Preclinical Development of *Crocus sativus*-Based Botanical Lead IIIM-141 for Alzheimer’s Disease: Chemical Standardization, Efficacy, Formulation Development, Pharmacokinetics, and Safety Pharmacology

Sonali S. Bharate, Vikas Kumar, Gurdarshan Singh, Amarinder Singh, Mehak Gupta, Deepika Singh, Ajay Kumar, Ram A. Vishwakarma, and Sandip B. Bharate

ABSTRACT: *Crocus sativus* L. (family: Iridaceae) has been documented in traditional medicine with numerous medicinal properties. Recently, we have shown that *C. sativus* extract (IIIM-141) displays promising efficacy in a genetic mice (5XFAD) model of Alzheimer’s disease (AD) (*ACS Chem. Neurosci*. 2017, 16, 1756). To translate the available traditional knowledge and the scientifically validated results into modern medicine, herein we aimed to carry out its preclinical development. IIIM-141 is primarily a mixture of crocins containing trans-4-GG-crocin (36 % w/w) as the principal component. The in vitro studies show that IIIM-141 has protective as well as therapeutic properties in assays related to AD. It induces the expression of P-gp, thereby enhancing the amyloid-β clearance from an AD brain. It also inhibits NLRP3 inflammasome and protects SH-SYSY cells against amyloid-β- and glutamate-induced neurotoxicities. In behavioral models, it decreased the streptozotocin-induced memory impairment in rats and recovered the scopolamine-induced memory deficit in Swiss albino mice at 100 mg/kg dose. The acute oral toxicity study shows that IIIM-141 is safe up to the dose of 2000 mg/kg, with no effect on the body weight and on the biochemical/hematological parameters of the rats. The repeated oral administration of IIIM-141 for 28 days at 100 mg/kg dose did not cause any preterminal deaths and abnormalities in Wistar rats. The pharmacokinetic analysis indicated that after oral administration of IIIM-141, the majority of the rats. The repeated oral administration of IIIM-141 for 28 days at 100 mg/kg dose did not cause any preterminal deaths and abnormalities in Wistar rats. The pharmacokinetic analysis indicated that after oral administration of IIIM-141, the majority of thecapsule formulation resulted in 3.3-fold enhancement in the area under the curve of crocetin and doubling of the crocetin/crocin ratio in plasma compared with the extract. The data presented herein will serve as the benchmark for further research on this botanical candidate.

INTRODUCTION

Alzheimer’s disease (AD) is a chronic neurodegenerative disorder of complex etiology with progressive neuronal damage resulting in dementia symptoms. The therapeutic interventions for the treatment of AD are mainly focused on three hallmarks, namely impaired neurotransmission, amyloid-β deposition, and tau hyperphosphorylation [formation of neurofibrillary tangles (NFTs)]. Apart from these primary hallmarks, in recent years, newer approaches are being explored such as targeting amyloid-β clearance via induction of P-gp, inhibition of NLRP3 inflammasome, or targeting glucose metabolism-related targets. Despite the extensive efforts on each of these hallmarks, till now only four medicines (donepezil, galantamine, rivastigmine, and memantine) are available in the market for treating this disease. All these drugs provide only symptomatic treatment, whereas none of them halts progression of the disease. Thus, identifying a disease-modifying therapy for AD is one of the major challenges for researchers. The undesirable outcomes of all recent clinical trials have indicated that no single strategy would efficiently work for treating AD. Therefore, a polypharmacology/multiple target approach has better chances of success in AD clinical trials. Increased consideration of the contributing role of neuroinflammation has necessitated the need for inhibiting the inflammasome along with enhancing amyloid-β clearance and providing protection to neurons. The polypharmacology...
involves either administering a combination of multiple drugs acting on different targets or identifying a candidate that displays a favorable effect on multiple targets of the disease. The latter strategy would have better chances of getting successful; however, it is highly challenging to identify such a candidate.

Botanical drugs or natural products have such a unique ability to display polypharmacological effects. *Crocus sativus* L. (also called as saffron or kesar; family: Iridaceae) is a traditional medicine being consumed by human beings since ancient times because of several health benefits. Saffron extracts have antioxidant, anti-inflammatory, antitumor, free radical scavenging, anti-convulsant, anti-atherosclerotic, and anti-Alzheimer activities. The saffron stigma contains a group of carotenoids including picrocrocin and crocins. Sixteen types of sugar moiety and the geometry of the 13−14 double bond (structures are provided in section S1 of the Supporting Information). The group of crocins and their aglycone metabolite, crocin, are mainly responsible for pharmacological activities associated with saffron.

Recently, we have shown that the administration of the saffron extract along with the food has resulted in a decreased load of amyloid-β plaques, upregulation of synaptic proteins, and reduced neuroinflammation in the brains of SXFAD mice. The history of its traditional usage, the documented health benefits, and the scientific data generated over a period of two to three decades indicate a strong potential for its translation to a modern drug. Thus, the present study is aimed to carry out its "preclinical development" as a botanical drug candidate. The hydroalcoholic extract of *C. sativus* stigma (IIIM-141) was studied for its chemical standardization (with five chemical markers), quality control (for aflatoxin/pesticide content and microbial load), in vitro efficacy in multiple targets, in vivo efficacy in anti-dementia models, acute/subacute toxicity in rodents, and preparation of the oral dosage form. The scalable sustained release (SR) formulation was prepared to achieve the SR of crocin (and its bioactive metabolite crocetin) over a longer period of time. All preclinical results of IIIM-141 are discussed in this paper.

## RESULTS

AD is a complex and incurable disease characterized by the presence of amyloid-β plaques, NFTs, and degeneration of neurons. The formation of amyloid-β plaques is the central event in the AD pathology. Therefore, numerous efforts have been made to target amyloid-β associated approaches, including reducing amyloid-β production, preventing amyloid-β aggregation, and facilitating amyloid-β clearance.

However, recently, it has been reported that amyloid-β centric approaches (primarily those to reduce amyloid-β burden) that reached phase III clinical trials have failed (semagacestat, tarenflurbil, and tramiprosate have been discontinued). These failures have raised a concern about targeting the production of amyloid-β plaques. The plethora of evidences suggests that a single approach is not successful for the treatment of AD. Additionally, the complex pathology of AD also suggests that it needs to be tackled via a multifaceted approach. IIIM-141 is a botanical extract based on *C. sativus*, which displays multiple pharmacological activities related to AD pathology. Crocin is the bioactive marker and the major component of the extract; therefore crocin enrichment (crocin-enriched fraction: IIIM-141-CEF) was also performed and was studied in the P-gp induction assay and formulation studies.

The present study was undertaken to develop IIIM-141 as a botanical product. Toward this objective, a systematic study was planned, which comprises the following subobjectives viz. (a) preparation, standardization, and quality control of the crocin-rich extract; (b) demonstrating in vitro/in vivo efficacy of the extract in various models; (c) development of suitable oral formulation of the extract; (d) pharmacokinetic analysis of the extract and formulation; and (e) acute and subacute oral toxicity study in rats. The data and results of these preclinical studies are provided in the following sections.

**Standardization, Characterization, Analytical Method Development, and Quality Control of IIIM-141.** The hydroalcoholic extract (IIIM-141) was prepared using the cold-maceration method. The IIIM-141 was then partitioned between water and ethyl acetate. The evaporation of the water layer to dryness yielded crocin-enriched fraction, IIIM-141-CEF. The high-performance liquid chromatography (HPLC) chromatograms of IIIM-141 and IIIM-141-CEF (at 50 μg/mL) are shown in Figure 1. Although a total of 16 crocins are reported in the literature, only 6−7 peaks could be seen in the HPLC chromatogram, with the peak appearing at *t*~*h*~ 3.9 min (crocin-1) being the major one. Doubling of the height of peaks in IIIM-141-CEF is indicative of the ~40−50% enrichment of crocin-1 content in IIIM-141-CEF. To chemically standardize the extract and fraction, five chemical markers were isolated from IIIM-141 using semipreparative HPLC. The isolated markers M1−M5 were characterized as trans-4-GG-crocin (M1, *t*~*h*~ 3.9 min), trans-3-GG-crocin (M2, *t*~*h*~ 4.9 min), trans-2-gG-crocin (M3, *t*~*h*~ 8.5 min), cis-4-GG-crocin (M4, *t*~*h*~ 9.2 min) and trans-crocin (M5, *t*~*h*~ 12.9 min). The liquid chromatography mass spectrometry (LC−MS) chromatograms of IIIM-141 and the mass spectrums of...
indicated that IIIM-141 does not show any toxicity up to 200 μg/mL in LS-180, SH-SY5Y, and THP-1 cells. It showed strong inhibition of the NLRP3 in Pseudomonas aeruginosa and the count for specific microbes such as Enterobacteriaceae, Escherichia coli, Salmonella spp., Staphylococcus aureus, and Pseudomonas aeruginosa were in the acceptable limits.

The chemical standardization of the botanical extracts with marker compounds assures reproducibility of the batches, quality, efficacy, safety, and acceptability of the botanical formulations. Therefore, these extracts were standardized using isolated four markers by the HPLC method. The % content of M1–M5 in IIIM-141 was found to be 36.1, 13.7, 6.0, 7.6, and 0.07 % w/w of the dry raw material. Furthermore, it was found that the enrichment step resulted in 1.75-times increase in the content of M1 (64 % w/w of M1 in IIIM-141-CEF) (Table 1). IIIM-141 was also studied for different quality control parameters, as depicted in Table 2. The content of aflatoxins (B1, B2, G1, and G2) and pesticides was below the detection limits. The total microbial count, the total yeast/mold count, and the count for specific microbes such as Enterobacteriaceae, Escherichia coli, Salmonella spp., Staphylococcus aureus, and Pseudomonas aeruginosa were in the acceptable limits.

**In Vitro Efficacy.** Initially, the toxicity window of the test item was determined in each cell line that was used. Results indicated that IIIM-141 does not show any toxicity up to 200 μg/mL in LS-180, SH-SYSY, and THP-1 cells. The effect of IIIM-141 on the NLRP3 inflammasome was studied in THP-1 cells. It showed strong inhibition of the NLRP3 inflammasome at a concentration of 25 μg/mL, as depicted in Figure 2a. IIIM-141 at 100 μg/mL showed 42% inhibition of interleukin (IL)-1β release in comparison to lipopolysaccharide (LPS) + nigericin (100%).

**Table 1. % Content of Marker Compounds in IIIM-141 and IIIM-141-CEF**

| marker compound         | retention time (min) | % content of marker compound in the extract (mean ± SD) | regression equation | R²  |
|-------------------------|----------------------|------------------------------------------------------|---------------------|-----|
| trans-4-GG-crocin       | 3.8                  | IIIM-141: 36.093 ± 3.249 IIIM-141-CEF: 64.148 ± 4.242 | y = 7863x            | 0.996 |
| trans-3-Gg-crocin (M2)  | 5.1                  | IIIM-141: 13.737 ± 1.110 IIIM-141-CEF: 18.741 ± 1.113 | y = 15192x           | 0.999 |
| trans-2-gg-crocin (M3)  | 9.2                  | IIIM-141: 5.979 ± 0.382 IIIM-141-CEF: 9.376 ± 0.194  | y = 12913x           | 0.998 |
| cis-4-GG-crocin (M4)    | 9.6                  | IIIM-141: 7.655 ± 0.345 IIIM-141-CEF: 8.717 ± 0.124  | y = 16186x           | 0.999 |
| trans-crocin (M5)       | 12.9                 | IIIM-141: 0.072 ± 0.003 IIIM-141-CEF: nd            | y = 22480x           | 0.995 |

**Table 2. Quality Control Parameters of IIIM-141 Extract**

| parameter                     | observed value | parameter                     | observed value |
|-------------------------------|----------------|-------------------------------|----------------|
| acid-insoluble ash (% w/w)    | 0.235          | total bacterial count         | 1.6 × 10⁻³ (<10⁻³) |
| loss on drying at 105 °C (%)  | 0.600          | total yeast, mold count       | Nil (<10⁻³)     |
| total ash (% w/w)             | 9.375          | Enterobacteriaceae            | <10 (<10⁻⁹)     |
| total sugar (% w/w)           | 34.667         | Escherichia coli              | absent          |
| crude fat (% w/w)             | 3.822          | Salmonella spp.               | absent          |
| protein (% w/w)               | 11.203         | Staphylococcus aureus         | absent          |
| extractive value (% w/w)      | 92.237         | Pseudomonas aeruginosa        | absent          |

*BDL, below detection limit.

As a positive control, which showed 90% inhibition of IL-1β release at 40 ng/mL, in comparison to LPS + nigericin (100%). In the P-gp induction assay, IIIM-141 treatment in LS-180 colon cancer cells at various concentrations ranging from 6.25 to 100 μg/mL led to a significant increase in the efflux of substrate rhodamine 123 dye, as determined by the decrease (by 16–40%) in intracellular % Rh123 levels (Figure 2b). Rifampicin was used as a positive control in this study. Rifampicin at 10 μg/mL showed a decrease in the intracellular accumulation of Rh123 levels (by 31%) in LS180 cells, in comparison with the control (100%). The Western blot results indicated that IIIM-141 induces P-gp expression by 3–8 folds in LS-180 colon cancer cells at concentrations ranging from 12.5 to 100 μg/mL (Figure 2c), which might be the possible explanation for the increased efflux of the Rh123 dye. Rifampicin at 10 μg/mL showed a 9-fold increase in the P-gp expression. The pure markers crocin and crocetin were also tested for P-gp induction activity in LS-180 cells. Similar to the IIIM-141 extract, both crocin and crocetin showed potent P-gp induction activity, as shown by reduced % intracellular Rh123 levels in LS-180 cells (Figure 2d). As crocin displayed a dose-dependent decrease in the intracellular accumulation of the Rh123 dye (Figure 2d), it was hypothesized that enriching the extract with crocin content would enhance its bioactivity. The crocin-1-enriched fraction IIIM-141-CEF (wherein, the crocin-1 content is enhanced by 1.75-fold) and the hydroalcoholic extract IIIM-141 showed 73 and 60% Rh123 levels in LS180 cells at 25 μg/mL, indicating the superiority of the enriched fraction.

The neuroprotective activity of IIIM-141 was assessed in human neuroblastoma SH-SYSY cells, wherein it completely protected the neuronal cells from amyloid-β (Figure 2e) and glutamate-induced toxicities (Figure 2f). Ginkgolide B, a known neuroprotective agent, was used as a reference standard in the neuroprotection assay.

**In Vivo Efficacy.** The in vivo efficacy of IIIM-141 was assessed in two behavioral models: (a) Morris water maze (MWM) test and (b) passive avoidance test. The MWM test assesses the ability of animals to locate the hidden platform in the pool while swimming. Prior to the study, animals were trained for this activity. As a part of the study design, the memory of the animals was impaired using streptozotocin (STZ). There was a significant increase in the latency time to locate the hidden platform in STZ-treated rats as compared with artificial cerebrospinal fluid (aCSF) (vehicle)-treated rats, suggesting memory impairment. The recovery of animals from this memory deficit by the test sample was assessed using the swimming test. The treatment of memory-impaired rats with IIIM-141 at 50 and 100 mg/kg dose on the 15–18th day of the study resulted in reversal of the STZ-induced memory deficit, as demonstrated by the reduction in the latency time.
The tracking of rats during the study and the effect of IIIM-141 on the latency time is depicted in Figure 3a, b, respectively. The % change in the transfer latency with respect to group G1 on day 4 is shown in Figure 3c. Briefly, IIIM-141 showed anti-dementia properties at a dose of 100 mg/kg dose. The effect of IIIM-141 was then assessed in another behavioral model (passive avoidance test), wherein the learning ability of animals to avoid an anxious event (electric shock) was studied. The study equipment consisted of two compartments, one light and other dark, containing a grid floor that is electrically connected by a sliding door. The reduction in transfer latency from the light compartment to the dark compartment indicated memory impairment. The administration of anticholinergic drug scopolamine results in memory impairment in mice, as demonstrated by a significant reduction in comparison to the control (Figure 3d). IIIM-141 at 100 mg/kg body weight for 7 days significantly recovered the memory, as shown by the increase in transfer latency, compared with scopolamine-treated mice (Figure 3e). Positive control rivastigmine at a dose of 2 mg/kg body weight resulted in a significant increase in the latency time (Figure 3d,e), indicating that memory has been restored in these animals. In summary, pretreatment of animals with IIIM-141 for 7 days at 100 mg/kg showed anti-dementia properties, as demonstrated by a significant improvement in scopolamine-induced memory deficit in passive avoidance test in Swiss albino mice (SAM).

Safety Studies. As per regulatory requirements, for a botanical drug candidate, acute oral toxicity and 28-day repeat dose toxicity of IIIM-141 was assessed by following the guidelines of Organisation for Economic Co-Operation and Development (OECD). In the acute toxicity study, the extract did not cause any type of toxicity, and all animals survived the study over a period of 14 days at a single dose of 2000 mg/kg. Furthermore, there was no change in the body weight of animals (Figure 4a) and no effect on biochemical and
hematological parameters (Figure 4b) even at this dose, except for a slight gastric distress and soft stools during the first hour of administration. The LD$_{50}$ of IIIM-141 in Wistar rats was found to be more than 2000 mg/kg of the body weight.

The 28-day repeat dose toxicity was performed at 25, 50, and 100 mg/kg doses. There was no specific treatment-associated preterminal deaths and abnormalities observed in the rats at any of these doses. All animals from the control and the treated dose groups survived throughout the dosing period of 28 days. Food consumption of the control and treated animals was found comparable throughout the dosing period. There was no effect on the body weight (Figure 4c,d), biochemical parameters (Figure 4e,f), and hematological parameters (Figure 4g,h) on day 29 in the IIIM-141-treated group in comparison to the control group. The results of safety studies are shown in Figure 4a–h.

Chemical and Enzymatic Hydrolytic Stability of Crocin. Crocin is the bioactive marker of this botanical extract, and hence its chemical and enzymatic stabilities were studied to identify a suitable formulation for the extract. The pharmacokinetic profile clearly indicated that developing SR formulations would be an ideal strategy for effectively delivering bioactive markers crocin and crocetin. The pH-dependent chemical stability of crocin (pH 1.2 to pH 10 buffer solutions) indicated that it is stable up to pH 8.0. However, it was isomerized to the cis form and hydrolyzed to its aglycone crocetin beyond pH 8.0. The hydrolysis of crocin to crocetin was also observed in rat plasma. The results of stability studies are summarized in Table 3.

Formulation Development, Evaluation, and Characterization. The pharmacokinetic analysis of crocin indicated that it reaches the blood circulation in its hydrolyzed form crocetin. The plasma half-life of crocetin is low, and it gets eliminated quickly from the blood.$^{19}$ The rationale for developing SR formulations of standardized extracts of C. sativus was to release crocin in the gastrointestinal tract (GIT) in a controlled manner. With this approach, crocin would be available over a longer period of time to the hydrolytic enzymes of GIT for bioactivation to its aglycone crocetin. The aglycone metabolite, being lipophilic in nature, would rapidly get absorbed from the GIT, resulting in its higher concentration in blood circulation.

The SR formulations were prepared by the wet granulation method. On the basis of the assay results, a formulation,
equivalent to 50 mg of crocin-1, was filled into an empty hard gelatin capsule, and in vitro dissolution was performed in triplicates. The formulation details and % crocin-1 content are in Table 4.

The results obtained from the in vitro dissolution study of formulations indicated the SR of crocin-1 (60–75% release) over a period of 24 h compared to the plain hydroalcoholic extract and the enriched fraction of C. sativus (100% release in 30 min). The dissolution profiles and results of formulations are shown in Figure 5 and Table 5. The dissolution results of hydroalcoholic (IIIM-141) and enriched fraction (IIIM-141-CEF) of C. sativus with their SR formulations, namely, CSHA-14 and CSE-2, respectively, were compared (Table 5). Briefly, the SR profile of CSHA-14 showed 2.8-fold improvement in the area under the curve (AUC) (0–24) with respect to IIIM-141 extract (172 vs 482 μg/mL h). Furthermore, T_max of CSHA-14 was 16 h compared to CSHA-1 (0.5 h), indicating the SR profile of SR formulation over the plain extract. A similar
observation was noted in the SR formulation (CSE-2) of crocin-1-enriched fraction (IIIM-141-CEF; CSE-1). The AUC\((0\rightarrow 24)\) of CSE-2 was 3.6-fold higher than that of CSE-1 (517 vs 143 \(\mu g/mL\) h). On the basis of these results, CSHA-14 and CSE-2 were considered as the optimum formulations. The in vitro dissolution results were then validated using the comparative oral pharmacokinetics of IIIM-141-CEF and its SR formulation CSE-2 in rats.

The compatibility of the extract with the polymers (HPMC-K4M and HPMC-K15M) used in the formulations was investigated using differential scanning calorimetry (DSC) and Fourier transform infrared (FT-IR) analysis (Figure 6a,b, respectively). The formulation ingredients, their 1:1 binary mixture, and SR formulations were investigated to identify any incompatibility with each other. The DSC and FT-IR examinations indicated no interaction between the extract and excipients and thus pointed out no incompatibility between them.

**Pharmacokinetic Study.** Before comparing the pharmacokinetics of the extract and its formulation, the oral bioavailability of pure markers, namely crocin and crocetin, was determined. The absolute oral bioavailability of crocin was found to be <1% because majority of the crocin gets hydrolyzed to crocetin when given perorally. There was minimum conversion of crocin to crocetin (parent to metabolite) upon intravenous (IV) administration of crocin.

**Table 3. Stability of Crocin in Different pH Buffers, Biological Fluids, and Enzymes**

| sr. no | medium                  | % remaining at zero time | % remaining after 24 h\((\pm SD)\) | isomerization to cis-form | conversion to crocetin |
|--------|-------------------------|--------------------------|-----------------------------------|---------------------------|------------------------|
| 1      | HCl buffer pH 1.2       | 100                      | 102.18                            | no                        | no                     |
| 2      | PBS pH 4.0              | 100                      | 94.9 \(\pm 12.4\)                 | no                        | no                     |
| 3      | PBS pH 6.8              | 100                      | 100                                | no                        | no                     |
| 4      | PBS pH 7.4              | 100                      | 98.7 \(\pm 1.7\)                  | no                        | no                     |
| 5      | alkaline borate buffer pH 8.0 | 100                  | 91.3 \(\pm 15.7\)                | no                        | no                     |
| 6      | alkaline borate buffer pH 9.0 | 100                  | 46.9 \(\pm 7.7\)                  | yes                       | yes                    |
| 7      | alkaline borate buffer pH 10.0 | 100                  | 38.3 \(\pm 7.0\)                  | yes                       | yes                    |
| 8      | rat plasma              | 100                      | 44.5 \(\pm 4.7\)                  | yes                       | yes                    |
| 9      | human saliva            | 100                      | 48.1 \(\pm 10.0\)                 | no                        | yes                    |
| 10     | SGF (pH 1.2)            | 100                      | 72.3 \(1 \pm 8.3\)                | no                        | no                     |
| 11     | SIF (pH 6.8)            | 100                      | 100                                | no                        | no                     |
| 12     | esterase                | 100                      | 72.4 \(\pm 4.6\)                  | no                        | no                     |
| 13     | lipase                  | 100                      | 58.0 \(\pm 13.1\)                 | no                        | no                     |

“\(^{a}\)The values represent an average of three independent determinations \(\pm\) standard deviation (SD).

**Table 4. Formulation Details and % Crocin-1 Content**

| formulation code | IIIM-141 | HPMC-K4M | HPMC-K15M | EC-10 | EUDRAGIT RS100 | crocin-1 content (% w/w \(\pm SD\)) |
|------------------|----------|----------|-----------|-------|----------------|-------------------------------------|
| CSHA-1           | 100      |          |           |       |                | 35.18 \(\pm 1.21\)                   |
| CSHA-14          | 60       | 40       |           |       |                | 21.91 \(\pm 0.05\)                   |
| CSHA-18          | 60       |          | 26.66     | 13.33 |                | 20.23 \(\pm 0.55\)                   |
| CSHA-19          | 60       |          | 30        | 10    |                | 20.93 \(\pm 0.97\)                   |
| CSHA-21          | 60       |          | 20        | 20    |                | 21.73 \(\pm 0.28\)                   |
| Hydroalcoholic Extract of C. sativus, IIIM-141 | **| ** | ** | ** | ** | ** |
| CSE-1            | 100      |          |           |       |                | 55.18 \(\pm 1.82\)                   |
| CSE-2            | 60       | 40       |           |       |                | 31.64 \(\pm 0.28\)                   |
| CSE-3            | 60       |          | 40        |       |                | 37.63 \(\pm 2.60\)                   |
| CSE-8            | 50       | 40       |           | 10    |                | 31.98 \(\pm 0.45\)                   |
| CSE-11           | 50       |          | 40        | 10    |                | 31.84 \(\pm 0.75\)                   |
| An Enriched Extract of C. sativus, IIIM-141-CEF | **| ** | ** | ** | ** | ** |

**Figure 5.** In vitro dissolution profiles of the developed SR formulations. (a) In vitro dissolution profile of hydroalcoholic extract (CSHA-1, IIIM-141) and its SR formulation CSHA-14 and (b) in vitro dissolution profile of crocin-1-enriched fraction (CSE-1, IIIM-141-CEF) and its SR formulation CSE-2.

**Table 5. Stability of Crocin in Different pH Buffers, Biological Fluids, and Enzymes**

| sr. no | medium                  | % remaining at zero time | % remaining after 24 h\((\pm SD)\) | isomerization to cis-form | conversion to crocetin |
|--------|-------------------------|--------------------------|-----------------------------------|---------------------------|------------------------|
| 1      | HCl buffer pH 1.2       | 100                      | 102.18                            | no                        | no                     |
| 2      | PBS pH 4.0              | 100                      | 94.9 \(\pm 12.4\)                 | no                        | no                     |
| 3      | PBS pH 6.8              | 100                      | 100                                | no                        | no                     |
| 4      | PBS pH 7.4              | 100                      | 98.7 \(\pm 1.7\)                  | no                        | no                     |
| 5      | alkaline borate buffer pH 8.0 | 100                  | 91.3 \(\pm 15.7\)                | no                        | no                     |
| 6      | alkaline borate buffer pH 9.0 | 100                  | 46.9 \(\pm 7.7\)                  | yes                       | yes                    |
| 7      | alkaline borate buffer pH 10.0 | 100                  | 38.3 \(\pm 7.0\)                  | yes                       | yes                    |
| 8      | rat plasma              | 100                      | 44.5 \(\pm 4.7\)                  | yes                       | yes                    |
| 9      | human saliva            | 100                      | 48.1 \(\pm 10.0\)                 | no                        | yes                    |
| 10     | SGF (pH 1.2)            | 100                      | 72.3 \(1 \pm 8.3\)                | no                        | no                     |
| 11     | SIF (pH 6.8)            | 100                      | 100                                | no                        | no                     |
| 12     | esterase                | 100                      | 72.4 \(\pm 4.6\)                  | no                        | no                     |
| 13     | lipase                  | 100                      | 58.0 \(\pm 13.1\)                 | no                        | no                     |
The comparative oral pharmacokinetic results indicate that when IIIM-141-CEF was administered in rats, the majority of crocin-1 was metabolized to crocetin. The AUC_{(0→∞)} of crocin-1 and crocetin in IIIM-141-CEF was observed to be 1360 and 5540 μg h/mL, respectively (crocetin/crocin-1 ratio in plasma was 4:1). When the equivalent dose of extract (IIIM-141-CEF) was administered as a SR formulation (CSE-2 formulation), the crocetin concentration in plasma increased by 3.3-fold. The AUC_{(0→∞)} of crocetin changed from 5540 to 18300 μg h/mL, and C_{max} also increased by 1.79-fold. Interestingly, the crocetin/crocin-1 ratio in plasma also increased from 4:1 to 9:1. This indicated that more amount of crocetin (a bioactive constituent) is available in blood circulation to exert the therapeutic effect at the site of action. Furthermore, the AUC_{(0→1)} ratio obtained from in vitro dissolution and pharmacokinetics was in agreement with each other (3.62 vs 3.30).

### DISCUSSION

Because of the complex disease pathology, the polypharmacology approach is desirable for the management of AD. In particular, natural products have always proved to be a valuable source of drugs for central nervous system (CNS) diseases. Furthermore, the CNS drugs have deep roots in traditional medicines, specifically in China, India, and South Africa. Hence, the crude drugs, which are being used in traditional systems of medicine, are worth validating scientifically to translate them into modern drugs. In this context, *C. sativus* is being used in Ayurveda and other folk medicines for treating various ailments. The strong history of its traditional usage and the available scientifically validated data for *A. philippica* have prompted us to develop it as a botanical drug. The active principles of this plant are carotenoid class of compounds, most of which are highly polar in nature and thus are water-soluble and get extracted in hydroalcoholic solution. Therefore, herein, a hydroalcoholic extract (IIIM-141) was studied. IIIM-141 is primarily a mixture of crocins, wherein crocin-1 (trans-GG-crocin) is the major component. It is present in 36.1 % w/ w of the dry plant material. The repeatability has been the major concern associated with botanical drugs; therefore IIIM-141 was standardized using four chemical markers viz. GG-crocin, 4GG-crocin, cis-3-GG-crocin, and trans-3-GG-crocin, and have been found to play an important role in pathogenic neuroinflammation in animal models of AD. As further, we have shown that IIIM-

### Table 5. In Vitro Dissolution Results of Hydroalcoholic Extract, Enriched Fraction, and Their Sustained Release Formulations

| Formulation code | T_{max} (h) | C_{max} (μg/mL) | AUC_{(0→1)} (μg h/mL) | AUC_{(0→∞)} (μg h/mL) |
|------------------|-------------|-----------------|------------------------|------------------------|
| CSHA-1 (IIIM-141) | 0.5         | 74.76           | 172.16                 | 1                      |
| CSHA-14          | 16          | 28.95           | 481.71                 | 2.8                    |
| CSHA-18          | 2           | 27.09           | 493.13                 | 2.86                   |
| CSHA-19          | 2           | 44.34           | 282.59                 | 1.64                   |
| CSHA-21          | 2           | 29.65           | 142.09                 | 0.83                   |
| Formulations of Hydroalcoholic Extract, IIIM-141-CEF | | | | |
| CSE-1 (IIIM-141-CEF) | 0.5 | 75.42 | 147.42 | 1 |
| CSE-2            | 16          | 27.93           | 517.08                 | 3.62                   |
| CSE-3            | 12          | 23.59           | 470.38                 | 3.3                    |
| CSE-8            | 16          | 21.43           | 386.74                 | 2.71                   |
| CSE-11           | 16          | 20.85           | 358.36                 | 2.51                   |

Figure 6. (a) DSC and (b) FT-IR spectra of the developed formulations, CSHA-14 and CSE-2.

at a dose of 5 mg/kg. However, crocin to crocetin conversion was 2-fold higher following oral administration of crocin at 50 mg/kg in male Sprague Dawley (SD) rats compared to its IV administration. The time versus plasma drug concentration curves of crocin after IV and oral administrations are shown in Figure 7a,b. Figure 7b clearly indicates that majority of crocin is converted to crocetin. The absolute oral bioavailability of crocin upon IV and oral administrations in male SD rats (5 vs 50 mg/kg, respectively) was found to be 48%. The elimination half-life (t_{1/2}) and the terminal plasma half-life were 1.60 and 2.08 h, respectively, as shown in Figure 7c.
141 along with its constituents, crocin and crocetin, also induces the expression of P-gp, an efflux transporter pump responsible for the transport of amyloid-β from the brain to the blood. The extract IIIM-141 protected human neuroblastoma cells from the toxicity of amyloid-β and glutamate at 10⁻⁵₀ μg/mL, indicating the potential of this botanical lead to be developed as a "dietary food supplement" or a "nutraceutical product."

Cognitive decline is a prime symptom of AD, and a large number of animal models have been established over the years that reiterates the aspects of the cognitive impairments seen in AD. Behavioral models particularly, MWM test¹⁷ and passive avoidance test,¹⁸ are the most commonly used models, which provide critical linkage with the pathology of AD. The MWM test uses rodents to study behavioral neurological parameters such as spatial learning and memory, and it strongly correlates with the N-methyl-D-aspartate (NMDA) receptor function and hippocampal synaptic plasticity.²³ The intra-cerebroventricular (ICV) injection of STZ resulted in the progressive deficits in memory, learning, and cognitive ability in rats, which is similar to sporadic AD.²⁴ In this test, the decrease in the latency time to locate the hidden platform indicates successful learning. The treatment of memory-impaired rats with IIIM-141 extract resulted in a decrease in STZ-induced memory impairment in rats at a dose of 100 mg/kg p.o. The passive avoidance test is a fear-aggravated test used to evaluate learning and memory in rodent models. Scopolamine, an anticholinergic drug, induces significant memory impairment and is associated with an increased latency time. IIIM-141 at 100 mg/kg dose was able to recover the animals from scopolamine-induced memory deficit. The results of these two studies further supports our earlier results obtained in the transgenic mice model¹⁴ and warrants the development of this botanical lead for the treatment of AD or other dementia-associated diseases. Furthermore, IIIM-141 displayed an excellent safety profile in acute oral toxicity as well as in 28-day repeat dose toxicity study. These results provide scientific validation for the therapeutic benefits and safe usage of this spice as a food ingredient, used particularly in Asian countries since ancient times.

The crocins are highly hydrophilic in nature because of the presence of multiple sugars at both the terminals of the aglycone crocetin. The therapeutic activity of the Crocus extract is attributed to the trans-crocetin. The enzymatic
bioactivation of crocin primarily occurs in the intestinal tract resulting in the formation of “crocetin” via hydrolytic cleavage of the glycosidic linkages.\(^{19a,20b,25}\) The aglycone crocin has been reported to inhibit the process of amyloid-\(\beta\) aggregation and increase the clearance of amyloid-\(\beta\)-42. It inhibits the formation of amyloid-\(\beta\) fibrils, destabilizes preformed amyloid-\(\beta\) fibrils, and stabilizes amyloid-\(\beta\) oligomers, thereby preventing their conversion into amyloid-\(\beta\) fibrils.\(^{26}\) Cathepsin B, a lysosomal cysteine protease of the papain family, has been reported to play an important role in the enhancement of amyloid-\(\beta\)-42 degradation in monocytes of AD patients via enhancing the cathepsin B levels. Crocetin also provides neuroprotection by reducing the production of various neurotoxic molecules from activated microglia.\(^{27}\) The pharmacokinetic studies\(^{20b,25}\) and the reports on pharmacological activities of “crocetin”\(^{26,28,29}\) have indicated that this aglycone is the bioactive metabolite responsible for all documented therapeutic benefits of saffron.

AD being the chronic disease, the treatment regime requires treatment for multiple days. Therefore, the availability of crocin in the blood circulation for longer duration would help in enhancing the treatment outcome. The formation of crocin occurs via enzymatic hydrolysis of crocin in the GIT. Crocin being highly hydrophilic in nature, it was thought that controlling its release in the GIT would result in the prolonged availability of crocin in the blood circulation. This would also avoid the elimination of crocin from the GIT itself, before absorption. Therefore, the SR formulations were prepared with the hypothesis that the controlled release of the crocin from the formulation would create a more favorable scenario for its bioactivation to its metabolite crocetin and is expected to produce a higher ratio of crocetin/crocin in the blood circulation. The comparative pharmacokinetic study conducted in rats have shown that the SR formulations have resulted in superior plasma exposure and higher ratio of the crocetin/crocin in the plasma (4:1 vs 9:1). The 3-fold improvement in plasma exposure by SR capsule formulation would result in 3-fold reduction in the effective dose of the extract in preclinical/clinical studies. The validation of SR formulations in the oral pharmacokinetics study has indicated that these formulations would be an advantage in terms of dose reduction, better therapeutic window, cost of therapy, and improvement in patience compliance.

### CONCLUSIONS

In the present study, the chemical standardization and quality control of anti-dementia/anti-Alzheimer botanical candidate IIIM-141 have been presented. The developed extract was found to be efficacious in in vitro and in vivo assays of dementia and AD and was found to be safe as per OECD Guidelines for the Testing of Chemicals. The newly developed SR capsule formulation showed superiority over the conventional formulation, as seen in the in vitro dissolution and in the oral pharmacokinetic study. Overall, the preclinical data presented herein warrant its clinical investigation. This data will serve as the benchmark for further research on this botanical product.

### EXPERIMENTAL SECTION

**General.** The authentic plant material of C. sativus (stigma) was purchased from Srinagar (Jammu and Kashmir, India). It was taxonomically characterized, and a voucher specimen was deposited in the Janaki Ammal Herbarium of the Indian Institute of Integrative Medicine. Crocin (CAS no. 42553-65-1) was procured from Sigma-Aldrich (St. Louis, MO). The raw material was stored at 2–8 °C until further use.

The anti-dementia studies were performed at Panacea Biotech Ltd., Mohali on commercial basis. The protocols of MWM test and passive avoidance test were approved by the Panacea Biotech Animal Ethical Committee (IAEC approval numbers: 411 and 407). The pharmacokinetic study was performed at Eurofins Advinus Ltd. Bangalore (IAEC approval number. ATL-43_PKM-035/Jun-2017).

**Chemicals and Reference Compounds.** Reference marker compounds, trans-4-GG-crocin (also named as crocin-1), trans-3-GG-crocin, trans-2-gg-crocin, and cis-4-GG-crocin, were isolated from the IIIM-141 extract using semipreparative HPLC. These markers were used for standardization of extracts by HPLC. Acetonitrile (SD Fine Chemicals), methanol (SD Fine Chemicals), formic acid (Ran kem), and ethanol were used in the semipreparative isolation of markers by HPLC. Polyvinylpyrrolidone (PVP)-K30 and ethyl cellulose 10 cps were procured from Sigma-Aldrich. HPMC-K4M and HPMC-K15M were obtained as gift samples from Colorcon Asia Pvt. Ltd. (Goa). Eudragit RS100 was received from Evonik Industries as a gift sample. Empty hard gelatin capsules were obtained from Natural Capsules Ltd. Bengaluru, India as a gift sample.

**Synthesis of trans-Crocetin.** The hydroalcoholic extract (IIIM-141; 3 g) of C. sativus was stirred in the alkali solution (10% w/v NaOH; 70 mL) at 80 °C for 5 h. The resulting mixture was then allowed to cool down to room temperature and neutralized using 1N HCl solution under ice-cold conditions. The resulting precipitate was filtered using a Whatman filter paper, and the residue was washed with water (3 × 150 mL). The obtained powder (340 mg) was a mixture of trans- and cis-crocetin in the ratio of 85:15 (ratio determined by HPLC; see section S3 of the Supporting Information). The silica gel column chromatography of this mixture of trans- and cis-crocetin using hexane–EtOAc as the mobile phase resulted in the isolation of trans-crocetin (ratio of trans-to cis-form as 98:2).

**Preparation, Chemical Standardization, and Quality Control of C. sativus Extract.** The hydroalcoholic extract of C. sativus stigma was prepared in three stages using the cold-maceration method. The first stage of extraction consisted of mixing ~100 g of raw material with 1000 mL of hydroalcoholic solution (EtOH/water—1:1). After 3 h of extraction, the resulting mixture was filtered to get the first intermediate hydroalcoholic extract and the residue. The residue obtained from the first and second stages was further extracted in the same way as stage 1. The hydroalcoholic extract obtained from all three stages were combined, and ethanol was evaporated using vacuo rotavapor at 40 °C. The concentrated mass was lyophilized to generate a dry powder of the hydroalcoholic extract (IIIM-141). The extractive value was found to be 50–55 % w/w of the dry raw material. To prepare crocin-1-enriched fraction (IIIM-141-CEF), the hydroalcoholic extract was partitioned between water and ethyl acetate. The aqueous portion was concentrated on a rotary evaporator to get a powder of crocin-1-enriched fraction (IIIM-141-CEF). The extractive value of IIM-141-CEF was found to be 25–30 % w/w of the dry raw material. The obtained extracts were pulverized, sieved through sieve #30, and stored in a vacuum
desiccator until further use. The extracts were standardized by HPLC using five markers viz. trans-4-GG-crocin (crocin-1), trans-3-Gg-crocin, trans-2-gg-crocin, cis-4-GG-crocin and trans-crocetin.

The simultaneous estimation of markers in IIIM-141 and IIIM-141-CEF was performed using a HPLC system consisting of an Intersil C8-3 (5 μ, 250 × 4.6 mm) column and a photodiode array detector. The detection wavelength was 440 nm. The flow rate was 1 mL/min with the injection volume of 10 μL. The total run time was 25 min with gradient elution using the mobile phase of acetonitrile (A) and 0.1 % v/v formic acid in water (B). The gradient (with respect to v/v % of B) was as follows: 0–2 min, 70; 2–7 min, 70 → 30; 7–15 min, 30; 15–20 min, 30 → 70, and 20–25 min, 70. Calibration curve for each marker in the concentration range of 0, 5, 10, 20, 40, 80, and 160 μg/mL was prepared. For simultaneous estimation of markers in IIIM-141 and IIIM-141-CEF, ~5 mg of extract was transferred in an Eppendorf tube containing 1 mL of MeOH, and the mixture was sonicated. The Eppendorf tubes were centrifuged at 16 000 RCF (G-force) for 10 min to separate the solid matrix, if any. The supernatant was injected into the HPLC system after diluting it with MeOH to get a concentration of 50 μg/mL. The % content of each marker was calculated using a regression equation obtained from the calibration curve of each reference standard of a marker. The data are shown in Table 1.

The extract was also evaluated for acid-insoluble ash, loss on drying at 105 °C, total ash, total sugar, crude fat, protein, moisture content, 50% alcohol-soluble extractive, aflatoxin/pesticide content,38 and microbial load using the procedures described previously.9,13 Aflatoxins content (B1, B2, G1, and G2) was determined using an Official Methods of Analysis of AOAC International.10–32 Microbial testing31 of the extract was performed as per British Pharmacopoeia33 to find out the total bacterial count, total yeast and mold count, and the count of Enterobacteriaceae and other Gram-negative bacteria (E. coli, Salmonella spp., S. aureus, and P. aeruginosa).

In vitro Efficacy. The mechanism of action of IIIM-141 was unraveled by performing various in vitro assays viz. NLRP inflammasome inhibition,8 P-gp induction,38 and neuro-protection34 against amyloid-β- and glutamate-induced toxicities. Before performing in vitro mechanistic studies, the toxicity window of the test item was determined in each cell line using the MTT assay.56

Inhibition of NLRP3 Inflammasome. Human monocytes, THP-1 cells were differentiated with phorbol 12-myristate 13-acetate (20 ng/mL) for 24 h. The media was changed after 24 h, and the cells were kept in rest of two days in complete Roswell Park Memorial Institute medium containing 10% heat-inactivated fetal calf serum. Cells were primed with LPS (1 μg/mL) for 4 h, followed by pretreatment with different concentrations of IIIM-141 for 30 min. Then, the cells were stimulated with 10 μM nigericin for 1 h. The supernatant was collected and stored at −20 °C for enzyme-linked immunosorbent assay of IL-1β. The secretion of IL-1β with nigericin was measured by BD OptEIA for IL-1β (human) and was considered as readout for NLRP3 activation.38

P-gp Induction Activity of IIIM-141 in LS-180 Cells. IIIM-141 was screened for its ability to induce P-gp using rhodamine 123 (Rh123) cell exclusion method in colorectal LS-180 cells (ECACC catalog number 87021202; passage number S2), as described earlier.34 In this method, P-gp function was evaluated for accumulation and efflux of rhodamine 123 (Rh123). Rifampicin was used as the positive control for the experiment. The Western blot analysis of IIIM-141 in LS-180 cells was also carried out as described earlier.34

Neuroprotection of SH-SY5Y Cells Against Amyloid-β- and Glutamate-Induced Toxicities. The neuroprotection activity of IIIM-141 was evaluated in differentiated SH-SY5Y cells, using the protocol described earlier.4,34 Briefly, 10 000 SH-SY5Y cells (passage no. 13) were cultured in MEM/F12 media into each well of a 96-well plate. Cells were further differentiated with 10 μM retinoic acid for 1 week. Six wells were used for each treatment, and the test samples were added 30 min prior to the treatment with amyloid-β or glutamate for 24 h. Ginkgolide B was used as a standard neuroprotective agent16 in this assay. MTT (1.2 mM) was added 4 h prior to the termination of the experiment.35 The cell viability of the vehicle-treated cells was considered as 100%, whereas the viability of the cells treated with test samples was calculated.

In Vivo Efficacy. The in vivo anti-dementia effect of IIIM-141 was assessed using two behavioral tests: (a) intra-cerebroventricular STZ-induced dementia in SD rats using MWM test,17,23 and (b) scopolamine-induced dementia in SAM using passive avoidance test.18

MWM Test in SD Rats. Animals were randomized into four groups (G1 to G4) based on body weight, with each group consisting of five to seven rats. The entire study duration was 18 days. On days 1 and 3, aCSF (147 mM NaCl, 2.9 mM KCl, 1.6 mM MgCl2, 1.7 mM CaCl2, and 2.2 mM dextrose) was injected to group G1 animals. STZ (3 mg/kg) was administered on days 1 and 3 through the ICV route to animals from G2–G5 groups. The treatment was performed from 15th–18th day, wherein the vehicle (0.25% carboxymethyl cellulose, p.o.) was administered to G1–G2 animals, and the test item IIIM-141 (50 and 100 mg/kg, p.o.) was administered to G3–G4 animals. The rats were subjected to MWM test, 30 min after dosing comprising 4 trials for 4 days, conducted on each day from the 15th to 18th day. The rats were put for 90 s in a circular water pool and were observed for their ability to locate the hidden platform in the pool. If the animal failed to locate the hidden platform in the stipulated time, they were gently pushed to the hidden platform and kept on the platform for 20 s. Time (seconds) to locate the hidden platform was recorded as “escape latency” (latency time). The escape latency was recorded for retention session (i.e., from day 16–18). The difference (% change) in escape latency was compared with the G1 group. The % change in the latency time was analyzed and compared with G1 by one-way analysis of variance (ANOVA) followed by Tukey test.

Passive Avoidance Test in SAM. Animals were randomized into five groups according to their body weight, with each group consisting of six to eight mice. The total study duration was 8 days, and the treatment plan was from the first to seventh day. The groups G1, G2, and G5 served as the negative control, vehicle control, and positive control, respectively. During the treatment plan, 0.25% CMC was administered to G1 and G2. Furthermore, 50 and 100 mg/kg of IIIM-141 were administered perpendicular to G3 and G4 animals. G5 was the positive control group, which received rivastigmine 2 mg/kg, p.o. On the seventh day, the groups G2–G5 received scopolamine (3 mg/kg) via intraperitoneal injection just after administration of the vehicle/test item. All animals were habituated to the experimental apparatus before the initiation of the experiment by placing them in the light compartment of the apparatus. After 5 s, the guillotine door
was opened and the animals were allowed to enter the dark compartment without giving the shock. Animals that took more than 100 s to enter the dark room were eliminated from the experiment. For acquisition trial, the animals were placed in the light compartment and allowed to enter the dark compartment through guillotine door. As soon as the animal entered the dark compartment, the door was closed, and the animal was delivered a foot shock (0.5 mA current) for the duration of 5 s. Animals were then removed from the apparatus after 20 s and placed temporarily in its home cage. The same procedure was repeated with an interval of 5 min between acquisition trials, till the animal remained in the light compartment for 120 s consecutively. During the retention trial, on day 2, retention of animals in the dark chamber was recorded without any shock. Increase in the transfer latency time from the light to dark chamber indicated learning in animals. The data were acquired and compared with the control as the transfer latency (in seconds), that is, the time taken by each mouse to move from the light to dark compartment. The % change in transfer latency as compared with the control on the retention trial was calculated. The G2 group which received scopalamine was compared with G1, whereas the groups G3−G5 were compared with G2. For statistical analysis, one way ANOVA followed by Tukey test was used.

Regulatory Safety Studies. The acute toxicity study of IIIM-141 was carried out in healthy female Wistar rats weighing 116−125 g, according to Fixed Dose Procedure adopted from OECD Guideline no. 420. A 28-day repeated dose oral toxicity study (as per OECD guideline no. 407) was then conducted to determine the toxicity profile of IIIM-141 when administered daily (at 25, 50, and 100 mg/kg) for 28 days in Wistar rats via the intended clinical route, which is oral.

Chemical and Enzymatic Hydrolytic Stability of Crocin. The hydrolytic stability of crocin was determined in various buffer solutions (pH 1.2 to 10.0), biorelevant media, namely, SGF (pH 1.2) and SIF (pH 6.8), rat plasma, human saliva, and in the presence of enzymes viz. esterase and lipase.

Formulation Development, In Vitro Dissolution, and Characterization. The polymeric granules of IIIM-141 with various SR polymers were prepared by weight granulation by mixing the extracts and polymers in a mortar and pestle in geometric proportions. HPMC-K4M, HPMC-K15M, ethyl cellulose 10 cps, and Eudragit RS100, alone and in combinations, were used as release retardants in the range of 70−30 % w/w. The obtained mixture was kneaded using 10 % w/v PVP-K30 in isopropl alcohol solution as a binder to form a dough. The dough was then passed through sieve #10, and the obtained granules were dried in a vacuum desiccator at room temperature. The dried granules were passed through sieve #10 and retained on #30. The granules were collected and stored in a desiccator at room temperature for further analysis. The polymeric granules were assayed using the developed HPLC method for crocin-1 content. The formulation details are given in Table 4. The granules equivalent to 50 mg of crocin-1 were filled into the hard gelatin capsules of size "0." The in vitro dissolution of these capsules was performed using a USP dissolution apparatus (Lab-India Dissolution Tester, model: DS 8000; type 1—basket) by buffer change method with 900 mL of dissolution medium. The extent of dissolution was quantified from the AUC ratio ([AUC(0–t),SR formulation]/[AUC(0–t),extract]). The optimized formulations CSHA-14 and CSE-2 were characterized using DSC and FTIR spectroscopy to find out the possibility of drug−excipient interaction, if any.

Pharmacokinetics of Crocin, Crocetin, IIIM-141-CEF, and SR Formulation. The pharmacokinetic study was performed in male SD rats. The oral bioavailability of crocin and crocetin was determined by administering them at 5 and 50 mg/kg intravenously and by oral route, respectively. The comparative oral pharmacokinetics of IIIM-141-CEF and CSE-2 was then performed. The plasma samples were analyzed for crocin-1 and crocetin contents using a fit-for-purpose LC−MS/MS method with a lower limit of quantification of 9.95 ng/mL. The pharmacokinetic parameters of crocin and crocetin were calculated using the noncompartmental analysis tool of validated Phoenix WinNonlin software (version 6.3).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00841.

1 Structures of chemical constituents of C. sativus and LC-MS and HPLC spectra (PDF)

AUTHOR INFORMATION

Corresponding Authors
*E-mail: ram@iiim.ac.in. Phone: +91 191 2569111. Fax: +91 191 2569333 (R.A.V.).

*E-mail: sbharate@iiim.ac.in. Phone: +91 191 2569006. Fax: +91 191 2569333 (S.B.B.).

ORCID
Sonali S. Bharate: 0000-0001-7267-5080
Deepika Singh: 0000-0001-5347-6713
Ram A. Vishwakarma: 0000-0002-0752-6238
Sandip B. Bharate: 0000-0001-6081-5787

Author Contributions
S.S.B. designed, executed, and interpreted the results of formulation development and its analysis. S.S.B. isolated marker compounds required for standardization of the extract using semiprep HPLC. S.S.B. and V.K. prepared the formulations and performed its analysis and also performed chemical and enzymatic stability of crocin; V.K. prepared extracts of C. sativus; G.S. and A.S. designed and executed safety pharmacology studies; D.S. performed quality control studies; M.G. and A.K. designed and executed in vitro inflammasome and P-gp assays; S.B.B. and R.A.V. designed, executed, and coordinated the whole study; and S.B.B., S.B.B., and R.A.V. contributed to manuscript writing.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
The authors acknowledge the financial support received from the CSIR 12th FYP project BSC-0205 and the CSIR Phytopharmaceutical Mission project HCP-0010. V.K. is thankful to the UGC for the research fellowship. S.S.B. is a Women Scientist (DBT-BioCARe) receiving the fellowship from the Department of Biotechnology Funded project GAP-2158. S.B.B. thanks CSIR-YSF (Young Scientist Award) Research Grant for the financial support to some of the
activities of this work. The authors thank the Analytical Department, IIMM for the analytical support. We acknowledge the support provided by Colorcon Asia Ltd., Evonik Industries, and Natural Capsules Ltd. by providing us the gift samples of polymers and empty hard gelatin capsules required for this research.

ABBREVIATIONS

aCSF, artificial cerebrospinal fluid; CAP, cellulose acetate phthalate; ECACC, European Collection of Authenticated Cell Cultures; HBSS, Hank’s buffered salt solution; HPMCP, hydroxy propyl methyl cellulose phthalate; HPMCK1SM, hydroxypropylmethylcellulose-K15SM; ICV, intra-cerebroventricular; LS-180, colon adenocarcinoma cell line; LRPI, low-density lipoprotein receptor-related protein 1; NLRP3, NOD-like receptor (NLR) subfamily; OECD, Organisation for Economic Co-operation and Development; PVP-K30, polyvinylpyrrolidone K 30; P-gp, p-glycoprotein; Rh123, rhodamine 123; SAM, Swiss albino mice; STZ, streptozotocin; THP-1, human monocyte cell line; TBST, mixture of trisbuffered saline (TBS) and Tween 20; STZ, streptozotocin

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