocalteu (F−C) reagent and fluorescein sodium salt were from Panreac Quimica S.A. (Barcelona, Spain). 2,2′-Azobis(amidinopropane) dihydrochloride (AAPH) was from Fluka Chemie (Buchs, Switzerland). Iscove's modified Dulbecco's medium (IMDM), RPMI medium, fetal calf serum (FCS), penicillin/streptomycin, and L-glutamine were purchased from Biochrom (Berlin, Germany). Ficoll-Paque Plus (1.077 g/mL) and Percoll (1.130 g/mL) were from Amersham Biosciences (Piscataway, NJ).

Preparation and Characterization of Plant Extracts. The ground plant material (2 g) was extracted with methanol (50 mL) in an ultrasonic bath for 15 min at room temperature. After the solvent was evaporated in vacuum, the extracts were purged with nitrogen and kept under −18 °C until use. Chemical characterization was achieved using high performance liquid chromatography−diode array detection (HPLC−DAD). The system consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA), a Midas autosampler (Spark, Emmen, The Netherlands), and a UV 6000 LP diode array detector (Thermo Separation Products). Elution was performed on a Nucleosil 100 5*μ* C18 column (250 mm × 4.6 mm i.d.) (Macherey-Nagel GmbH & Co. KG, Düren, Germany) under conditions described in the literature (*13, 15, 16*). Detection of the major compounds present in saffron, oregano, and turmeric extracts was achieved at 440, 330, and 420 nm, respectively. The data were processed using the ChromQuest Version 3.0 software (Thermo Separation Products).

Preparation of Crocetin (CRT). Crocetin was prepared from an aqueous extract of *Crocus sativus* L. by acid hydrolysis according to the following procedure: Saffron powder (4 g) was weighed into a glass centrifuge tube to which water was added (70 mL). The mixture was vortexed (1 min), kept in the dark (10 min), agitated again (1 min), and finally centrifuged (4000 rpm, 15 min). The supernatant was acidified to pH 0.10 (±0.03) by the addition of concentrated H_2SO_4 and then was heated at 90 °C for 5 h, cooled, and filtered through Whatman Filter paper No. 3. Hydrolysis of crocins was monitored periodically by TLC of the hydrolysate (elution solvents petroleum ether/acetic acid 1:1, v/v). CRT (red powder) was purified after repeated washes with deionized water (at least 3 × 100 mL until a colorless eluent was discarded) and lyophilized. Additional purification was obtained when CRT (red powder) was dissolved in pyridine, which in turn was evaporated to dryness using a rotary evaporator. The purity and structure of the isolated CRT were analyzed by FT-IR, and the results were in agreement with those reported in the literature (*17*).

Isolation of Human Monocytes. Monocytes were isolated from heparinized whole blood from healthy human donors as described previously (*18*). In brief, heparinized whole blood was diluted with phosphate buffered saline (PBS) (1 mM EDTA, pH 7.2) and underlayered with the use of an 18-gauge spinal needle with Ficoll-Paque Plus (1.077 g/mL) in 50 mL falcon tubes. After centrifugation (400*g*/20 min/RT/no brake), the peripheral blood mononuclear cell (PBMC) layer was collected and put in new, clean 50 mL falcon tubes. There followed three washes with PBS (1 mM EDTA, pH 7.2). The PBMCs were then diluted with complete IMDM and overlayered on 46% Percoll in 50 mL falcon tubes. After centrifugation (550*g*/30 min/RT/no brake), the monocyte layer was collected, diluted with PBS, and washed twice with PBS before use in experiments (10^6 cells/mL). Monocyte purity in the final samples was measured on a Beckman Coulter EPICS XL-MCL flow cytometer using CD14 antibody and was found to be >85%.

Folin−Ciocalteu Assay. The reducing power of saffron (S), oregano (O), and turmeric (T) extracts as well as of the pure compounds was estimated as proposed elsewhere (*19*). For each plant extract, the addition level used was 500 mg/L (S500, O500, and T500). In the case of saffron, a 10-fold higher level (5000 mg/L, S5000) was also tested. The F−C values of plant extracts were expressed as Trolox equivalents (mM) on the basis of a linear curve constructed for Trolox. For pure compounds, a series of methanolic solutions of 0.5−2.0 mM were prepared. Graphs of antioxidant concentration vs absorbance were then constructed and the slope of the linear curve derived from the

constructed graphs was calculated and represented the F−C value. For each compound and each concentration, measurements were carried out in triplicate.

$ABTS^{•+}$ Assay. The $ABTS^{•+}$ solution was prepared by reaction of 5 mL of a 7 mM aqueous ABTS solution and 88 μL of a 140 mM (2.45 mM final concentration) potassium persulphate ($K_2S_2O_8$) solution (*20*). After storage in the dark for 16 h, the radical cation solution was further diluted in buffer (PBS, pH = 7.4) until the initial A_{734} value was 0.70 ± 0.05 au. A portion of methanolic solution (15, 30, and 45 μL) of S500, S5000, O500, and T500 or of Trolox (5, 10, and 15 μL) was added to the radical solution (2 mL). A_{734} values were recorded at 0 and after 6 min of reaction. Linear regression plots of antioxidant concentration versus %Inh values (%Inh = $(A_{0min} - A_{6min})/A_{0min}) \times 100$ were then constructed. ABTS values were those of the slope of the linear curve derived from the constructed plots. The concentration of Trolox with a %Inh value equivalent to that of a 1 mM antioxidant solution was calculated from three point graphs. The results were expressed as Trolox equivalent values. All tests were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions.

ORAC Assay. Evaluation of peroxyl radical scavenging activity was carried out as proposed by Nenadis et al. (*21*). In a 5-mL volumetric flask, 4 mL of an 8.6 nM fluorescein solution (preincubated at 37 °C for 15 min) prepared daily from a stock solution (0.11 mM) was transferred. Then, 250 μL of S500, S5000, O500, and T500 or phosphate buffer for the blank reading was added. The methanol extracts were diluted with a 75 mM phosphate buffer (PB), pH = 7.0 (1:20, v/v) prior to addition to the reaction mixture. The reaction started with the addition of 120 μL of a 125 mM AAPH solution. Then, the volume was brought up to 5 mL with buffer solution (pH = 7.0), and the reaction mixture was vortexed for 0.5 min. The fluorescence was recorded every 0.5 min ($\lambda_{excitation}$ = 490 nm, $\lambda_{emission}$ = 515 nm) until no fluorescence was recorded on a Shimadzu RF 1501 spectrofluorometer (Kyoto, Japan). During the whole experimental procedure, the temperature was maintained at 37 °C and the reaction mixture was magnetically stirred. For each extract, measurements were made in triplicate. The net area under the curve (AUC) was obtained by subtracting that of the blank (AUC_{blank}) from that of the respective tested compound (AUC_{test}). Calculations were carried out by means of the RF 1501-PC software (Shimadzu, Kyoto, Japan). The results were expressed as Trolox equivalents.

$DPPH^•$ Assay. Estimation of the radical scavenging activity was based on the procedure described in ref *22*. Briefly, an aliquot (0.1 mL) of the methanolic solution of S500, S5000, O500, and T500 extracts, CRT (290−1160 μM), rosmarinic acid (145−435 μM), curcumin (290−725 μM), and Trolox (290−870 μM) was added to the solution of $DPPH^•$ (2.9 mL, 100 μM). The $[DPPH^•]_0$ reduction was monitored by absorbance measurement at 516 nm until steady state and the $[DPPH^•]_{steady}$ was estimated as described previously (*23*). A_{516} values were automatically recorded on a UV-1601 Shimadzu spectrophotometer (Kyoto, Japan) and were corrected for radical decay using blank methanol solutions. The reaction solution was thermostatted at 25 ± 0.5 °C by an outer water-circulating bath. Measurements were performed in triplicate. In the case of plant extracts, the activity was expressed in terms of %RSA values calculated according to the equation %RSA = $[([DPPH^•]_0 - [DPPH^•]_{steady})/[DPPH^•]_0] \times 100$. For the pure compounds, the parameters EC_{50} (efficient molar ratio necessary to decrease the initial $[DPPH^•]$ by 50%), $T_{EC_{50}}$ (reaction time needed to reach the steady state at EC_{50}), and AE (antiradical efficiency, AE = $1/(EC_{50} \times T_{EC_{50}})$) were calculated. Moreover, the reaction of saffron extracts and CRT with the $DPPH^•$ was monitored as suggested by Kanakis et al. (*24*). The UV−vis spectra of the test solution were recorded at time intervals of 5 min for 60 min, in the region 200−700 nm. Second derivative spectra were calculated, and the area of the $DPPH^•$ peak at 516 nm was measured with the aid of the Shimadzu UVPC 1601 software. The %RSA values were calculated according to the equation %RSA = $[(B_{control} - B_{sample})/B_{control}] \times 100$, where $B_{control}$ is the area of the $DPPH^•$ peak of the solution not containing the test extract/compound and B_{sample} is the area of the respective peak of the test solution.

Phosphatidylcholine Liposome Oxidation. Lecithin was suspended in double-distilled water (8 mg/mL) by stirring first with a glass rod and then using sonication for ∼5 min. Liposome formation was achieved through further sonication with a rod (UP 200S, Dr. Hielscher, GmbH, Berlin, Germany) (2.5 min for 10 mL of the liposome sample). Upon completeness of the liposome preparation, aliquots (0.5 mL) of methanolic solutions of S500, S5000, O500, and T500 and CRT (30−150 μM) were added into Erlenmeyer flasks (100 mL). Trolox was used for comparison. Liposomes were weighed into the flasks and diluted with double distilled water to a final lecithin concentration of 0.8% w/w. The samples were then set for 2 min in a bath-type sonicator and oxidized by addition of cupric acetate (3 μM) in a shaker at 37 °C in the dark. The course of oxidation was spectrophotometrically monitored through measurement of conjugated diene formation at 234 nm (*19*). All measurements were performed in triplicate.

Bulk Oil Stability Test. Aliquots of the purified olive oil (1.6 g) were distributed in a series of open dark glass bottles of pharmacopoeia quality (18 mm i.d.). Oil samples containing Trolox (15 mg/kg), saffron (1000 and 10000 mg/kg), oregano, and turmeric extracts (1000 mg/kg) were stored at 62 °C in the dark. The process of oxidation was followed by periodic measurements of peroxide values (PV).

Intracellular Superoxide Anion ($O_2^{•-}$) Detection. Monocytes (10^6 cells/mL) were incubated with 25 μM HE for 20 min at 37 °C and were then washed three times with PBS (pH 7.2). Cells were then incubated in the presence of plant extracts (10^{-5} to 10 μg/mL) or pure compounds (3×10^{-5} μM) for 3 h at 37 °C followed by incubation in the presence of insulin (50 μU/mL) for 30 min at 37 °C. The control samples were incubated in parallel. After two washes with PBS were performed, fluorescence was measured under continuous magnetic stirring at 20 °C in a 3 mL quartz cuvette using a Shimadzu (RF-5000) thermostatic spectrofluorophotometer with the excitation and emission wavelengths set at 396−520 nm and 510−610 nm, respectively (*25, 26*). Plant extracts were tested in monocytes from three different cell donors. Each sample was then analyzed in duplicate on each preparation of monocytes (n = 2). For pure compounds, one cell donor was used and each analysis was conducted in triplicate (n = 3). The cell experiments were performed in an incubation chamber under standard atmospheric pressure conditions.

Statistical Analysis. Evaluation of statistical significance of differences was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with the aid of the SPSS 14.0 for Windows (SPSS Inc.) statistical program.

RESULTS AND DISCUSSION

UV−vis and RP-HPLC analysis of saffron, oregano, and turmeric extracts confirmed their high content in crocins, rosmarinic acid, and curcumin, respectively (see Supporting Information), in line with the literature (*12, 13, 15*) and consequently their suitability for the purpose of the study.

Assessment of antioxidant activity is a multidimensional approach, as was clearly stated by Frankel several years ago (*27*). Consequently, various analytical methods have to be employed in the evaluation of antioxidant potential of plant materials important to food, pharmaceutical, or other uses. In our study, complementary information was obtained using the F−C assay as a means of evaluating the reducing power of saffron extract while $ABTS^{•+}$, ORAC, and $DPPH^•$ assays were chosen as the most frequently used ones. Data on the capacity of two saffron extracts at two different concentrations, 500 and 5000 mg/L, to reduce the F−C reagent as well as to scavenge free radical species using the above assays are presented in **Table 1**. In the same table, results are also given for the activity of oregano and turmeric extracts (500 mg/L).

On the basis of the information given in **Table 1**, saffron extract (S500) exhibited almost no reducing power or radical scavenging capacity. This became more evident when the activity of oregano and turmeric extracts was examined on the same dry weight basis. O500 and T500 extracts were found to

Table 1. Reducing and Radical Scavenging Activity of Plant Extracts Using Various Assays[a]

	reducing activity	radical scavenging activity		
plant extract (mg/L)	F−C[c] (mM Trolox)	$ABTS^{•+c}$ (mM Trolox)	ORAC[c] (μM Trolox)	$DPPH^{•b,c}$/%RSA (t_R in min)
S500	0.46 ± 0.03[a]	<5	0.13 ± 0.01[a]	0 (−)
S5000	4.99 ± 0.04[b]	12.9 ± 1.1[a]	2.47 ± 0.16[b]	0 (−)
O500	1.90 ± 0.02[c]	15.5 ± 1.3[b]	1.82 ± 0.01[c]	39.7 ± 0.8[a] (2.7 ± 0.1)
T500	1.61 ± 0.01[d]	11.0 ± 0.1[c]	0.68 ± 0.09[d]	28.5 ± 1.9[b] (2.9 ± 0.0)

[a] Values within the same column with different lowercase letters as superscripts are significantly different at $p < 0.05$. [b] Percent radical scavenging activity values at t_R = time required to reach steady state (minutes). [c] Each value is the mean of triplicate determinations ± SD.

be active under all experimental conditions. The order of activity was oregano > turmeric > saffron. Quite high differences in the magnitude of activity were only traced under the ORAC conditions. Hence, the potency of S500 toward the $ABTS^{•+}$ was found to be only 2−3 times weaker than that of O500 or T500 but 13-fold and nearly 5-fold weaker toward the AAPH-derived peroxyl radicals, respectively. Our finding could be associated with differences in the prevailing mechanism and kinetics of reactions taking place under the $ABTS^{•+}$ and ORAC assay conditions (*28*). When a 10-fold higher concentration of saffron extract (S5000) was used, the F−C, $ABTS^{•+}$, and ORAC values were increased by almost 10, 2, and 17 times, respectively indicating a dose−response dependence in activity. It is worth noting that in comparison to T500, S5000 was found to be approximately ∼3-fold more efficient in reducing the F−C reagent, of equal activity toward the $ABTS^{•+}$, and ∼4-fold more potent under ORAC conditions. Similar observations were made regarding the activity of O500. Our findings using the F−C, $ABTS^{•+}$, and ORAC assays for the S5000 extract were considered interesting bearing in mind the qualitative differences in the composition of the studied plant extracts.

More specifically, O500 performance is due to the high antiradical potency of rosmarinic acid. The presence of dihydroxyl- (catechol) groups ortho to the aromatic rings of the latter greatly contributes to stabilization of the phenoxyl radical generated after abstraction of a hydrogen atom. Similarly, the presence of high levels of curcumin in T500 is responsible for its activity. Curcuminoids possess ortho-hydroxy-methoxyl groups and extended conjugation that are essential characteristics for antiradical activity. On the other hand, *trans*- and *cis*-crocins, prevailing in saffron extract, can be considered as "atypical" carotenoids mainly due to their fine water-solubility properties, related with the presence of bulky sugars esterified to the terminal carboxylic groups of the carbon skeleton. Although the importance of such characteristics for the activity of the above carotenoids has not been elucidated yet, it is accepted that the mechanistic paths for scavenging of free radicals are similar to those of the most known nonpolar carotenoids (e.g. refs *9*and *29*). Our finding that saffron extract showed a comparable activity to those of oregano and turmeric only when used at multifold higher concentration indicates that the activity of crocins, though dose-dependent, is rather poor. Exclusion of the impact of analytical interferences to the above found order and magnitude of activity was also considered. Thus, blank test solutions of saffron extract at pH 7.0 in the absence of fluorescein were examined under the ORAC conditions ($\lambda_{excitation}$ = 490 nm and $\lambda_{emission}$ = 515 nm). No interfering fluorescence was recorded. Moreover, interaction of AAPH-derived peroxyl radicals with the sugar moieties attached to the terminal carboxylic groups of crocetin is not expected, as has been reported in the case of glucose under the ORAC conditions (*30*).

Concerning the $DPPH^•$ assay, both S500 and S5000 were found to be inert toward this synthetic radical. This result was not in line with those of Assimopoulou et al. (*10*), who have reported a quite high $DPPH^•$ scavenging activity of saffron extracts (∼51% inhibition at a 2500 mg/L level of addition). Although the $DPPH^•$ assay protocols used in both studies were similar, differences in results could be attributed to the extract preparation procedure or the duration of the monitoring period. Time is considered as a critical parameter of the $DPPH^•$ assay for the evaluation of the activity of different plant extracts given that these complex matrices contain radical scavengers of different kinetic behavior. For example, in the present study, the %RSA values of oregano and turmeric extracts were 40 and 30, respectively, within ∼3 min (**Table 1**), while saffron extracts were not found to scavenge $DPPH^•$ even after 60 min (data not shown). Verification of this finding was achieved through calculation of the second derivative spectra of the reaction mixture at different time intervals (see Supporting Information). No measurable changes were observed within the 60 min monitoring period.

Inhibition or induction of hydroperoxide formation during accelerated oxidation of lipid substrates, which may be related with metal reducing or chelating properties, is an antioxidant/prooxidant index of high importance for the potential uses of plant extracts in food preparations (*27*). Therefore, all of the extracts were tested under accelerated oxidation conditions in bulk oil (62 °C) and in Lecithin liposome (37 °C, in the presence of Cu^{2+}) systems. Higher levels of the extracts (1000 and 10000 mg/kg) were used in the case of the bulk oil stability test based on previous experience on oregano extract activity under similar experimental conditions (*12*). The obtained data are given in **Figure 2**. Saffron extract activity is discussed with reference to those of oregano and turmeric extracts and also to that of Trolox at the appropriate addition level.

Under the experimental conditions, oregano, turmeric, and Trolox were found to be equally effective for the period of 25 days of purified olive oil storage whereas saffron exhibited neither antioxidant nor prooxidant activity at the same level of addition (1000 mg/kg) (**Figure 2A**). The latter showed a weak antioxidant activity only when added at a 10-fold higher level. Similar was the trend observed in the multiphase system (**Figure 2B**). It has to be stressed that saffron extract activity was weaker than that found in the bulk oil. No concentration-dependent effect could be clearly assigned in this case. The results in **Figure 2** point out that when saffron is used as a spice imparting coloring in food preparations on a *quantum Satis* basis, it cannot be expected to exert chain-breaking effects to the substrates.

So far, documentation on the ex vivo antioxidant effect of saffron extracts or of its active constituents (*1−3, 6, 7*) indicated that possibly the above in vitro tests are not appropriate for the evaluation of the antioxidant potential of saffron extract. Investigation using a single-cell model system (human monocyte) was considered to be the next tool to employ because it simulates physiologically relevant conditions (*31*). The protocol for the detection of intracellular ROS production using 2′,7′-dichlorofluorescein diacetate (DCF-DA) as a fluorescent probe for monitoring oxidation (*32*) was not found to be applicable

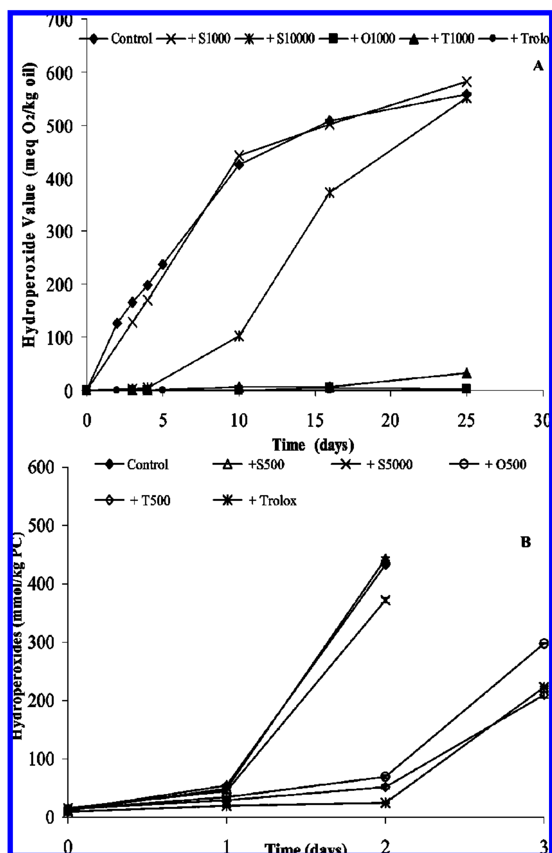

Figure 2. (**A**) Purified olive oil oxidation (62 °C) in the presence of plant extracts (1000 or 10000 mg/kg) or Trolox (15 mg/kg). The values are means of duplicate determinations. (**B**) Copper-induced liposome oxidation (37 °C) in the presence of plant extracts at various concentrations or Trolox (30 μM). The values are means $\pm$ SD (n = 3).

due to interferences with saffron extract constituents. The probe of choice was dihydroethidium, which is widely used as an intracellular $O_2^{\bullet-}$-specific probe, since it may penetrate cell membranes (*32*). Recent considerations about the specificity of the particular protocol were taken into account (*25, 26*). The properties of saffron extract to suppress the production of $O_2^{\bullet-}$ from human monocytes under normoxic conditions as well as after stimulation by the addition of insulin (50 μU/mL) were monitored at excitation/emission wavelengths set at 520/610 nm, the most frequently used detection wavelengths, but also at 396/510 and 480/ 567 nm (*25, 26*). Saffron properties were studied for three different extract concentrations (10^{-5}, 10^{-2}, and 10 mg/L), and in each case, the activity was compared to that of the respective oregano and turmeric extracts (**Figure 3**).

In contrast to what was observed under the conditions of the cell-free assays, saffron extracts were shown to suppress the intracellular production of $O_2^{\bullet-}$ to the same extent as the two herbal extracts. The levels of the radical detected into the monocytes incubated with the lowest saffron extract concentration were restrained only by 13% compared to those found in control cells. A much higher reduction in $O_2^{\bullet-}$ levels (42−57%) was evidenced upon addition of saffron extract in the range 10^{-2} to 10 mg/L, and the same dose-dependent trend was observed for turmeric extract. The respective oregano one showed a somewhat lower activity as the % $O_2^{\bullet-}$ levels were reduced by 37−48% compared to those of the control cells. As expected, addition of insulin was found to highly promote the generation of $O_2^{\bullet-}$ (**Figure 3**). Incubation of insulin-stimulated monocytes with saffron extracts (10^{-2} to 10 mg/L) resulted in a marked reduction of $O_2^{\bullet-}$ (41−44%), almost counteracting the negative effect of insulin. This performance was similar to that of the respective turmeric extracts (reduction by 44−49%) and somewhat better than that of oregano ones (24−36%). A similar trend was observed when the intracellular production of $O_2^{\bullet-}$ was monitored at other excitation/emission wavelengths (396/510 and 480/567 nm; see Supporting Information).

Various mechanisms of action, other than direct scavenging of $O_2^{\bullet-}$, may be related with the antioxidant effect of saffron extracts observed in the monocyte system. Interaction of crocins or of crocetin with enzymes such as superoxide dismutase (SOD) or peroxidases or with signal transduction pathways regulating the production of free radicals in the monocyte cannot be excluded (*6, 7*). Saffron extracts, isolated crocins, crocetin, and crocetinates have been reported to exert activity related with direct or indirect suppression of ROS (*6−8*). In an effort to understand which of these mechanisms plays the most significant role to the cellular antioxidant activity of saffron extracts, we further examined the behavior of crocetin, bearing the responsible carotenoid backbone structure. Its activity was examined in parallel with that of rosmarinic acid and curcumin as well as that of Trolox, a common reference antioxidant. Each of these pure compounds was used at the level of 3×10^{-5} μM, which was selected after preliminary experimentation. The results are presented in **Figure 4**. A comparative study of crocetin,

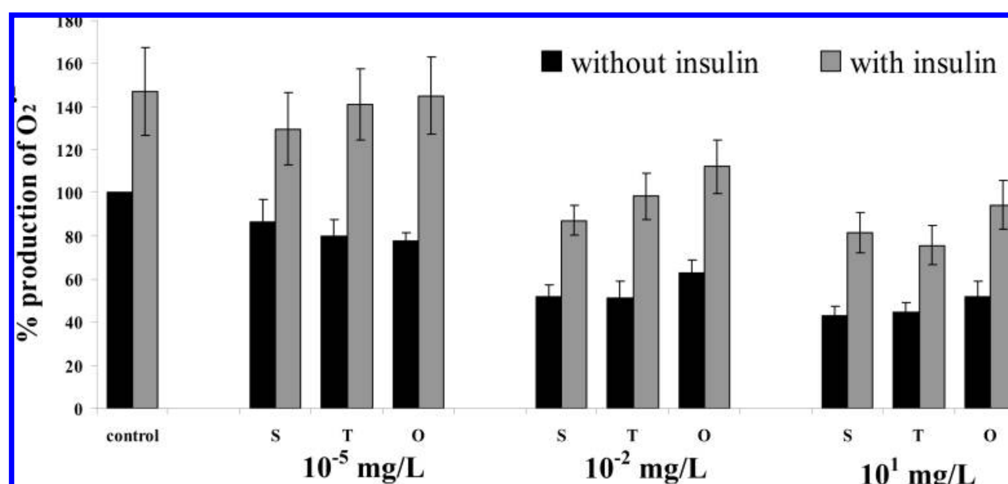

Figure 3. Effect of saffron (S), oregano (O), and turmeric (T) extracts (10^{-5}, 10^{-2}, 10^{1} mg/L) on the levels of $O_2^{\bullet-}$ produced from monocytes ($\lambda_{excitation}$ = 520 nm, $\lambda_{emission}$ = 610 nm, as described under Materials and Methods). The values are means $\pm$ SD (n = 3).

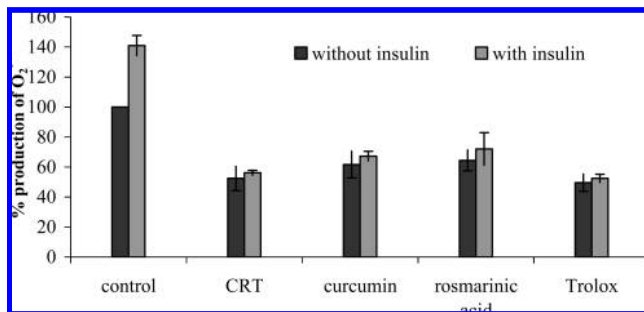

Figure 4. Effect of crocetin (CRT), rosmarinic acid, curcumin, and Trolox (3×10^{-5} μM) on the production of $O_2^{\bullet-}$ from monocytes ($\lambda_{excitation}$ = 520 nm, $\lambda_{emission}$ = 610 nm, as described under Materials and Methods). The values are means ± SD (n = 3).

rosmarinic acid, and curcumin activity was also carried out using the F−C, DPPH$^{\bullet}$, and Lecithin liposome tests.

Pure compounds showed similar trends in activity in the presence or absence of insulin, in line with observations made on the efficiency of respective extracts. The fact that the intracellular performance of CRT was comparable to that of Trolox, rosmarinic acid, and curcumin, does not necessarily confirm direct radical scavenging properties, since different mechanistic routes may be responsible in each case. This view was further supported when the compounds were tested using the above-mentioned cell-free in vitro assays. Thus, on the basis of the F−C values, rosmarinic acid was the most active one (F−C value: 2.14 ± 0.18); curcumin was less potent (F−C value: 0.56 ± 0.03), while CRT presented a rather weak reducing activity (F−C value: 0.29 ± 0.05), nearly equivalent to that of Trolox (F−C value: 0.27 ± 0.03), a known weak reductant (*21*). This is a new piece of information for the reducing activity of crocetin. Similar observations were made for the order of activity toward the DPPH$^{\bullet}$, as evidenced by the corresponding EC_{50} values (rosmarinic acid, 0.09; Trolox, 0.20; curcumin, 0.24; CRT > 0.5). For all of the studied compounds, $T_{EC_{50}}$ values ranged between 3 and 6 min, indicating that reactions with radicals follow a rapid kinetics and that time is not a critical factor in the evaluation of crocetin activity toward the DPPH$^{\bullet}$.

Rosmarinic acid and curcumin, possessing free phenolic −OH groups, were highly active under all assay conditions, as expected. The fact that, under the cell-free conditions assayed, crocetin, representing the backbone of saffron carotenoids, showed a weak reactivity toward free radicals, is supportive of the view that this feature of crocins is responsible for the low potential of saffron extract evidenced in this study. Still, the presence of mono-, di-, and trisaccharide moieties in crocins may also contribute to the observed inactivity of saffron extracts, possibly due to steric hindrance phenomena. The high polarity of crocins could also be a limiting factor. Investigation of crocetin performance in liposome systems revealed a dose−response dependence (**Figure 5A**), obviously much weaker than that of Trolox, rosmarinic acid, and curcumin for the same levels of addition (**Figure 5B**). Taking into account our findings about the respective activity of saffron active constituents toward lipid peroxyl radicals (see **Figure 2B**), it seems that the presence of sugar moieties attached to the terminal −COOH groups of the crocetin skeleton is critical for the distribution of these constituents in the bilayers of Lecithin liposomes. The same moieties might also play a role in the penetration of cell membranes. For example, the membrane-stabilizing effect of

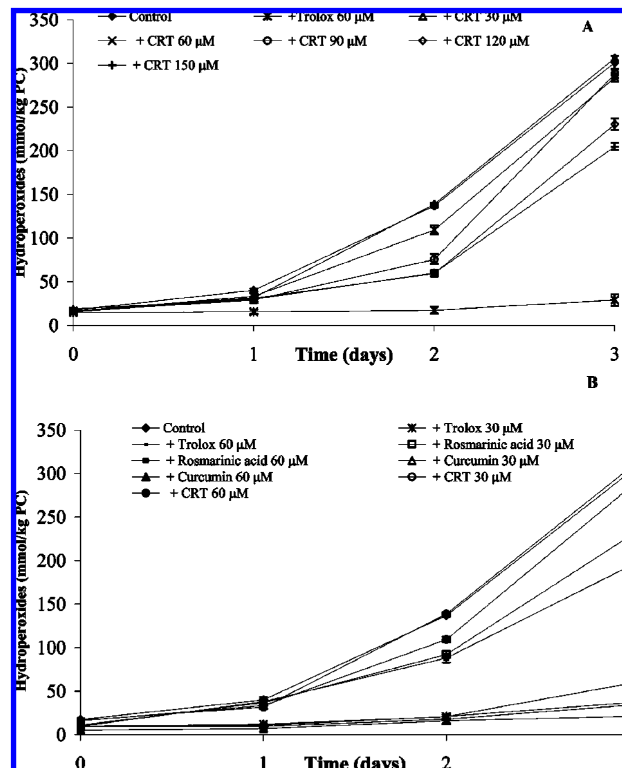

Figure 5. Copper-induced liposome oxidation (37 °C) in the presence of (**A**) crocetin (CRT) at 30−150 μM and (**B**) rosmarinic acid, curcumin, and CRT at the same levels of addition. The values are means ± SD (n = 3).

crocins, reported by Ochiai et al. (*6*) was clearly assigned to their powerful antioxidant activity, similar to that of α-tocopherol.

In summary, our findings point out that saffron extracts exhibit a remarkable intracellular antioxidant activity that cannot be revealed using assays repeatedly applied to the evaluation of phenolic-type antioxidants present in food or biological fluids. In the human monocyte system—employed for the first time for saffron—its methanol extract, rich in crocins or even isolated crocetin, was found to reduce $O_2^{\bullet-}$ production as effectively as the selected well-known phenolic antioxidants. Our finding reinforces the perception of saffron bioactivity through antioxidant mechanism of action. The latter needs further substantiation by in vivo studies.

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Supporting Information Available: HPLC-DAD analysis of (A) saffron, (B) oregano, and (C) turmeric rhizome methanol extracts at 440, 330, and 420 nm, respectively (as described under Materials and Methods). UV−vis spectra of the solutions containing saffron extract and/or DPPH$^{\bullet}$ (a) zero order and (b) second order derivative. Effect of saffron (S), oregano (O), and turmeric (T) extracts (10^{-5}, 10^{-2}, 10^{1} mg/L) on the levels of $O_2^{\bullet-}$ produced from monocytes at (a) $\lambda_{excitation}$ = 396 nm, $\lambda_{emission}$ = 510 nm and (b) $\lambda_{excitation}$ = 480 nm, $\lambda_{emission}$ = 567 nm. This material is available free of charge via the Internet at http://pubs.acs.org.

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