TRPA1 mediates the antinociceptive properties of the constituent of *Crocus sativus* L., safranal

Simone Li Puma | Lorenzo Landini | Sergio J. Macedo Jr | Viola Seravalli
Ilaria M. Marone | Elisabetta Coppi | Riccardo Patacchini | Pierangelo Geppetti
Serena Materazzi | Romina Nassini | Francesco De Logu

1Department of Health Sciences, Section of Clinical Pharmacology and Oncology, University of Florence, Florence, Italy
2Department of Pharmacology, Federal University of Santa Catarina, Florianópolis, Brazil
3Department of Health Sciences, Section of Paediatrics, Midwifery, Gynaecology and Nursing, University of Florence, Florence, Italy
4Department of Neuroscience, Psychology, Drug Research and Child Health, Section of Pharmacology and Toxicology, University of Florence, Florence, Italy
5Department of Pharmacology, Chiesi Farmaceutici SpA, Parma, Italy

Correspondence
Romina Nassini, Department of Health Sciences, University of Florence, Florence, Italy.
Email: romina.nassini@unifi.it

Funding information
Ministry for University and Scientific Research (MIUR) Rome, Italy, Grant/Award Number: PRIN 201532AHAE_003; Regione Toscana

Abstract
Safranal, contained in *Crocus sativus* L., exerts anti-inflammatory and analgesic effects. However, the underlying mechanisms for such effects are poorly understood. We explored whether safranal targets the transient receptor potential ankyrin 1 (TRPA1) channel, which in nociceptors mediates pain signals. Safranal by binding to specific cysteine/lysine residues, stimulates TRPA1, but not the TRP vanilloid 1 and 4 channels (TRPV1 and TRPV4), evoking calcium responses and currents in human cells and rat and mouse dorsal root ganglion (DRG) neurons. Genetic deletion or pharmacological blockade of TRPA1 attenuated safranal-evoked release of calcitonin gene-related peptide (CGRP) from rat and mouse dorsal spinal cord, and acute nociception in mice. Safranal contracted rat urinary bladder isolated strips in a TRPA1-dependent manner, behaving as a partial agonist. After exposure to safranal the ability of allyl isothiocyanate (TRPA1 agonist), but not that of capsaicin (TRPV1 agonist) or GSK1016790A (TRPV4 agonist), to evoke currents in DRG neurons, contraction of urinary bladder strips and CGRP release from spinal cord slices in rats, and acute nociception in mice underwent desensitization. As previously shown for other herbal extracts, including petasites or parthenolide, safranal might exert analgesic properties by partial agonism and selective desensitization of the TRPA1 channel.

KEYWORDS
calcitonin gene-related peptide, neurogenic inflammation, pain, safranal, transient receptor potential ankyrin 1

1 INTRODUCTION

*Crocus sativus* L., known as saffron crocus, belongs to the family of *Iridaceae* and is commonly used for flavouring and colouring food preparations. Saffron extracts contain three main bioactive constituents: the carotenoid crocin, responsible for its typical colour, the monoterpene aldehyde picrocrocin, and the volatile compound safranal, which accounts for its special flavour. Saffron has been reported to possess beneficial effects against depression, sexual dysfunction, premenstrual syndrome and weight loss. Although clinical trials reported headache as one possible adverse effect of saffron, in Indian traditional medicine saffron has been...
used to treat headache. Preclinical studies focused on the pharmacological activity of saffron and its purified constituents, suggesting antioxidan
ty, anti-inflammatory and antinociceptive properties of the golden spice. Antinociceptive effect of safranal has been demonstrated in models of inflammatory pain, such as formalin, acetic acid or carrageenan, and in models of neuropathic pain, including chronic constriction injury and nerve crush injury. However, the underlying mechanisms responsible for the analgesic action of saffron have not been yet elucidated.

Transient receptor potential (TRP) channels are pleiotropic excitatory ion channels present in a large variety of cells. Some TRPs are highly expressed by specific subpopulations of primary sensory nociceptive neurons, where they contribute to sensing noxious chemical, mechanical and thermal stimuli. In particular, the TRP ankyrin 1 (TRPA1) is coexpressed with the vanilloid type 1 (TRPV1, the capsaicin receptor), or the vanilloid type 4 (TRPV4) by a subpopulation of nociceptors, which contain and release the proinflammatory neuropeptide calcitonin gene-related peptide (CGRP) and substance P (SP). CGRP and SP released from peripheral endings of primary afferents mediate neurogenic inflammation and CGRP is now recognized to play an essential role in migraine pain. TRPA1 is activated by a number of exogenous irritant and pungent agents, including allyl isothiocyanate (AITC), cinnamaldehyde and formalin. Notably, reactive oxygen, nitrogen and carbon species gate TRPA1 by reacting with specific cysteine/lysine residues, thus mediating pain and neurogenic inflammation.

Here, we evaluated whether the three main constituents of saffron affect the function of TRP channels expressed in nociceptors, and in particular that of TRPA1. We found that safranal and its precursor, picrocrocin, but not crocin, selectively activate TRPA1, but not TRPV1 or TRPV4, safranal being far more effective than picrocrocin. In addition, safranal behaves as a partial agonist on TRPA1 as compared to the full agonist, AITC. Finally, safranal induces selective desensitization of the TRPA1 channel, thus attenuating neuronal excitation that results in nociception and CGRP release. This unforeseen TRPA1-desensitization mechanism could possibly explain the analgesic effects attributed to saffron.

2 MATERIALS AND METHODS

2.1 Animals

In vivo experiments and tissue collection were carried out according to the European Union (EU) directive guidelines (2010/63/EU) and Italian legislation (DLgs 26/2014) for animal care procedures. Studies were conducted under the University of Florence research permit #194/2015-PR. Animals were housed in a temperature- and humidity-controlled vivarium (12-hour dark/light cycle, free access to food and water) and were allowed to acclimatize for at least 7 days prior to experimentation. Animal studies were reported in compliance with the ARRIVE guidelines. A group size of six animals for behavioural experiments was determined by sample size estimation using G’Power (v3.1) to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20% respectively. Allocation concealment was performed using a randomization procedure (http://www.randomization.org).

The following mouse strains were used: C57BL/6 mice (male, 20-25 g; 5 weeks; Envigo, Milan, Italy); littermate TRPA1 wild-type (Trpa1+/+) and TRPA1-deficient (Trpa1−/−) mice (25-30 g, 5-8 weeks) generated by heterozygotes on a C57BL/6 background (B6.129P-Trpa1<sup>tm1Kyok</sup>/J, Jackson Laboratories, Bar Harbor, ME, USA); wild-type (Trpv4<sup>+/+</sup>) and TRPV4-deficient (Trpv4<sup>−/−</sup>) mice (25-30 g, 5-8 weeks), generated by heterozygotes on a C57BL/6 background and TRPV1-deficient mice (Trpv1<sup>−/−</sup>; B6.129X1-Trpv1<sup>tm1Ad</sup>/J) backcrossed with C57BL/6 mice (Trpv1<sup>−/−</sup>) for at least 10 generations (Jackson Laboratories; 25-30 g, 5-8 weeks). Sprague-Dawley rats (male, 75-100 g, Envigo) were also used. Animals were killed with inhaled CO₂ plus 10%-50% O₂.

2.2 Cell culture

Naïve untransfected HEK293 cells (American Type Culture Collection, Manassas, VA, USA; ATCC® CRL-1573®) were cultured according to the manufacturer’s instructions. HEK293 cells were transiently transfected with the cDNAs (1 µg) coding for wild-type (wt-hTRPA1) or mutant 3C-hTRPA1 (C619S, C639S, C663S, K708Q; 3C-k-Q hTRPA1-HEK293) using the jetPRIME transfection reagent (Polyplus-transfection®, SA, Strasbourg, France) following the manufacturer’s protocol. HEK293 cells stably transfected with cDNA for human TRPA1 (hTRPA1-HEK293), or with cDNA for human TRPV1 (hTRPV1-HEK293), or with cDNA for human TRPV4 (hTRPV4-HEK293) were cultured as previously described. Human foetal lung fibroblasts (IMR90; American Type Culture Collection; ATCC® CCL-186®), which express the native TRPA1 channel, were cultured as previously described. Cells were plated on glass-coated (poly-L-lysine, 8.3 µmol/L) coverslips and cultured for 1-2 days before being used for recordings.

2.3 Isolation of primary sensory neurons

Primary dorsal root ganglion (DRG) neurons were isolated from adult Sprague-Dawley rats and Trpa1<sup>−/−</sup> and Trpa1<sup>+/+</sup> mice, and cultured as previously described. Briefly, DRG were bilaterally excised and inhaled CO₂ plus 10%-50% O₂. Animals were killed with inhaled CO₂ plus 10%-50% O₂.
Neurons were then plated on glass coverslips coated with poly-L-lysine (8.3 μmol/L) and laminin (5 μmol/L).

### 2.4 Cellular recordings

Mobilization of intracellular calcium ([Ca^{2+}]_i) was measured in untransfected or transfected HEK293 cells, in IMR90 and in DRG neurons. Cells on coated coverslips were loaded with 5 μmol/L Fura-2 AM-ester (Alexis Biochemicals, Lausen, Switzerland) added to the extracellular solution (37°C) containing the following (in mmol/L): 2 CaCl₂, 5.4 KCl, 0.4 MgSO₄, 135 NaCl, 10 D-glucose, 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After 40 minutes loading, coverslips with cells were washed and then transferred to a chamber on the stage of an Olympus IX81 microscope for recording. Cells were excited alternatively at 340 and 380 nm and recorded with a dynamic image analysis system (Xcellence Imaging software; Olympus srl, Milan, Italy). Results were expressed as the percentage increase in R_{340/380} over the baseline (% Change in R_{340/380}), and each effect was normalized to the patch clamp recordings, coverslips with cells were transferred to a recording chamber (1 mL volume), mounted on the platform of an inverted microscope (Olympus CKX41) and superfused at a flow rate of 2 mL/min with a standard extracellular solution at pH 7.4 (adjusted with NaOH) containing (in mmol/L): 10 HEPES, 10 D-glucose, 147 NaCl, 4 KCl, 1 MgCl₂, and 2 CaCl₂. Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) were pulled with a Sutter Instrument puller (model P-87) to a final tip resistance of 4-7 MΩ. Pipette solution used for HEK293 cells contained (in mmol/L): 134 K-glutamate, 10 KCl, 11 EGTA, 10 HEPES (pH adjusted to 7.4 with KOH). When recordings were performed on rat DRG neurons, the extracellular solution contained 5 mmol/L CaCl₂, and pipette solution contained (in mmol/L): 120 CsCl, 3 Mg₂ATP, 10 BAPTA, 10 HEPES-Na (pH adjusted to 7.4 with CsOH). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), stored and analyzed with a pClamp 9.2 software (Axon Instruments). All the experiments were carried out at room temperature (20-22°C). Currents were detected as inward currents activated on cell superfusion with the various stimuli in the voltage-clamp mode (holding potential of −60 mV). Cell membrane capacitance was calculated in each cell throughout the experiment by integrating the capacitive currents elicited by a ±10 mV voltage pulse. Peak currents were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results. Signals were sampled at 1 kHz and low-pass filtered at 10 kHz.

Cells and neurons were challenged with safranal (0.1-300 μmol/L), picrocrocin (1-300 μmol/L) and crocin (30-200 μmol/L). Allyl-isothiocyanate (AITC, 1-10 μmol/L) and GSK1016790A (0.05-0.1 μmol/L) were used to induce TRPA1 and a TRPV4 selective response respectively. Capsaicin (0.1-1 μmol/L) was used to induce a TRPV1 selective response and to identify capsaicin-sensitive neurons. Buffer solution containing dimethyl sulfoxide (DMSO, 1%) was used as vehicle. The activating peptide for human proteinase-activated receptor 2 (hPAR2-AP; 100 μmol/L) or KCl (40-80 mmol/L) were used to elicit a TRP-independent cellular response. Some experiments were performed in the presence of TRPA1, TRPV1 and TRPV4 selective antagonists, HC-030031 (50 μmol/L), capsazepine (10 μmol/L) and HC-067047 (30 μmol/L) respectively, or their vehicles (0.5% or 0.1% or 0.3% DMSO respectively).

### 2.5 Organ bath assay

Rat urinary bladder was excised from rat, and longitudinal strips were suspended at a resting tension of 1 g in 10-mL organ bath bathed in aerated (95% O₂ and 5% CO₂) Krebs-Henseleit solution maintained at 37°C containing (in mmol/L): 119 NaCl, 25 NaHCO₃, 1.2 KH₂PO₄, 1.5 MgSO₄, 2.5 CaCl₂, 4.7 KCl and 11 D-glucose. After 40 minutes of equilibration, tissues were challenged twice with capsaicin (CCh, 1 μmol/L), with a 45-minute washing out period between the two administrations. Motor activity was recorded on a force transducer isometrically (Harvard Apparatus, Ltd, Kent, UK).

Tissues were challenged with safranal (10-300 μmol/L), AITC (100 μmol/L), GSK1016790A (10 μmol/L) and capsaicin (0.3 μmol/L) or their vehicles. In some experiments, tissues were pre-exposed to HC-030031 (50 μmol/L), capsazepine (10 μmol/L), HC-067047 (30 μmol/L) or a combination of NK1 and NK2 receptor antagonists, L-733,060 and SR48968 respectively (both 1 μmol/L). Some preparations were desensitized by treatment with a high concentration of capsaicin (10 μmol/L for 20 minutes, twice) or were exposed to safranal (300 μmol/L for 20 minutes, twice) before the challenge with other stimuli. Responses were expressed as percentage (%) of the maximum contraction, induced by CCh (1 μmol/L).

### 2.6 CGRP-like immunoreactivity assay

For CGRP-like immunoreactivity (CGRP-LI) outflow, 0.4-mm slices of rat or mouse spinal cord were superfused with an aerated (95% O₂ and 5% CO₂) Krebs-Henseleit solution modified with 0.1% bovine serum albumin plus the angiotensin-converting enzyme inhibitor, captopril (1 μmol/L) and the neutral endopeptidase inhibitor, phosphoramidon (1 μmol/L) to minimize peptide degradation. Fractions (4 mL) of superfusate were collected at 10-minute intervals before, during and after administration of the stimuli and then freeze-dried, reconstituted with assay buffer, and analysed for CGRP-LI using a commercial enzyme-linked immunosorbent assay kit (Bertin Pharma, Montigny le Bretonneux, France). Detection limits of the assays were 5 pg/mL. Stimuli did not cross-react with CGRP antiserum. Tissues were stimulated with safranal (10-300 μmol/L) or its vehicle (1% DMSO). Some tissues were pre-exposed to capsaicin (10 μmol/L, 30 minutes) or superfused with a calcium-free buffer containing EDTA (1 mmol/L). Some preparations were pre-exposed to TRPA1 antagonist, HC-030031 (50 μmol/L), capsazepine (10 μmol/L) or HC-067047 (30 μmol/L). Other tissues were pre-exposed to safranal (100 μmol/L, 30 minutes) and then, after a prolonged washing (40 minutes), stimulated with AITC (50 μmol/L) capsaicin (0.1 μmol/L) or GSK1016790A (10 μmol/L). Results were expressed as femtograms of peptide per gram of tissue.
2.7 | Acute nociceptive response

The acute nociception was assessed in C57BL/6, Trpa1+/− and Trpa1−/−, Trpv1+/− and Trpv1−/−, or Trpv4+/− and Trpv4−/− mice, after intraplantar (i.pl.) injection (20 μL/paw) of safranal (0.2-20 nmol), AITC (10 nmol), capsaicin (0.2 nmol), GSK1016790A (2 nmol) or their vehicle (7% and 0.5% DMSO). Immediately after injection, mice were placed individually in plexiglas chambers and the amount of time (seconds) spent licking and shaking the injected paw was recorded for a 5-minute time period, as previously described.31 Nociception induced by safranal (20 nmol) was also evaluated 60 minutes after safranal i.g. administration. AITC (10 nmol), capsaicin (0.2 nmol), GSK1016790A (2 nmol) or their vehicle (7% and 0.5% DMSO). Immediately after injection, mice were placed individually in plexiglas chambers and the amount of time (seconds) spent licking and shaking the injected paw was recorded for a 5-minute time period, as previously described.31 Nociception induced by safranal (20 nmol) was also evaluated 60 minutes after intraperitoneal (i.p.) treatment with HC-030031 (100 mg/kg) or 30 minutes after capsazepine (4 mg/kg) or HC-067047 (10 mg/kg) or their vehicle (all, 4% DMSO plus 4% tween 80 in isotonic saline, 0.9% NaCl). In another experimental setting, safranal (0.5-1 mg/kg, i.g.) was administered each day for 5 consecutive days.8 Each day, 60 minutes after safranal i.g. administration, AITC (10 nmol), capsaicin (0.2 nmol) or GSK1016790A (2 nmol) or their vehicles (0.5% DMSO) were administered (20 μL, i.p.) and acute nociceptive response was recorded.

2.8 | Drugs and reagents

HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-(4-isopropylphenyl) acetamide] was synthesized as previously described.32 If not otherwise indicated, all other reagents were obtained from Sigma-Aldrich (Milan, Italy).

2.9 | Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student’s t test for comparisons between two groups and the one-way ANOVA, followed by the post-hoc Bonferroni’s test for comparisons of multiple groups (GraphPad Prism version 5.00, San Diego, CA, USA). A P < 0.05 was considered statistically significant. Data are reported as mean ± SEM.

3 | RESULTS

3.1 | Safranal and picrocrocin, but not crocin, selectively activate the human TRPA1 channel

Safranal and picrocrocin evoked a concentration-dependent calcium response in hTRPA1-HEK293 (EC50 17 ± 0.3 μmol/L and 56 ± 0.3 μmol/L respectively), but not in naïve HEK293 cells (Figure 1B). The selective TRPA1 antagonist HC-030031 attenuated the response evoked by safranal and picrocrocin and AITC (Figure 1B). Crocin, the third main constituent of saffron was not investigated in this test because of its intense yellow colour which interferes with the recording system. Thus, the ability of crocin to target TRPA1 was analysed by whole-cell patch-clamp electrophysiology. Crocin did not evoke any measurable inward current in hTRPA1-HEK293, compared to its vehicle (Figure 1C).

Further investigation was limited to safranal, the most potent of the three compounds. The non-electrophilic agonist, menthol, evoked a robust calcium response in 3CK-Q hTRPA1-HEK293 cells, which express a mutant form of the TRPA1 receptor, lacking three key cysteine (C619, C639, C663) and one lysine (K708) residues, which are required for channel activation by electrophilic agonists23,33 (Figure 1D). However, safranal failed to induce any calcium response in 3CK-Q hTRPA1-HEK293 cells (Figure 1D). In isolated from Trpa1−/− mice, but not in neurons isolated from Trpa1+/− mice (Figure 2C), while the response to capsaicin was unchanged in both mice strains.

3.2 | Safranal selectively excites TRPA1 in rodent sensory neurons

Exposure to safranal of cultured rat DRG neurons evoked a concentration-dependent calcium response in a subset of cells, identified as nociceptors by their ability to respond to KCl, capsaicin and AITC31 (Figure 2A). In rat DRG neurons maximum calcium responses to safranal or AITC were 35.8% ± 8.5% and 56.0% ± 6.0% of ionomycin (n = 25, P < 0.01) respectively, and EC50 were 38 ± 0.03 μmol/L and 5 ± 0.3 μmol/L respectively. Calcium responses were attenuated by HC-030031, and unaffected by the respective TRPV1 and TRPV4 selective antagonists, capsazepine and HC-067047 (Figure 2A). Safranal or AITC elicited inward currents in rat DRG neurons, that were blocked by HC-030031 (Figure 2B), which, however, did not affect currents evoked by capsaicin, indicating selectivity (Figure 2A, B). Safranal and AITC produced calcium responses in DRG neurons isolated from Trpa1−/− mice, but not in neurons isolated from Trpa1+/− mice (Figure 2C), while the response to capsaicin was unchanged in both mice strains.

3.3 | Safranal causes neuropeptides release and acute pain via TRPA1 activation in nociceptors

There is evidence that TRPA1 is localized in peptidergic primary sensory neurons.35,36 Central and peripheral endings of primary sensory neurons are widely expressed in most tissues and organs, including rat urinary bladder and rat or mouse spinal cord, where upon stimulation they release proinflammatory neuropeptides, such as CGRP.
and SP. SP released upon stimulation of TRPV1 or TRPA1 results in a contractile response of isolated strips of rat urinary bladder that is mediated by activation of the NK1 and NK2 receptors for SP in bladder smooth muscle cells.37

**FIGURE 1** Safranal (SFR) and picrocrocin (PICR) selectively target the human TRPA1 channel. A. Chemical structures of SFR, PICR and crocin (CRO). B. Concentration response curves of the calcium mobilization evoked by SFR and PICR in hTRPA1 HEK293 cells. Representative traces and pooled data of calcium response evoked by SFR, PICR and AITC in hTRPA1 HEK293 pre-exposed to HC-030031 (HC03, 30 μmol/L) or its vehicle (-) and in naïve HEK293 cells. C. Representative traces and pooled data of whole-cell patch-clamp inward currents evoked by CRO and AITC (100 μmol/L) in hTRPA1 HEK293. D. Pooled data of calcium responses evoked by SFR and AITC (100 μmol/L) in hTRPA1 HEK293. E. Pooled data of calcium responses evoked by SFR and PICR in IMR90 cells. Representative traces and pooled data of calcium responses evoked by SFR, PICR and AITC pre-exposed HC03 (30 μmol/L) or its vehicle (-) in IMR90 cells. HC03 does not affect the response evoked by the hPAR2-AP (100 μmol/L). Veh is the vehicle of SFR, dash (-) indicates the vehicle of HC03. Data are mean ± SEM of n > 20 cells from 4 to 6 independent experiments (B, D, E) and n > 3 cells from 3 to 5 independent experiments (C). *P < 0.05 vs veh, §P < 0.05 vs SFR, PICR or AITC; one-way ANOVA with Bonferroni post-hoc correction.

Safranal caused a concentration-dependent contractile response of isolated strips of rat urinary bladder with a slightly lower potency than AITC (EC50s were 76 ± 0.07 μmol/L and 56 ± 0.02 μmol/L respectively) (Figure 3A). Efficacy of safranal was also lower than
that of AITC (maximum response, 15.5% ± 3.0% of carbachol, n = 6, and 35.1% ± 6.2% of carbachol respectively, n = 6, P < 0.05) (Figure 3A). The response of safranal was attenuated by pre-exposure to a combination of NK1 and NK2 receptor antagonists (L-733,060 and SR48968 respectively), by pre-exposure to a high concentration of capsaicin able to desensitize the nociceptors and by pre-exposure to the TRPA1 selective antagonist, HC-030031, while it was unaffected by pre-exposure to capsazepine (TRPV1 antagonist) and HC-067047 (TRPV4 antagonist) (Figure 3A). This finding supports the hypothesis that safranal behaves as a partial agonist at the TRPA1 channel.

Safranal elicited a concentration-dependent increase in CGRP-LI outflow from rat spinal cord slices which was absent in a calcium-free medium or in tissues pre-exposed to a desensitizing concentration of capsaicin and in the presence of HC-030031 (Figure 3B). Safranal-evoked CGRP release was unaffected by capsazepine or HC-067047 (Figure 3B). Exposure to safranal increased CGRP outflow from dorsal spinal cord slices obtained from Trpa1+/− mice, but not from tissues taken from Trpa1−/− mice (Figure 3C). Thus, safranal elicits CGRP release from a subset of TRPV1-positive neurons via a neurosecretory process, mediated by TRPA1.
These ex vivo findings were replicated in an in vivo setting. Injection of safranal (0.2-20 nmol) into the mouse paw (20 μL, i.pl.) evoked a dose-dependent acute nociceptive response (Figure 3D). The response evoked by the highest dose (20 nmol) of safranal was attenuated by pretreatment with systemic administration HCl-030031 (100 mg/kg, i.p.) and was absent in Trpa1−/− mice (Figure 3E,F). Selectivity of safranal action for TRPA1 was strengthened by the observation that the nociceptive response evoked by i.pl. safranal (20 nmol) in Trpv1−/− and Trpv4−/− mice was maintained in Trpv1−/− and Trpv4−/− mice (Figure 3G,H). Similarly, the nociceptive response to safranal was unaffected by pretreatment with TRPV1 and TRPV4 antagonists (capsazepine and HC-067047 respectively) (Figure 3E).

### 3.4 In vitro and in vivo exposure to safranal attenuated TRPA1-mediated responses

In hTRPA1-HEK293 transfected cells, inward currents evoked by AITC underwent a concentration-dependent attenuation after pretreatment with safranal (Figure 4A), whereas KCl-evoked currents were not affected (Figure 4A). In cultured rat DRG neurons, pre-exposure to safranal reduced inward currents evoked by AITC, but not currents evoked by capsaicin, suggesting that safranal promotes selective TRPA1 desensitization (Figure 4B). Specific desensitization of TRPA1 was also observed in organ bath experiments. Pre-exposure to safranal reduced contractile responses of rat urinary bladder strips evoked by safranal and AITC, but not those evoked by capsaicin, GSK1016790A or carbachol (Figure 4C). Finally, pre-exposure to safranal was able to markedly reduce CGRP release from rat spinal cord evoked by AITC, without affecting the release evoked by capsaicin or GSK1016790A (Figure 4D).

To test whether pre-exposure to safranal could desensitize TRPA1-mediated responses in vivo, safranal was administered by the intragastric route (i.g.) at two different doses (0.5-1 mg/kg). Single i.g. administration of either doses (day 1) did not affect the ability of local (i.pl.) AITC, capsaicin or GSK1016790A to evoke acute nociceptive responses (Figure 5A-C). After administration of the two doses of safranal for 3 consecutive days the nociceptive responses evoked by i.pl. AITC were slightly reduced, but not those evoked by capsaicin and GSK1016790A (Figure 5A-C). After administration of the two doses of safranal for 5 consecutive days the nociceptive

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**FIGURE 3** Safranal (SFR) activating TRPA1 in nociceptors causes neuropeptides release and pain. A. Concentration response curves of the contractile response induced by SFR and AITC in rat urinary bladder. Pooled data of the contractile response evoked by SFR in rat urinary bladder pre-exposed to a combination of NK1/NK2 receptor antagonists (NK1/2 RA: L-733,060 and SR48968, both 1 μmol/L), to a high concentration of capsaicin (CPS des; 10 μmol/L), HC-030031 (HC03, 50 μmol/L), capsazepine (CPZ; 10 μmol/L), HC-067047 (HC06; 30 μmol/L) or their vehicles (.). B, Pooled data of CGRP release evoked by SFR from rat dorsal spinal cord after calcium removal (Ca2+ free), or after capsaicin desensitization (CPS des; 10 μmol/L), or exposed to HC03 (50 μmol/L), CPZ (10 μmol/L) or HC06 (30 μmol/L). C) CGRP release evoked by SFR from dorsal spinal cord from Trpa1+/− and Trpa1−/− mice. D) Dose-dependent acute nociceptive response evoked by intraplantar (i.pl.) injection (20 μL) of SFR in C57BL/6 mice. E) Pooled data of the acute nociceptive response evoked by SFR (20 nmol, i.pl.) after intraperitoneal (i.p.) HC03 (100 mg/kg), CPZ (4 mg/kg), HC06 (10 mg/kg) or their vehicles (. 4% DMSO, 4% tween 80 in 0.9% NaCl). F-H, Acute nociceptive response evoked by SFR (20 nmol, i.pl.) in Trpa1+/− and Trpa1−/−, Trpv1+/− and Trpv1−/−, and Trpv4+/− and Trpv4−/− mice. Veh is the vehicle of SFR. Dash (−) indicates vehicles of treatments. Data are mean ± SEM of 4-6 independent experiments (A-C) and n = 6 mice per group (D-H). *P < 0.05 vs Veh; †P < 0.05 vs SFR; one-way ANOVA with Bonferroni post-hoc correction.
responses evoked by i.pl. AITC were markedly attenuated, the effect of the highest dose of safranal being more pronounced (Figure 5A). Nociceptive responses evoked by both capsaicin and GSK1016790A were unaffected (Figure 5B,C).

4 | DISCUSSION

Present results show that two of the three main constituents of saffron, safranal and its precursor, picrocrocin, target the mouse, rat and human (native and recombinant) TRPA1, whereas the third constituent, crocin, was completely inactive. The lower potency of picrocrocin compared to safranal may depend on various factors. The presence of two \( \beta \) substituents on the double bond and chemical instability as compared to safranal\(^ {38} \) could explain the lower reactivity of picrocrocin. The failure to stimulate TRPV1 or TRPV4 channels indicates that nociceptor activation by safranal is selectively mediated by TRPA1. Interestingly, safranal failed to evoke any calcium response in HEK293 cells transfected with mutant TRPA1 (3CK-Q) channel, indicating that, similarly to other electrophilic and reactive agonists,\(^ {23,33} \) channel activation by the saffron constituent is mediated by specific cysteine/lysine residues. Both potency and efficacy of the calcium response evoked by safranal in cultured rat DRG neurons were lower than that of AITC. In particular, the lower efficacy supports the hypothesis that safranal may act as a partial TRPA1 agonist.

Peptidergic sensory neurons expressing TRPA1 exert a dual afferent and efferent role, because upon stimulation they can both signal pain to the brain and release from their peripheral terminals the neuropeptides SP and CGRP, which mediate neurogenic inflammatory responses.\(^ {13,39} \) One of these responses is the SP-mediated contraction of rat urinary bladder smooth muscle,\(^ {40} \) which can be also produced by TRPA1 activation in intramuscular sensory nerve terminals.\(^ {37} \) Here, we found that safranal produced a contraction mechanistically similar to that induced by AITC, because it was abrogated after exposure to a high, desensitizing, concentration of capsaicin, in the presence of a TRPA1 selective antagonist and in the presence of a combination of NK1 and NK2 receptor antagonists. Notably, the efficacy of safranal to target TRPA1 in the urinary bladder was lower than that of AITC, further supporting a possible partial agonism of the compound.

Results obtained in vitro were recapitulated in an in vivo setting, where the proalgesic action of safranal was mediated exclusively by TRPA1. Intraplantar injection of safranal elicited a concentration-dependent acute nociceptive response that was TRPA1-dependent, being selectively attenuated by TRPA1 antagonism and gene deletion. However, there is also indication that safranal has beneficial effect in certain pain conditions.\(^ {3,41} \) In particular, safranal attenuated mechanical alldynia and thermal hyperalgesia in a chronic constriction injury model,\(^ {10} \) suppressed the second phase of the orofacial pain induced by formalin\(^ {42} \) and the late phase of the carrageenan-induced paw edema.\(^ {8} \) The apparent contradiction between these findings and the ability of safranal to excite the

**FIGURE 4** Safranal (SFR) causes desensitization. A, Representative traces and pooled data of whole-cell patch-clamp inward currents of the concentration dependant desensitization induced by SFR in response to AITC and KCl in hTRPA1 HEK293. B, Representative traces and pooled data of whole-cell patch-clamp inward currents of the desensitization induced by SFR in response to AITC and capsaicin (CPS) in rat DRG (rDRG) neurons. C, Pooled data of the desensitization induced by SFR in the contractile response evoked by SFR, AITC, CPS, GSK and carbachol (CCh). D, Pooled data of the desensitization induced by SFR in CGRP-LI release from rat spinal cord evoked by AITC, CPS and GSK. Veh is the vehicle of SFR. Data are mean ± SEM of n > 3 cells from 3-5 independent experiments (A, B) and 4-6 independent experiments (C, D). \( ^{4} P < 0.05 \) vs AITC, SFR; one-way ANOVA followed by Bonferroni test. \( ^{5} P < 0.05 \) vs AITC and SFR, Student’s t test.
proalgesic TRPA1 may be explained considering that pre-exposure to safranal of cultured DRG neurons selectively attenuated AITC-evoked responses, without affecting TRPA1-independent responses. In vitro results were recapitulated by in vivo experiments, where repeated administration (5 days) of i.g. safranal reduced the acute nociceptive responses evoked by local AITC, leaving unaffected the nociceptive responses evoked by TRPV1 or TRPV4 stimulation. Thus, safranal attenuates the afferent function of nociceptors apparently by promoting a process of homologous desensitization of the TRPA1 channel, which might contribute to the antinociceptive properties of saffron. More recently, it has been demonstrated that TRPA1 is expressed by Schwann cells, where it can amplify and sustain macrophage-dependent neuropathic pain.19 Thus, it is possible that safranal exerts its partial agonist and desensitizing activities at the Schwann cell TRPA1, contributing in this manner to reduce neuropathic pain.

Clinical investigations with saffron has reported headache as a possible adverse reaction.4 In contrast with this observation, saffron has been used by Indian traditional medicine to treat headaches.5 TRPA1 is preferentially expressed by peptidergic sensory neurons, and upon its activation evokes the simultaneous release of the proinflammatory and proalgesic neuropeptides, SP and CGRP.21,43 CGRP is now considered a major contributor of migraine pain as small molecule CGRP receptor antagonists and monoclonal antibodies against CGRP or its receptor have marked beneficial effects in migraine.13,15 The observation that in strips rat urinary bladder and in rat dorsal spinal cord slices pre-exposure to safranal reduced AITC-evoked SP-mediated contractile responses and CGRP release respectively, without affecting TRPA1-independent responses further suggests that safranal attenuates the efferent and pro-migraine function of peptidergic nociceptors.

We previously showed that upon in vitro or in vivo exposure to other herbal preparations, such as isopetasin, contained in butterbur [Petasites hybridus (L.) Gaertn.],29 and parthenolide, a major constituent of Tanacetum parthenium,30 TRPA1-expressing trigeminal neurons undergo concentration- or dose-dependent desensitization. However, while isopetasin and parthenolide evoked non-selective desensitization of peptidergic nociceptors as they also attenuated responses mediated by TRPV1 and TRPV4 activation,29,30 safranal seems to selectively reduce responses elicited by TRPA1 agonism. The mechanism of the selective activity of safranal is unknown. Different pharmacokinetic properties or distinct activation of intracellular signalling mechanisms may be the causes of the diverse ability of the three herbal derivatives to desensitize the channel, and these deserve further investigation. Nevertheless, safranal, one major constituent of saffron extract, targets TRPA1 with a lower potency than full agonists and attenuates responses mediated by TRPA1 activation by other stimuli. The dual action of safranal on TRPA1 might contribute to the reported either detrimental or beneficial actions of the compound in animal models of pain and of saffron in humans.

ACKNOWLEDGMENTS

We thank A.H. Morice (University of Hull, UK) for the hTRPA1-HEK293 cells, M.J. Gunthorpe (GlaxoSmithKline, UK) for the hTRPV1-HEK293 cells, N.W. Bunnett (Monash Institute of Pharmaceutical Sciences, Australia) for the hTRPV4-HEK293 cells, D. Julius (UCSF, CA USA) for the human TRPA1 wild-type and human TRPA1 mutant (C619S, C639S, C663S, K708Q) cDNAs, and G. Cirino (University of Naples, Italy) for PAR2 selective agonist, SLIGKV-NH2.

We also thank Mary K. Lokken for her expert English revision. This work was supported by grants from Regione Toscana, grant Nutraceuticals 2014, "POFCADT"; Ministry for University and Scientific Research (MiUR) Rome, Italy Grants PRIN 201532AHAE_003 (to P.G.).
CONFLICT OF INTEREST

RP is fully employed at Chiesi Farmaceutici SpA, Parma, Italy. The other authors declare no competing financial interests.

AUTHOR CONTRIBUTION

SLP, LL, EC, RP, PG, SM, RN and FDL designed experiments and interpreted results. SLP, LL, SJM and VS, performed calcium experiments. SLP, EC and RP., performed electrophysiological experiments. SLP, LL, SJM, IMM, EC and SM, performed neurochemical in vitro assays. SLP, LL, SJM, IMM, SM, RN, and FDL, performed in vivo experiments. RP, PG, RN and FDL wrote the manuscript.

ORCID

Ramina Nassini https://orcid.org/0000-0002-9223-8395

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Li Puma S, Landini L, Macedo SJ Jr, et al. TRPA1 mediates the antinociceptive properties of the constituent of Crocus sativus L., safranal. J Cell Mol Med. 2019;23:1976–1986. https://doi.org/10.1111/jcmm.14099