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BRIEF COMMUNICATION

Saffron and retinal neurodegenerative diseases: Relevance of chemical composition

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Abstract

Saffron is an ancient spice largely used in traditional medicine. It has been found to be effective in treatment of retinal neurodegenerative diseases like age-related macular degeneration and Stargardt. In the present manuscript, it is shown that saffron's neuroprotective power is strongly related to the bioactivity of all its chemical components. Nuclear magnetic resonance spectroscopy and "in vitro" experiments confirm the relevance of crocins for saffron efficacy. These results underline the importance of strictly defining the chemical composition of the natural compounds in saffron to optimize their effectiveness in the treatment of diseases.

KEYWORDS
"in vitro" test, neuroprotection, NMR, saffron

1 | INTRODUCTION

Saffron is a multifunctional spice in terms of its use in human consumption. Its culinary applications are well known worldwide, and it has been used since very ancient times. A plethora of other applications is also known. It was already adopted in late Ptolemaic Egypt, where saffron was used for textile dying, in aromatic fragrances preparation and in a multitude of medicinal applications. Some studies suggest that saffron was first cultivated in Greece, and used by royals such as Alexander the Great, who used to bathe in saffron infusions to heal faster from battle wounds, as well as Cleopatra, who used it to make her baths more pleasurable.

Written records on the medical uses of saffron date back to the time of Hippocrates and Galen. Nowadays, new medicinal applications have emerged, widening the potentiality of its beneficial effects on human health. Advanced pharmacological studies have, in fact, highlighted its numerous beneficial health effects including cardioprotective, antidepressant, anticarcinogenic, anti-asthma and neuroprotective (see for review Srivastava et al., 2010). Antinociceptive and anti-inflammatory activities were reported from stigmas and petals of saffron (Hosseinzadeh & Younesi, 2002). Additionally, a new role in immunomodulatory and anti-inflammatory activity in Sars-Cov 2 treatment of saffron due to the crocins content have been suggested in managing pre- and post-infection treatments (Husaini et al., 2021; Kordzadeh et al., 2020).

In this manuscript, a focus on saffron neuroprotective activity is reported, particularly in retinal neurodegenerative diseases. Recent studies highlighted its applicability as neuroprotection in age-related macular degeneration (AMD) (Falsini et al., 2010; Maccarone et al., 2008; Marangoni et al., 2013; Piccardi et al., 2012) and Stargardt disease (Piccardi et al., 2019). It has to be noted that saffron treatment is more efficient than the Age-Related Eye Disease Studies protocol in long-term treatment of AMD patients (Bisti et al., 2020), suggesting further complex modes of action not

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only limited to antioxidant activity. Microarray experiments (Natoli et al., 2010) provided evidence that saffron treatment is able to modulate gene expressions counteracting the effects induced by light damage (LD). In addition, saffron modulates a big number of non–coding genes, such as those expressing microRNA which affect the transcription. Moreover, saffron was able to protect retinal photoreceptors from stress by acting on P2X7 receptors (Corso et al., 2016).

These are just some examples of the potential of saffron treatment, which was proven to work at many functional levels (Di Marco et al., 2019; Maccarone et al., 2016; Maggi et al., 2020) suggesting an influence of this spice in increasing tissue resilience (Stone et al., 2018). Original results on AMD patients have been confirmed in other clinical trials (Broadhead et al., 2019; Lashay et al., 2016; Riazi et al., 2017); some cases show less efficacy most likely due to the quality of saffron used. Interestingly, starting from experiments in animal models it was immediately clear that different saffron batches showed different efficacy. However, only parallel experiments, linking data from "in vitro" and "in vivo" experiments and analytical investigations, have been able to disclose the critical chemical differences supporting functional efficacy. Ultimately, it has been established that only a specific chemical composition of saffron resulted to be effective (Di Marco et al., 2019; Maggi et al., 2020).

The chemical composition of saffron is closely linked to its neuroprotective activity, and this has led to the deposition of an international patent, to which a quality mark of saffron is attached (REPRON®). In the present manuscript, qualitative and quantitative evaluation of the metabolite content (spectroscopic) of REPRON® and not-REPRON® saffron samples have been performed putting lights on the "minimum" metabolite requirement to be effective (bioactive).

In addition, the results of tests performed on various cell lines using different saffron samples (with different chemical composition) are discussed.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human neuroblastoma cell line SH-SY5Y purchased from American Type Culture Collection were grown in standard culture medium Ham's F10/Dulbecco's Modified Eagle Medium (DMEM; 1:1) supplemented with 2 mM L-glutamine and 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded in 12-well plates at a density of 12×10^4 cells per well and allowed to attach overnight at 37°C. All culture media components were purchased from Sigma Aldrich.

2.2 | Cells treatment

Cells were incubated with 50 μM $CdCl_2$ for 45 min to 2 h in medium without additional serum. After incubation cells were washed twice with phosphate buffered saline (Sigma) and fresh medium containing serum with and without saffron at a final concentration of 25 or 50 μg/ml. Stigmas of different saffron (REPRON® and not REPRON®) were dissolved in sterile water at concentration of 5 mg/ml, 16–18 h before cell treatment. Pre-treatment with saffron at indicated concentration was done for 1 h at 37°C. Cultures were kept in the incubator for another 48 h. Viable cells were counted in triplicate in each sample using trypan blue exclusion or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Data are the mean ± standard error (*SE*) from at least five experiments. Statistical significance was estimated by unpaired Student's *t*-test.

2.3 | NMR sample preparation

A total of 10 mg of saffron ground stigmas was extracted with deuterated dimethylsulphoxide (DMSO-d_6, 600 μl), stirred (vortex) for 3 min at room temperature. After 10 min, centrifugation at 12,100*g* for 10 min was performed, and 500 μl of the supernatant was used for the nuclear magnetic resonance (NMR) analysis. Standard of kaempferol 3-O-sophoroside (purity 99.7% by high performance liquid chromatography [HPLC]) was purchased by Nature Standard Shanghai Standard Technology Co. Ltd.

2.4 | NMR spectrum acquisition and processing

^{1}H-NMR spectra were recorded on Bruker AV600 spectrometer (Bruker Biospin GmbH) operating at 14.09 T and equipped with a 5-mm inverse probe with z-gradient at 300 K. The spectra were acquired with a spectral width of 10,000 Hz and 32 K data points using the mono-dimensional version of Nuclear Overhauser Effect Spectroscopy (NOESY) sequence. The residual water suppression was achieved by applying a presaturation scheme with low-power radiofrequency irradiation for 1.2 s. A resolution enhancement function with an exponential multiplication of 0.3 Hz for the line broadening was applied; ^{1}H-NMR spectra were carefully phased and baseline-adjusted with the TOPSPIN3.0® software (Bruker BioSpin GmbH, version 1.3). The spectra were referenced to the solvent signal at 2.50 ppm and subjected to manual bucketing in the range of 0.00–10.50 ppm according to the resonance assignment; the bucket normalization was performed with respect to solvent integral value using the ACD/Spec Manager (ACD Labs, version 11). Standard of kaempferol 3-O-sophoroside (purity 99.7% by HPLC) was purchased by Nature Standard Shanghai Standard Technology Co., Ltd.

3 | RESULTS AND DISCUSSION

3.1 | Saffron chemical composition

To investigate the role of saffron in neuroprotective activity, a deep analysis of the metabolite profile is worthwhile. First of all, the term "saffron" is generally an abused word, which has been

adopted sometimes improperly. *Crocus sativus* L. (Iridaceae) is an aromatic plant, and its flower is undoubtedly the most important element. This is constituted by different parts: stigmas (the most interesting one, there are three of them in each flower), tepals (which are enriched in anthocyanins and flavonoids and there are six of them in each flower) and stamens (also named styles or anther, and generally three of them are in each flower). Stigmas are dried to obtain the spice and the adoption of different drying procedures would result in products of different commercial quality (Carmona et al., 2005; Gregory et al., 2005; Pei et al., 2021). The principal chemical components of stigmas are picrocrocin, crocins (the glucoside esters of crocetin) and safranal. These compounds have been already disclosed by several authors, with different analytical methods. This has allowed the determination of different isomers of the main components and different compounds according to the sensitivity/resolution of the applied analytical method used. These components are used to define the commercial quality of saffron according to ISO procedures (ISO3632-1, ISO3632-2-2003, International Organization for Standardization) by measuring their absorbance in aqueous solution at 440 nm (crocins), 257 nm (picrocrocin) and 330 nm (safranal). It has been already pointed out that these procedures have several limitations, one overall is concerned with the limited solubility of safranal in water and the large amount of samples required to complete the ISO determinations.

Safranal is the volatile mono-terpenoid aldehyde (2,6,6-trimeth yl-1,3-cyclohexadien-1-carboxaldehyde) responsible for the aroma of saffron. Safranal is derived from picrocrocin by acidic hydrolysis or by the action of glucosidase, which generates safranal after the hydrolysis process obtained upon heating. Besides safranal, other major constituents of saffron's aroma are 3,5,5-trimethyl-2-cycloh exene-1-one (isophorone), 4-hydroxy-2,6,6-trimethyl1-cyclohexene -1-carboxaldehyde, 3,5,5-trimethyl-3-cyclohexen-1-one (an isomer of isophorone), 2,6,6-trimethyl2-cyclohexene-1,4-dione and 2,6,6 -trimethyl-1,4-cyclohexadiene1-carboxaldehyde (an isomer of safranal). Some authors (Kanasawud & Crouzet, 1990) suggested its presence reasonably derived from the carotenoids degradation due to the combined action of heat and oxygen. Several isomers of safranal have been identified by gas chromatography–mass spectrometry (GC–MS) investigations (Tarantilis & Polissiou, 1997), like 3,5,5 -trimethyl-2-cyclohexen-1-one, namely isophorone; 3,5,5-trimethyl-3-cyclohexen-1-one, isomer of isophorone; 2,6,6-trimethyl-2-cycloh exen-1,4-dione and 2,6,6-trimethyl-1,4-cyclohexadiene-1- carboxaldehyde. Furthermore, few new isomers have been identified as potential precursors of 2,6,6-trimethylcyclohexa-1,3-dienecarbaldehy de (safranal) and its carboxylic derivative (Straubinger et al., 1998). More recently D'Auria et al. (2004) investigated the volatile components of three Italian samples of different origins (Campania, Sardinia, Abruzzo) and one Iranian sample with solid-phase microextraction (SPME)-GC–MS. They revealed 18 new components. Interestingly, the analyses indicated that saffron from different cultivation sites have some peculiarities due to the presence of some unusual components.

Picrocrocin is the second most abundant apocarotenoid of saffron, a mono-terpene glucoside (4-glucopyranosyloxy-2,6,6-trime thyl1-cyclohexene-1-carboxaldehyde) which is responsible for the bitterness. It is derived by oxidative degradation of zeaxantine by means of a carotenase action, leading to crocetin-aldehyde and picrocrocin.

The colouring power of saffron is mainly due to the presence of crocins, which are the most prominent glycosylated derivatives of the C20-dicarboxylic acid crocetin, (8,8′-diapocarotene-8,8′-dicarboxylic acid). The main pigment of saffron was identified by Kuhn in late 1931 as the di-gentiobiosyl ester of crocetin (Kuhn & L'Orsa, 1931). Later on, additional glycosylated carotenoid derivatives have been isolated and characterized with different analytical techniques (Wittwer & Pfander, 1975a, 1975b). Those derivatives were identified as crocetin mono-(β-D-glucosyl) ester, crocetin di-(β-D-glucosyl) ester, crocetin mono-(β-gentiobiosyl) ester and crocetin (β-D-glucosyl) (β-gentiobiosyl) ester. Additionally, 13Z-crocin has been isolated from saffron and characterized by spectroscopic methods (ultraviolet/visible [UV/vis], NMR) and elemental analysis Speranza et al. (1984). In more recent decades, through the use of more sophisticated and sensitivity improved techniques, new glycosylated crocetins have been characterized (Carmona et al., 2006). In particular *trans* and *cis* triglucoside, neapolitanoside derivatives have been revealed in addition to gentiobioside and glucoside derivatives (Tarantilis et al., 1994; Tarantilis et al., 1995), for a total amount of 16 esters, also in comparison with crocetin esters observed in *Gadenia Jasminoides* Ellis fruits.

According to Sánchez et al. (2008) and Assimiadis et al. (1998) the major crocetin esters present in *C. sativus* stigma are, in decreasing amount, *trans* crocetin di-(β-D-gentiobiosyl) ester (T1) accounting for more than 60% of the total crocins content in aqueous extracts, *trans*-crocetin (β-D-gentiobiosyl) (β-D-glucosyl) ester (T2), *cis*-crocetin di-(β-D-gentiobiosyl) ester (C1) and *cis*-crocetin (β-D-gentiobiosyl) (β-D-glucosyl) ester (C2). The first three crocins account for more than 95% of the total crocetin esters.

The relative percentage of these compounds resulted to be strongly dependent from the origin, with a general amount of 10% in dry saffron for crocins, 4% for picrocrocin and safranal representing 70% of the volatile fraction.

The chemical composition of saffron, as shown in previous studies, is strictly correlated to some bioactive properties of this spice. The chemical analysis (qualitative and quantitative) of a large number of saffron samples, carried out in parallel with the treatment of animal models, showed that the neuroprotective activity of this spice depended on the content of some of its active compounds, in particular on the concentration of the two most abundant crocins (*trans*-crocetin di-(β-D-gentiobiosyl) ester (T1) and *trans*-crocetin (β-D-gentiobiosyl) (β-D-glucosyl) ester (T2)). The concentration of the two crocins was expressed as milligram of crocin contained in 100 g of dry spice (Sánchez et al., 2008). This allowed us to identify two threshold values of concentration of the two crocins (17% mg/g T1 and 8% mg/g T2) below which saffron has no neuroprotective activity. These results allowed to file an international patent, to which a

quality mark of saffron is attached (REPRON®) (see figure 2 in Maggi et al., 2020).

Taking into account the composition of the main metabolites, the use of NMR spectroscopy would lead to the evaluation of all the different metabolites detectable in extracts of stigmas, in their relative abundance. In this view, DMSO extracts have been evaluated for the quantification of the identified metabolites, including glycoside esters.

The analysis of ^{1}H-NMR spectra of REPRON® and not REPRON® samples, revealed several differences in compounds abundance, according to previous assignments (Cagliani et al., 2015). Among those, the most relevant compounds have been quantified and represented in Figure 1. These compounds are glycosylated flavonoid (kaempferol), kaempferol 3-O-S being the largest abundant form (resonance assignment confirmed on the basis of the standard compound) and kaempferol 3-O-S,7-O-G also present with a lower abundance, as described by Carmona et al. (2006). *Trans* and *cis* crocins can also be detected unambiguously, as well as picrocrocin, thanks to selective NMR signals described in the figure caption. These three selected compounds revealed to be largely present in REPRON® with respect to not-REPRON® samples.

3.2 | Saffron effects on cell lines

Among saffron constituents, crocins and crocetin showed high radical scavenging activities followed by safranal. Numerous studies "in vitro" describe the benefit of crocins treatment against oxidative stress. In microglia cells, the oxidative stress is always related to diabetic retinopathy (DR) and is inhibited by crocins treatment through the activation of the phosphoinositide 3-kinases/Akt signaling pathway (Yang et al., 2017). Oxidative stress is also one of the major factor that induces the apoptosis of retinal ganglion cells that can lead to glaucoma. Lv et al. (2016) found that crocins prevent H_2O_2-induced damage to mitochondrial pathway and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). In addition, safranal decreases retinal degeneration in the P23H rat model of autosomal dominant retinitis pigmentosa (Fernández-Sánchez et al., 2012).

The quality mark of saffron (REPRON®) is related to its chemical composition and in particular to the amount of crocins. We decided to test the neuroprotective activity of REPRON® compared to not-REPRON® saffron on the human neuroblastoma cell line SH-SY5Y.

Cells were stressed with the neurotoxic agent cadmium. In neuronal cells, cadmium induces oxidative stress, which produces protein damage and subsequently neurodegeneration (Figueiredo-Pereria et al., 1998; Williams, 1995). Cadmium is known to enhance the production of free radicals in the brain which may potentially damage both neurons and oligodendrocytes (Hossain et al., 2009) and interferes with the cellular defence mechanism against oxidation (Nair et al., 2013).

SH-SY5Y cell line was exposed to $CdCl_2$ under two different experimental conditions and cell viability was assessed using the trypan blue dye or MTT assay. One set of experiments was carried out treating cell cultures with saffron before the addition of $CdCl_2$ and the other one after the administration of $CdCl_2$. Since cadmium concentrations between 25 and 100 μM induce mainly apoptotic mechanism of cell death (Nair et al., 2013), neuroblastoma cells were incubated with 50 μM of $CdCl_2$ for 45, 60, 90 and 120 min in a serum-free medium and cell viability was assessed after 2 days recovery period in the standard medium with or without saffron. As illustrated in Figure 2a cell survival in presence of 50 μM $CdCl_2$ decreases with the increase of exposure (from 50% to 10% with respect to the control cultures): 45 min treatment with Cd in a serum-free medium was already sufficient to induce the death of half of the cells, while after 2 h of incubation, cell mortality increased up to 92%. Moreover, it has been observed (Figure 2) that treatment of SH-SY5Y cells with 25 μg/ml of saffron REPRON® for 2 days, significantly prevented cell death caused by cadmium. Also in this case, the protective effect of saffron depended on the duration of the stress treatment, becoming ineffective at incubation time >120 min. Saffron pre-treatment also resulted in increased cell viability; however, the effect was less effective than saffron post-treatment and was statistically significant only for a short period of time of cadmium incubation.

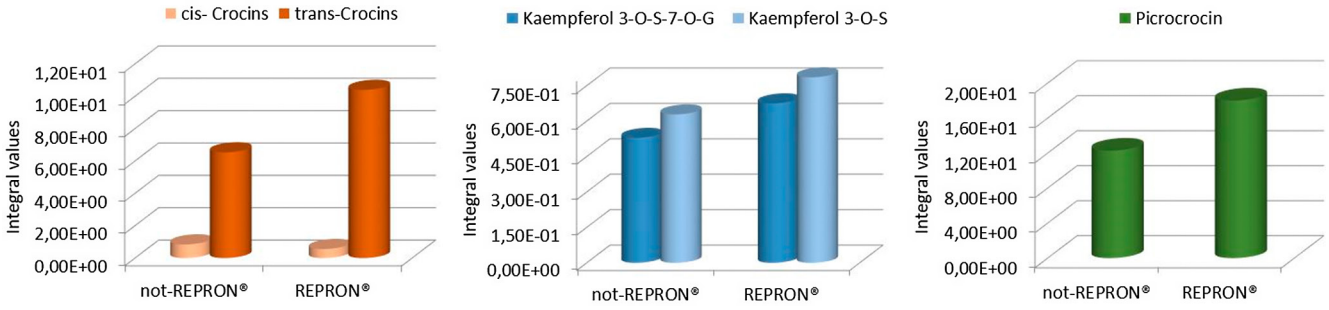


FIGURE 1 NMR quantification (integral values referred to solvent signal) of cis-crocins, trans-crocins, kaempferol 3-O-Sophoroside (kaempferol 3-O-S), kaempferol 3-O-Sophoroside 7-O-Glucoside (kaempferol 3-O-S,7-O-G) and picrocrocin identified in DMSO extracts of REPRON® and not-REPRON® saffron samples. Representative NMR signals for each metabolite have been considered: 7.46–7.51 ppm (cis-crocins), 7.29–7.40 ppm (trans-crocins), 8.02–8.05 ppm (kaempferol 3-O-S), 8.05–8.09 ppm (kaempferol 3-O-S,7-O-G), and 1.17–1.20 ppm (picrocrocin). DMSO, dimethyl sulphoxide; NMR, nuclear magnetic resonance.

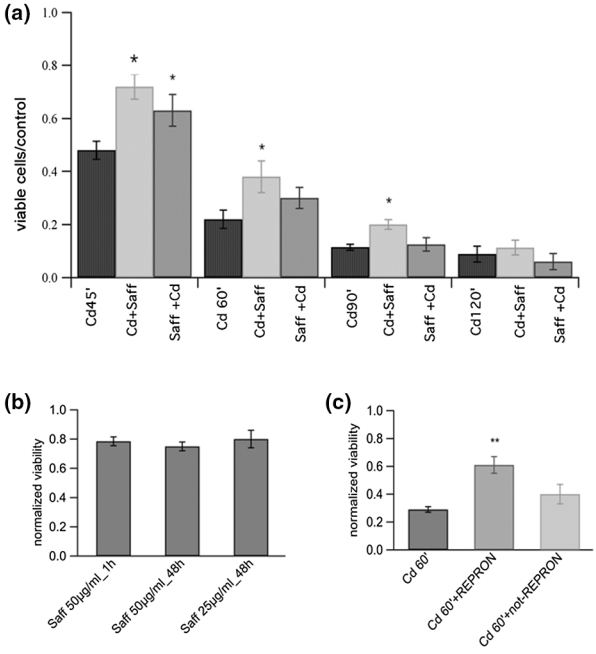
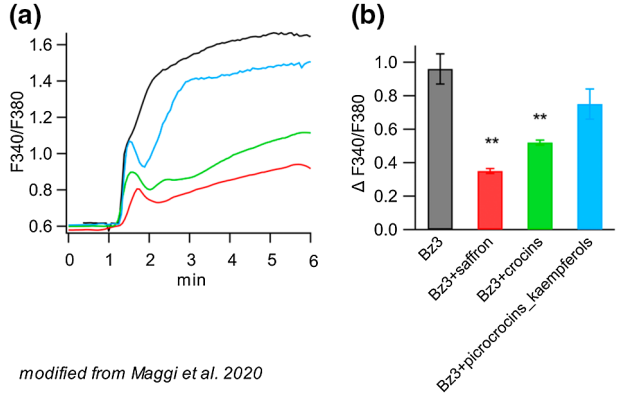


FIGURE 2 The effects of saffron on Cd-induced toxicity on SH-SY5Y neurons. (a) Cells were treated with Cd 50 µM. An amount of 25 µg/ml of saffron was applied before and together with Cd treatment. Cell viability was determined in triplicate for each sample using trypan blue exclusion or MTT assay 48 h after Cd removal. Saffron pre-treatment time was 1 h. Experimental values are the mean ± *SE* from at least five experiments. Statistical analysis was performed with unpaired Student's *t*-test (*) $p < 0.05$, (**) $p < 0.01$. (b) Apoptotic saffron effect on SH-SY5Y neuroblastoma. Cells were treated with 25 and 50 µg/ml of saffron for 2 days and with 50 µg/ml for 1 h. (c) Effect of REPRON® and not-REPRON® saffron on Cd-induced toxicity. Experimental conditions as in (a). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.



modified from Maggi et al. 2020

FIGURE 3 Effect of saffron components on the BzATP-induced internal calcium increase. (a) Representative traces of the fluorescence ratio, indicative of internal calcium variation, in response to application of 3 µM BzATP alone (black trace) or to application of BzATP plus 25 µg/ml saffron (red trace), 25 µg/ml crocins (green trace), and 25 µg/ml picrocrocins kaempferols (blue trace). In each experiment, saffron and both fractions were applied 5 min before the application of BzATP indicated by arrow. (b) The histogram reports the mean ± *SE* of the observed internal calcium variation ((**) $p < 0.01$).

Control experiments on saffron's effect on SH-SY5Y neuroblastoma cells were also done. Cells not stressed with cadmium were exposed to different concentrations of saffron for the whole experiment (48 h). The results show an unexpected apoptotic effect of saffron on neuroblastoma cells (Figure 2b). Cell viability decreases by about 20%–25% in the presence of 25 µg/ml of the aqueous saffron extract. As observed from the figure, the action of saffron is dose- and time-independent: with the increasing concentration of saffron the decrease in cell viability is negligible, being effective within 1 h. This apoptotic effect on neuroblastoma cells may probably be linked to the anticancer activity of saffron as it has been suggested as a natural agent in cancer therapy.

To investigate if the neuroprotective action of saffron could be related to its particular composition we compared the effect obtained in the presence of REPRON® saffron with that obtained with not-REPRON® saffron. Cells were incubated for 1 h with 50 µM of $CdCl_2$ and 25 µg/ml of both type of saffron. Cell viability was assessed after 2 days from treatment. As observed from Figure 2c REPRON saffron was more effective in comparison to other saffron: cell viability increased from 30% to 60% and only to 40% with other saffron.

However, the neuroprotective activity of saffron is not only due to the characteristic of its carotenoid content but it appears to be related to a synergistic activity of all chemical components and involves complex modes of actions. Some years ago our group found a new mechanism for the beneficial action of saffron that involves the purinergic receptor P2X7 (Corso et al., 2016). P2X7R belongs to the family of the P2X cation channels which are activated by extracellular ATP (Surprenant et al., 1996). Among these channels P2X7R has the unique feature of being activated by millimolar concentration of ATP, and therefore it plays a key role in many pathophysiological processes when large amounts of ATP are produced (Volonte et al., 2012). Following ATP gating, distinct signalling pathways are triggered, such as the secretion of pro-inflammatory cytokines or modulation of cell death. P2X7R has been always associated with multiple pathologies, including chronic inflammation, cancer, neuropathic pain and neurodegeneration (Pannicke et al., 2000). In the retina this receptor is widely expressed in Muller glia as well as retinal ganglion cells (Mitchell et al., 2009) and photoreceptor cells (Puthussery & Fletcher, 2004). Activation of P2X7R induces apoptosis of retinal ganglion cells (Zhang et al., 2005). Notomi et al. (2013) suggested that photoreceptors cells apoptosis induced by an excess of extracellular ATP involves P2X7R activation with caspase-8, -9 cleavage and mitochondrio-nuclear translocation of apoptosis-inducing factor. It is not surprising then, that P2X7R has become a particularly relevant therapeutic target to treat retinal diseases (Fletcher et al., 2019).

When we tested REPRON® saffron on mice primary retinal culture and on the photoreceptor 661W cell line, we observed that saffron was able to reduce ATP-induced cytotoxicity. A sustained activation of the P2X7Rs by high levels of ATP or by the agonist Bz-ATP induces calcium entry into the cells which can reach dramatic levels

leading the cells to apoptosis. We found that saffron decreased calcium influx induced by P2X7R activation into photoreceptor-derived 661W cells. Moreover, saffron treatment reduced apoptosis of photoreceptor cells.

Recently, in order to investigate the interaction of P2X7R with saffron main constituents, saffron extract was separated into two fractions, one containing kaempferol derivatives and picrocrocin and the other containing crocins (Maggi et al., 2020). Both fractions tested on P2X7 receptor and gave different results: crocins were still able to significantly reduce calcium entry into the cell while the fraction containing kaempferol derivatives and picrocrocin did not (Figure 3). Cytotoxicity also decreased with crocins treatment. However, neuroprotection exerted by crocins resulted in less efficacy than that induced by the entire saffron extract, confirming the fact that often the beneficial activity of saffron is related to the synergistic activity of its essential substances. Similar results are confirmed in animal model (Bisti et al., 2020).

The use of saffron, therefore, can be a valuable aid in the treatment of pathologies involving P2X7R. Targeting P2X7R in the retina is reported to have potential in the treatment of glaucoma, DR and AMD (Fletcher et al., 2019; Tassetto et al., 2021; Yang, 2017), by blocking NLRP3 inflammasome and preventing interleukin (IL)-1β and IL-18 release. Further, the important role of P2X7R in the immune system as an important regulator of T cell functions opens a new perspective on the use of saffron in immunological diseases.

3.3 | General considerations

Saffron was originally tested in an animal model where retinal neurodegeneration was induced by exposing albino rats to high intensity light (Maccarone et al., 2008). The starting hypothesis was that the carotenoid derivatives (mainly crocins) contained in saffron, were able to reduce the oxidative stress induced by membrane lipid peroxidation, thanks to their radical scavenger propensity. In parallel β-carotene from *Dunaliella salina* was tested. Both the treatments were able to reduce neuronal death, but saffron was also able to maintain retinal function. Interestingly, saffron modulated the activation of self protective mechanisms, like fibroblast growth factor 2 (Fgf2), which on one side reduces neuronal death but on the other blocks the transfer of visual information from photoreceptors to second order neurons. This initial observation led to the idea to perform microarray experiments in retinas of treated and untreated rats (Natoli et al., 2010). It became clear that saffron was able to modulate gene expression and a big number of non–coding genes, probably microRNA, which are able to affect the transcription. This could be the explanation of the original results showing that while LD increases the synthesis and release of Fgf2 proteins in the outer nuclear layer (ONL), saffron does not modulate Fgf 2 gene but blocks the final process. Thus, when animals with retinal neuro degenerative-induced disease are treated with saffron, they showed no Fgf2 protein in the ONL and a partially preserved visual response. This was the first example of the potentiality of saffron treatment.

Subsequently, based on the results obtained by looking at gene modulation (Natoli et al., 2010), a series of experiments have been planned to verify possible ways of action. Many pathways appear to be involved, from the maintenance of the extracellular matrix to modulation of microglia migration, Muller cells activity and release of pro-inflammatory cytokines (Bisti et al., 2020; Di Marco et al., 2013; Di Marco et al., 2014; Di Marco et al., 2019). In addition several membrane receptors are directly modulated by saffron (Corso et al., 2016; Maccarone et al., 2016). Altogether experimental results suggest an integrated activity of saffron components able to modulate neuro inflammation.

The relevant role of saffron treatment in coping with retinal neurodegenerative diseases related to oxidative stress and neuroinflammation has been documented in double blind-placebo control clinical trials in AMD (Falsini et al., 2010; Lashay et al., 2016) and Stargardt (Piccardi et al., 2019) disease. Long-term effects have been documented (Piccardi et al., 2012) together with the potentiality of saffron treatment in patients with risk genotypes (Marangoni et al., 2013). Many reviews reported critical analysis of the clinical and preclinical data obtained (Bosch-Morell et al., 2020; Heitmar et al., 2019). A second point rose from initial experiments: how critical is the chemical composition of saffron to optimize treatment and obtain better and reproducible results. To exploit this point, parallel experiments have been performed: saffron treatment in neuro-degenerating retinas and analytical chemical analysis of saffron used (see figure 2 in Maggi et al., 2020). Experimental evidence confirmed that by doubling the dose of not-REPRON® saffron, the concentrations of active principles (from which the neuroprotective activity of the spice depends) did not reach the threshold value defined in REPRON® (ratio among components remains invariant). High quality not-REPRON® saffron have neuroprotective activity but it does not reach the efficacy of REPRON® even at higher dose. Increasing the dose might not help, moreover natural substances might become toxic when administered in very high quantities.

4 | CONCLUSION

Experimental results reported in this manuscript confirm that chemical composition is critical for saffron efficacy in neuroprotection and all the components appear essential to reach the best protection (Bisti et al., 2020; Maggi et al., 2020) and increased tissue resilience (Stone et al., 2018). Altogether, all data obtained confirm the driving idea that testing natural compounds which include many molecules has to be done in parallel by different disciplines to precisely define the chemical characteristics of the active compound to optimize the results and offer patients a well-characterized and most effective product.

AUTHOR CONTRIBUTIONS

Conceptualization: Maria A. Maggi, Roberto Consonni, Laura R. Cagliani, Silvia Bisti, Cristiana Picco. Investigation: Maria A. Maggi, Roberto Consonni, Laura R. Cagliani, Gianfranco Prestipino, Silvia

Bisti, Cristiana Picco. Acquisition of data: Maria A. Maggi, Roberto Consonni, Laura R. Cagliani, Cristiana Picco: Data interpretation: Maria A. Maggi, Roberto Consonni, Laura R. Cagliani, Silvia Bisti, Cristiana Picco. Drafting of the manuscript Maria A. Maggi, Roberto Consonni, Laura R. Cagliani, Gianfranco Prestipino, Silvia Bisti, Cristiana Picco. Critical revision of the manuscript: Maria A. Maggi, Roberto Consonni, Laura R. Cagliani, Silvia Bisti, Cristiana Picco.

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CONFLICT OF INTEREST

S. Bisti and M. A. Maggi are inventors of the following international patent.: "Compositions based on saffron for the prevention and/or treatment of degenerative eye disorders", 2015 (W02015/145316) and is owned by Hortus Novus srl, to which is linked a mark (Repron TM) that attests the quality of ophthalmic saffron. S. B. holds a non-remunerative relationship with Hortus Novus srl.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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