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ADME/PK Insights of Crocetin: A Molecule Having an Unusual Chemical Structure with Druglike Features

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ABSTRACT: Crocetin is a promising phyto-based molecule to treat Alzheimer's disease (AD). The chemical structure of crocetin is incongruent with various standard structural features of CNS drugs. As poor pharmacokinetic behavior is the major hurdle for any candidate to become a drug, we elucidated its druggable characteristics by implementing in silico, in vitro, and in vivo approaches, as limited ADME/PK information is available. Results demonstrate several attributes of crocetin based on rules of drug-likeness, lipophilicity, pK_a, P-gp inhibitory activity, plasma stability, RBC partitioning, metabolic stability, CYP inhibitory action, blood-brain barrier (BBB) permeability, oral bioavailability, and pharmacokinetic interaction with marketed anti-Alzheimer's drugs (memantine, donepezil, galantamine, and rivastigmine). However, aqueous solubility, chemical stability, plasma protein binding, and P-gp induction are some concerns associated with this molecule that should be taken into consideration during its further development. Overall results indicate favorable ADME/PK behavior and potential druggable candidature of crocetin.

1. INTRODUCTION

Crocin, a water-soluble carotenoid, is found in the stigmas of *Crocus sativus* Linne and the fruits of *Gardenia jasminoides* Ellis.[1] It has been explored for medicinal purposes against diverse disease conditions, including neurodegeneration.[2−4] It is reported that it can counteract microglial activation, mitochondrial dysfunction, and neuroinflammation, which are associated with the onset and progression of critical brain pathological conditions like Alzheimer's disease (AD).[5] Chemically, crocin is the diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin.[6] Because of its chemical nature, only trace amounts of crocin were detected in the systemic circulation after oral administration. In contrast, crocetin (aglycone of crocin) was exposed 56−81-fold higher than crocin.[7] It indicates that crocetin is possibly the active molecule behind the pharmacological actions of crocin. Moreover, crocetin has also been explored for its medicinal use, especially for its neuroprotective features. Several research works demonstrate its promising action to manage neurodegenerative diseases, including AD. AD is the most prevalent neurodegenerative disease. However, hardly any disease-modifying therapy is available to cure or successfully manage the pathological conditions of AD. In this direction, literature evidence at the in vitro/ex vivo level advocates that crocetin has multifaceted pharmacological actions for managing AD (Figure 1).[8−12] Additionally, crocetin is found to be beneficial at the preclinical level due to its anti-inflammatory and memory-enhancing properties.[13,14] However, therapeutic effi-

cacy at the preclinical/clinical level against AD is yet to be established to strongly support its candidature as a therapeutic option for AD.

Plant-based natural products have immense potential to become drugs as a plethora of marketed drugs originate from plants either in a beeline or in a roundabout way.[15] Overwhelmingly, phyto-based drugs in the domain of AD therapy are not an exception.[16] The history of traditional usage, the acknowledged health benefits, and the scientific data gathered over a long period indicate its potential to transform into a drug. The key issue associated with developing a new drug is its unfavorable pharmacokinetics.[17] Renaissance of a phytochemical to become a drug, a molecule with efficient action must reach its target in the body at adequate concentration and stay there in a bioactive form for a sufficient period in order to obtain the expected biological effects. Therefore, absorption, distribution, metabolism, and excretion (ADME) characteristics of any new chemical entity are assessed in the early steps of drug development. It is beneficial to fasten the drug discovery process, trim down the attrition rate at the later stage of drug development, as well as scrimp,

Figure 1. Pictorial representation for pharmacological actions of crocetin against Alzheimer's disease at the in vitro/ex vivo level.

and save both time and money.[18] Despite various beneficial pharmacological actions of crocetin against AD, little information is known about its pharmacokinetic behavior.[7,19−22] Hence, evaluating its drug-likeness via in silico, in vitro, and in vivo approaches has become imperative to strengthen its candidature.

2. RESULTS AND DISCUSSION

2.1. Chemical Structure-Based Druggable Features of Crocetin. CNS drug discovery is a significant challenge for researchers due to its low success rate. Plant-based natural products have the legacy of discovering new drugs for CNS disorders and maintaining the same trajectory.[16] The present study deals with the druggability of crocetin. However, several facts postulate its nondrug-likeness under the CNS category. Crocetin is chemically a polyunsaturated conjugated acid, i.e., structurally similar to fatty acids (Figure 2). Due to the
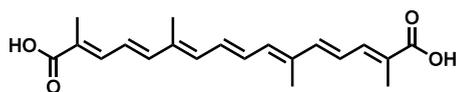
Figure 2. Chemical structure of crocetin.

presence of seven conjugated double bonds and two carboxylic acids, it may be prone to oxidation and acidic degradation, leading to instability. Polyene structure is uncommon among the USFDA-approved drugs and lacks in the CNS class as well. A few antifungal drugs (amphotericin B, nystatin, and natamycin) are from the class of polyenes, which are associated with poor oral bioavailability and severe toxicity.[23] The presence of heteroatoms, especially nitrogen, is desirable for BBB permeability attributes, as evidenced by marketed CNS drugs.[24] However, the chemical structure of crocetin is deficient in any heteroatoms except four "O" atoms. In

addition, fewer hydrogen bond donors may also lower the chances of BBB permeability, as evidenced by codeine (BBB−) vs morphine (BBB+).[16] Most CNS category drugs, however, are basic in nature and have a pK_a value >4.[25] The predicted pK_a value dictates its inability to cross the BBB (Table 1). Availability of rings (higher number/fused ring) and stereocenters are important aspects for any class of drugs toward selectivity/binding affinity/potency of the molecule. However, the chemical structure of crocetin does not have such attributes (Table 1). Chemical structure-based computational tools help to filter out nondrug-like molecules. In this context, we evaluated various physicochemical properties of crocetin with the help of extensively used computational tools and freely available software to evaluate its drug-likeness. In silico analysis of crocetin drug-likeness classified it as a nondrug (Table 1). Generally, for CNS drugs, optimum lipophilicity ($1 < \log D > 3$) should be there to facilitate their uptake in CNS cells and tissue.[16] In silico data of crocetin for $\log P/\log D$ are not encouraging as a candidate for CNS class. Any CNS class of drugs abundantly contains chiral centers, thereby having an increased number of stereoisomers responsible for their selectivity/efficacy,[26] in contrary crocetin structure is deficient of this feature (Table 1). Therefore, the chemical structure of crocetin is incongruent with the various standard structural features of CNS drugs.

2.2. Drug-Likeness of Crocetin. In spite of implementing successful strategies, more than 90% of clinical drug development has failed in the last decades.[27,28] Poor drug-likeness is one of the crucial bottlenecks for such clinical failures. Although it is not mandatory to follow the rules of drug-likeness as there are drugs available in the market that violate one/more than one criterion(s) of the major rules of drug-likeness, like antibiotics (glycopeptides/polymyxins/rifamycins), antifungals (polyenes/echinocandins), cardiac glycosides (digoxin/digitoxin) and many more violate the Lipinski's rule

Table 1. In Silico Prediction for Physicochemical Properties of Crocetin Using Various Software

parameter	optimum range	result
molecular formula		$C_{20}H_{24}O_4$ [a,b]
molecular weight	100 to 600	328.40[a]; 328.17[b,c]; 328.41[d]
number of heavy atoms	<36	24[a]
number of aromatic heavy atoms		0[a]
number of rotatable bonds	0 to 11	8[a,c,d]
number of H-bond acceptors	0 to 12	4[a−c]; 2[d]
number of H-bond donors	0 to 7	2[a−d]
number of rings	0 to 6	0[c]
number of atoms in the biggest ring	0 to 18	0[c]
number of heteroatoms	1 to 15	4[c]
number of rigid bonds	0 to 30	9[c]
number of stereocenters	≥2	0[b,c]
molar refractivity	40 to 130	98.48[a]
topological polar surface area	0 to 140	74.60[a,c]; 57.33[b]
pK_a		<0.0/4.38[b]
log P	0 to 3	3.00 (consensus)[a]; 4.96[b]; 1.37[c]; 4.61[d]
log D	1 to 3	−0.29[c]
log S	−4.0 to 0.5	−3.36; soluble (ESOL)[a]; −4.34[b]; −3.69[c]; −2.83[d]; −0.97[e]
drug-likeness model score	0−2 (drugs); < 0 (nondrugs)	−1.20[b]

[a]SwissADME. [b]molsoft. [c]ADMETlab. [d]pkCSM. [e]admetSAR.

Table 2. In Silico Prediction for Drug-Likeness of Crocetin Using Various Software

parameter	optimum range	result
Lipinski's rule	MW ≤ 500[b]	yes; 0 violation[a]
	log P ≤ 5	accepted[c]
	H-bond acceptors ≤10	
	H-bond donors ≤5	
Ghose's rule	−0.4 ≤ log P ≤ 5.6	yes[a]
	160 ≤ MW ≤ 480	
	40 ≤ molar refractivity ≤130	
	20 ≤ total number of atoms ≤70	
Veber's rule	number of rotatable bonds ≤10	yes[a]
	PSA ≤ 140	
Egan's rule	−1.0 ≤ log P ≤ 5.8	yes[a]
	TPSA ≤ 130	
Muegge's rule	200 ≤ MW ≤ 600	yes[a]
	−2.0 ≤ log P ≤ 5.0	
	TPSA ≤ 150	
	number of rings ≤7	
	number of carbons >4	
	number of heteroatoms >1	
	number of rotatable bonds ≤15	
	H-bond acceptors ≤10	
	H-bond donors ≤5	
Pfizer's rule	log P > 3	accepted[c]
	TPSA < 75	
GSK's rule	MW ≤ 400	accepted[c]
	log P ≤ 4	
Golden triangle rule	200 ≤ MW ≤ 500	accepted[c]
	−2.0 ≤ log D ≤ 5.0	

[a]SwissADME. [b]molsoft. [c]ADMETlab.

of 5.[29] Basically, in silico approaches are being used nowadays that help medicinal chemists to filter out the odd candidates at the early stages of development. In the current study, crocetin as a test compound abides by the set criteria of the widely recognized rules for drug-likeness (Table 2). Therefore, nonviolation of any rule indicate the druggability of crocetin.

In addition, we also evaluated various pharmacokinetic properties of crocetin in order to check the resemblance between in silico (Tables 1 and 3) versus in vitro/in vivo results. Relevant information is discussed in the respective sections of in vitro or in vivo data.

2.3. Aqueous Solubility of Crocetin. Solubility is one of the critical physicochemical properties of a molecule in order to achieve its desired concentration in systemic circulation for effective pharmacological activity. Basically, it affects the dissolution process (rate-limiting step for absorption) that aids in the absorption of any molecule. Therefore, we determined the equilibrium solubility of crocetin in the various biorelevant media using the widely accepted shake-flask method.[30] Chlorzoxazone (high aqueous solubility, >100 μg/mL), budesonide (moderate aqueous solubility, 10−100 μg/mL), and astemizole (low aqueous solubility, <10 μg/mL) were used as standards (Figure 3A−C), and results are matched with the reported literature.[31,32] The measured solubility of crocetin in the various experimental media is in the following order (Figure 3D): SGF/FaSSGF/FeSSGF (<0.5 μg/mL) < Water (0.6 μg/mL) < FaSSIF (3.2 μg/mL) < FeSSIF (6.5 μg/mL) < SIF (12.0 μg/mL) < PBS (13.9 μg/mL). Thus, results suggest that crocetin belongs to the

category of low aqueous soluble compound. Moreover. we observed that its solubility increases in the biorelevant media with increasing pH. The water solubility of crocetin was predicted by various software (except molsoft) to be in the optimum range (soluble), which deviates from the experimental value (Table 1). Poor water solubility of crocetin is obviously an undesirable physicochemical property to be a drug. Considering the CNS class of drugs, ziprasidone is an antipsychotic drug with low aqueous solubility.[33] However, the marketed anti-AD drugs have aqueous solubility from sparingly to very soluble.[34−37] In this context, BCS class II & IV drugs also suffer from low aqueous solubility. Various formulation strategies are generally adopted to improve the solubility of such drugs.[38]

2.4. Lipophilicity of Crocetin. The lipophilicity characteristic of any molecule is one determinant that influences its oral absorption process. Therefore, the lipophilicity of crocetin was examined in the form of log P and log D by adopting a widely used shake-flask method.[39] The results of log P and log D for testosterone and paracetamol as high and low lipophilicity standards, respectively, have a good agreement with the reported value (Figure 3E,F).[40] The log P and log D of crocetin were found to be 1.23 ± 0.04 and 0.70 ± 0.01, respectively (Figure 3E,F). It should be noted that considering the low solubility of crocetin, we used it after being dissolved in DMSO, which could have led to an underestimation of its lipophilicity due to cosolvency. The experimental log P value is closely matched with only the predicted value of 1.37 from ADMETlab software (Table 1). Based on our experimental results, crocetin has the optimum lipophilicity (0 < log P < 3),

Table 3. In Silico Prediction for ADME/PK Parameters of Crocetin Using Various Software

parameter	optimum range	result
GI absorption		high[a]
intestinal absorption		0 to 0.1 (probability of intestinal absorption <30%)[c]; 95.25%[d]; 0.86 (probability)[e]
Caco-2 permeability	>−5.15	−5.51[c]; 0.73 (log P_{app})[d]; 0.67 (log P_{app})[e]
P-gp inhibitor		0−0.1 (probability)[c]; no[d]; no (probability: 0.94−0.96)[e]
plasma protein binding (%)	<90	92.92[c]
fraction unbound in plasma	low: <5%; middle: 5−20%; high: >20%	5.73%[c] 0.108[d]
CYP1A2 inhibitor		no[a]; 0.5−0.7 (probability)[c]; no[d]; no (probability: 0.96)[e]; no[f]; 0.21 (probability)[g]
CYP2C19 inhibitor		no[a]; 0.1−0.3 (probability)[c]; no[d]; no (probability: 0.95)[e]; no[f]; 0.22 (probability)[g]
CYP2C9 inhibitor		no[a]; 0.3−0.5 (probability)[c]; no[d]; no (probability: 0.87)[e]; no[f]; 0.13 (probability)[g]
CYP2D6 inhibitor		no[a]; 0.7−0.9 (probability)[c]; no[d]; no (probability: 0.92)[e]; no[f]; 0.19 (probability)[gg]
CYP3A4 inhibitor		no[a]; 0−0.1 (probability)[c]; no[d]; no (probability: 0.91)[e]; no[f]; 0.12 (probability)[g]
CYP inhibitory promiscuity		low (probability: 0.96)[e]
BBB permeability	−1 < log BB < 0.3	yes[a]; 0−0.1 (probability)[c]; −0.143 (log BB)[d]; 0.65 (probability)[e]
BBB score	6-high; 0-low	2.96[b]
CNS permeability (log PS)	log PS ≥ −2.00	−2.188[d]
half-life ($T_{1/2}$, h)	long: >3 h; short: <3 h	>3 h (probability: 0.74)[c]
volume distribution (L/kg)	0.04−20	0.56[c]
		−1.35 (log L/kg)[d]
clearance (mL/min/kg)	high: > 15; moderate: 5−15; low: < 5	0.74[c]; 1.20 (log mL/min/kg)[d]
bioavailability score ($F_{10\%}$)		0.85 (probability in rat)[a]

[a]SwissADME. [b]molsoft. [c]ADMETlab. [d]pkCSM. [e]admetSAR. [f]CYPrules. [g]CYPlebrity.
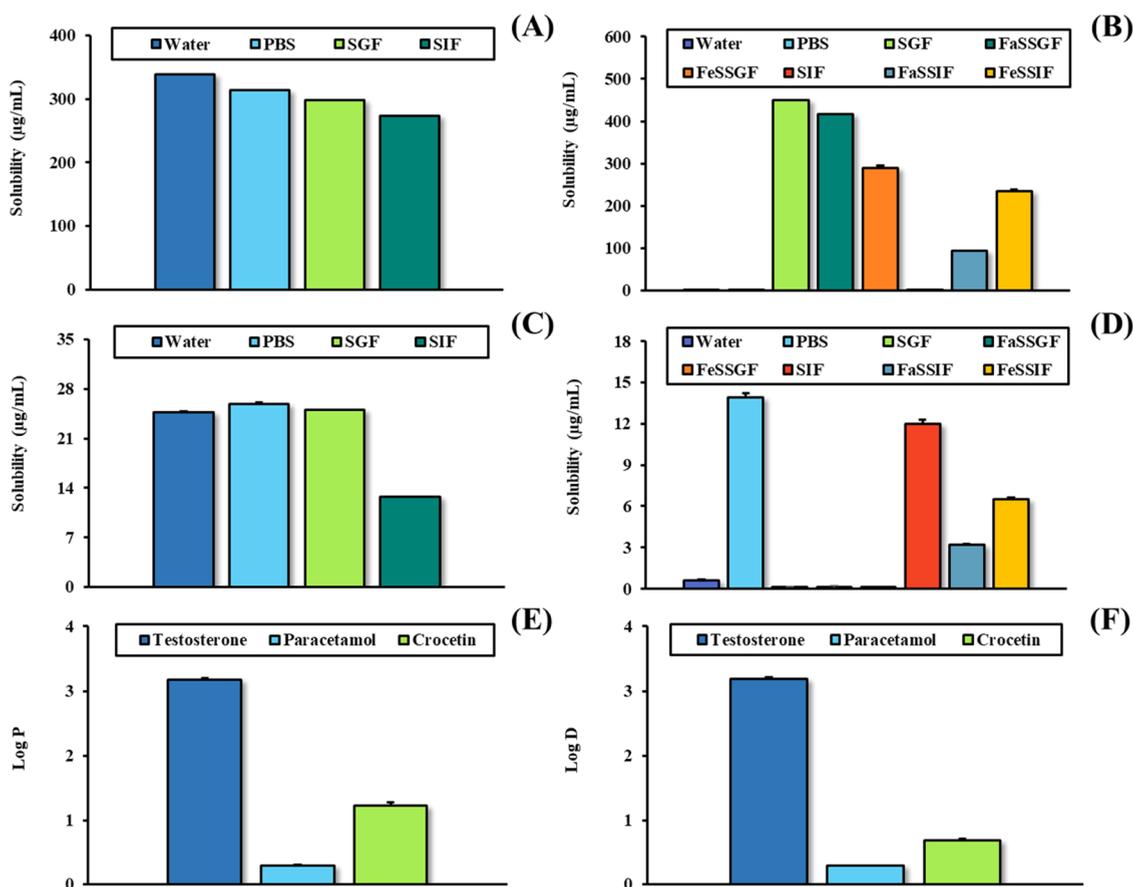
Figure 3. Solubility of chlorzoxazone/standard (A), astemizole/standard (B), budesonide/standard (C), and crocetin (D) in the various biorelevant media. log P (E) and log D (F) of crocetin along with testosterone/standard and paracetamol/standard. Data are represented as the mean ± SEM ($n = 3$).

which can be favorable for oral absorption through the GI tract.

2.5. Ionization constant (pK_a) of Crocetin. The pK_a is one of the key physicochemical properties of any molecule that dictates the degree of ionization (nonionizable/ionizable
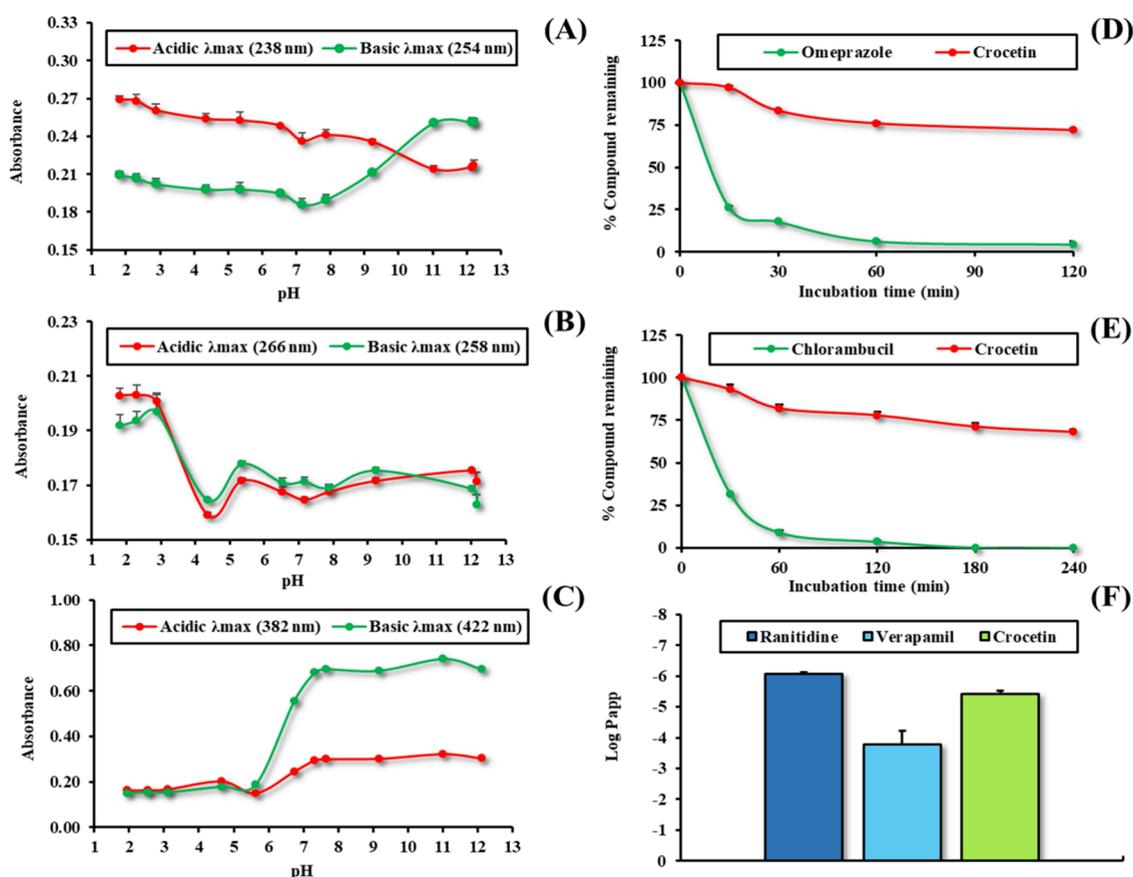
Figure 4. pK_a data of paracetamol/standard (A), isoniazid/standard (B), and crocetin (C). Chemical stability data of crocetin in SGF (D) and SIF (E) along with omeprazole/standard and chlorambucil/standard. Permeability data of crocetin along with ranitidine/standard and verapamil/standard (F), respectively. Data are represented as mean ± SEM (n = 3).

species) at a particular pH and subsequently influences the absorption phenomenon. Therefore, we measured the pK_a of crocetin with an extensively used UV-metric method by analyzing changes in multiwavelength UV spectra during acid−base titration of the test sample.[41] Paracetamol and isoniazid were used as standards (Figure 4A,B), and results are matched with their reported values in the literature.[42] The pK_a of crocetin was found to be 5.35 (Figure 4C). The predicted pK_a value is lower than that of the experimental outcomes (Table 1). The experimental results suggest that it belongs to the category of weak acid. Thus, it is expected to be predominantly in the un-ionized form in the stomach and in the small intestine to become readily available for absorption, depending on its permeability characteristics. Additionally, a weakly acidic compound tends to be more soluble at pH values higher than its pK_a value, and this is closely correlated to its increased solubility behavior in the higher pH of the dissolution media (Figure 3D). Thus, the pK_a value of crocetin indicates its brain-permeable nature as it is evident that an acidic molecule having a pK_a value <4 does not cross the BBB.

2.6. Chemical Stability of Crocetin. Compounds designed for oral therapy should be sufficiently chemically stable in SGF or SIF to be an acceptable route of administration. Therefore, we evaluated the chemical stability of crocetin in SGF and SIF based on the extensively used substrate degradation approach.[39] Omeprazole and chlorambucil were used as standards for SGF and SIF, respectively (Figure 4D,E), and the results corroborate with the reported literature.[43,44] Crocetin was intact up to 72% in SGF and 68%

in SIF at the experimental time frame of 2 and 4 h, respectively (Figure 4D,E). Any candidate is generally recognized as stable if its degradation does not exceed 15% and affects the extent of its absorption. Therefore, crocetin is a candidate with reasonable instability (SGF/SIF), which would make it a limiting factor for its adequate oral absorption. Therefore, the concern of chemical stability must be taken into account during the development process. For instance, marketed drugs from the class of penicillin and proton pump inhibitors are chemically unstable (acid labile), thus, pro-drug strategies (esterifies to NCE) and formulation (enteric-coated) approaches are used, respectively.[45,46]

2.7. GI Permeability of Crocetin. Permeability is one of the rate-limiting steps for the oral absorption of a molecule from the GI tract into the systemic circulation. Therefore, we determined the permeability of crocetin using the conventionally used parallel artificial membrane permeability assay (PAMPA) model.[47] Verapamil (high-permeable) and ranitidine (low-permeable) were used as standards (Figure 4F), and the results are consistent with their reported values.[48,49] Crocetin exhibited a log P_{app} value of −5.41 ± 0.13, which indicates crocetin as a low-permeability compound (log P_{app} < −5) (Figure 4F). However, predicted results dictate the higher chances of their absorption through the GI tract (Table 3). Lautenschlager et al. reported a similar line of observation, where crocetin was able to permeate the Caco-2 barrier with a high velocity (P_{app} = 2.6 ± 0.6 × 10^{-5} cm/s) and further concluded that crocetin can absorb by passive transcellular diffusion within a short time interval over the intestinal
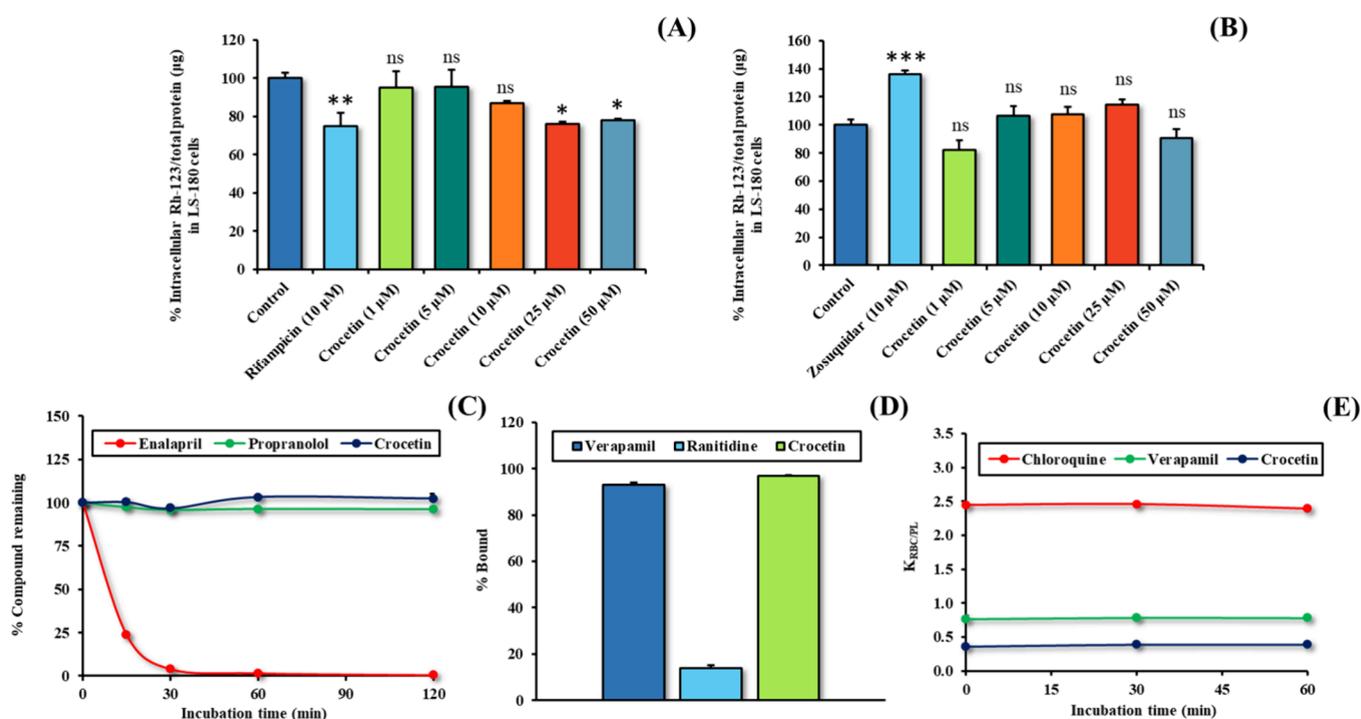
Figure 5. P-gp induction (A) and P-gp inhibition (B) data of crocetin along with rifampicin/standard and zosuquidar/standard, respectively; statistical significance $p < 0.05(*)/0.01(**)/0.001(***)$ with respect to control, ns denotes statistically not significant. Data are represented as mean $\pm$ SEM ($n = 2$). Plasma stability (C) data of crocetin along with enalapril/standard and propranolol/standard. Plasma protein binding (D) data of crocetin along with verapamil/standard and ranitidine/standard. RBC partitioning (E) data of crocetin along with chloroquine/standard and verapamil/standard. Data are represented as mean $\pm$ SEM ($n = 3$).

barrier.[50] Most in silico data on Caco-2 permeability also point toward its high permeability (Table 3). The present results can possibly linked to the limitation of our experimental in vitro model, which dictates only the transcellular passive diffusion process. Therefore, absorption of crocetin from the GI tract may also occur via alternative processes like carrier-mediated uptake, active transport mechanisms, and so on, subject to further studies at the in vivo level.

2.8. P-gp Induction Ability of Crocetin. P-gp is one of the most well-recognized efflux transporters in several tissues, including the intestine and brain. Therefore, we assessed the P-gp induction potential of crocetin using the in vitro model based on intracellular accumulation of Rh123 in LS180 cells.[51] As a standard, rifampicin exhibited significant induction of P-gp, i.e., a decrease in intracellular accumulation of Rh123 in treated cells compared to the control (Figure 5A). The results of the standard are in line with the reported value.[47] Crocetin displayed significant induction (22−24%) of the P-gp at the concentration level of 25−50 μM (Figure 5A). Thus, the results indicate that crocetin could induce P-gp. Therefore, it may lead to transporter-mediated drug interactions as evident for carbamazepine and phenobarbital from the CNS class of drugs.[52] On the other hand, P-gp is one of the crucial Aβ transport proteins located at the endothelial cells of the BBB, and a decrease in the P-gp expression has been found in the brain of Alzheimer's disease patients.[53] Anti-Alzheimer drugs like donepezil, galantamine, and rivastigmine are known to induce P-gp.[54] Therefore, the P-gp induction potential of crocetin may be beneficial to increase Aβ clearance, an attractive therapeutic approach under investigation to prevent and/or delay the onset of AD.[55,56]

2.9. P-gp Inhibition Ability of Crocetin. Alike P-gp induction, inhibition of P-gp has shown to be mainly responsible for several drug interactions.[57,58] Therefore, we evaluated the P-gp inhibition activity of crocetin using a similar in vitro model based on intracellular accumulation of Rh123 in LS180 cells.[51] As a standard, zosuquidar showed significant inhibition of P-gp, i.e., an increase in intracellular accumulation of Rh123 in treated cells compared to the control (Figure 5B). Results corroborate the reported findings.[39] Crocetin exhibited no marked P-gp inhibitory activity at the concentration level of 1 to 50 μM (Figure 5B). A similar forecasting on the inability of crocetin to inhibit P-gp was obtained from in silico data (Table 3). Therefore, the results suggest that crocetin could be safely coadministered with the drug (P-gp substrate) and is unlikely to precipitate any P-gp inhibition-mediated drug interaction.

2.10. Plasma Stability of Crocetin. In pursuance of observing desired pharmacological effects, any candidate should be stable in plasma, except prodrugs, which are commonly susceptible to hydrolysis by plasma enzymes for converting to their pharmacologically active form. Therefore, we elucidated the stability of crocetin in rat plasma using enalapril and propranolol as the standards (Figure 5C). The results of positive control (enalapril) and negative control (propranolol) are aligned with the reported literature.[59,60] Crocetin displayed a negligible degradation (<5%) over the experimental time frame (Figure 5C). Hence, the results reveal that crocetin is highly stable in plasma and remains intact there.

2.11. Plasma Protein Binding of Crocetin. The extent of binding of the drug to plasma protein is linked to the free drug concentration in plasma and its distribution into the
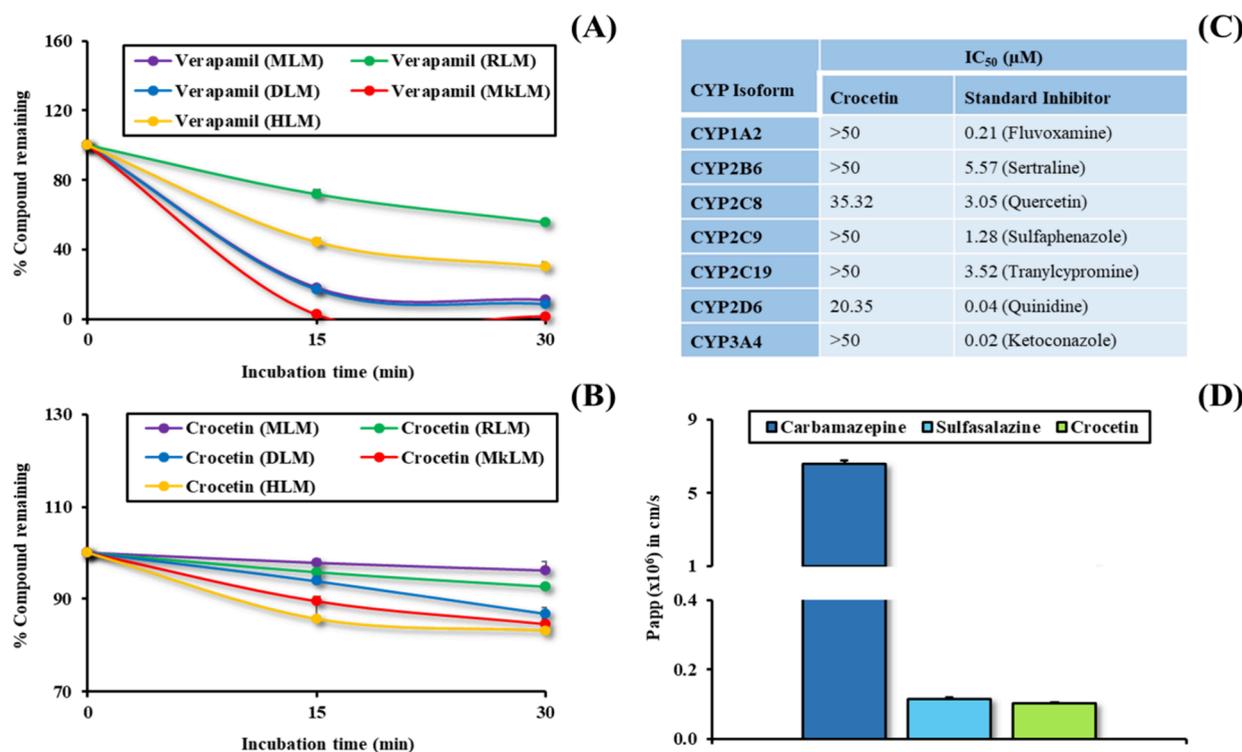
Figure 6. Metabolic stability data of verapamil/standard (A) and crocetin (B) in various liver microsomes. CYP inhibition data of crocetin along with the standard for various CYP isoforms (C). Brain permeability data of crocetin along with carbamazepine/standard and sulfasalazine/standard (D). Data are represented as mean $\pm$ SEM (n = 3).

various tissues. Therefore, we determined the plasma protein binding of crocetin using a well-accepted rapid equilibrium dialysis method.[61] Plasma protein binding of verapamil (high protein bound) and ranitidine (low protein bound) as standards were evaluated, and the results corroborate with the reported values (Figure 5D).[62,63] Crocetin exhibited plasma protein binding of 97.00 $\pm$ 0.03% (Figure 5D). The obtained in vitro plasma protein binding data corresponds to the in silico findings (Table 3). Plasma protein binding is commonly categorized as medium to low (<85%), high (85–98%), and very high (>98%).[64] The results indicate that crocetin belongs to a high plasma protein binding class. It is known that bioefficacy is determined by the concentration of the free drug, not the fraction of the free drug.[65] For instance, despite a high plasma protein-bound nature, CNS class of drugs such as diazepam, sertraline, donepezil, etc., are available in the market for therapeutic use.[62] Therefore, the plasma protein binding of crocetin must be taken care of during dose selection in order to achieve desired bioefficacy.

2.12. RBC Partitioning of Crocetin. RBC partitioning of a molecule is an integral part of the preclinical investigation, as the penetration of a drug into the RBC, to a considerable extent, can affect activity or cause toxicity. In addition, it also affirms the choice of the appropriate matrix for pharmacokinetic investigations in the in vivo studies. Thus, we assessed the RBC partitioning of crocetin using rat blood and plasma to check its propensity to accumulate in RBCs. Chloroquine (high RBC partition) and verapamil (low RBC partition) were used as standards, and the results were found to be well within the literature-reported range (Figure 5E).[44,66] The blood-to-plasma ratio ($K_{RBC/Plasma}$) of crocetin ranged from 0.36 $\pm$ 0.01 to 0.39 $\pm$ 0.04 at 0 and 60 min, respectively (Figure 5E). A molecule with a blood-to-plasma ratio of >1 is considered as a

high RBC partitioned compound, and therefore, it can be illustrated that crocetin has a minimal tendency for partitioning into RBC.

2.13. Metabolic Stability of Crocetin. The liver is the primary site of drug metabolism in the body. Hepatic CYP-mediated metabolism clears approximately 60% of marketed drugs from the body.[67] Therefore, we assessed the metabolic stability of crocetin in various liver microsomes by a classical substrate depletion approach.[68] The results of metabolic stability for verapamil as a positive control in MLM, RLM, DLM, MkLM, and HLM are consistent with the reported literature (Figure 6A).[69−71] Furthermore, no significant depletion was observed in samples without NADPH (negative control). Crocetin was found to remain intact >83%, depending on the species of microsomes (Figure 6B). Thus, results infer that crocetin has adequate microsomal stability at the in vitro level irrespective of the source of microsomes. Hence, the results reveal that crocetin has sufficient metabolic stability and is subject to subsequent in vivo studies in order to elucidate any effect of hepatic blood flow on its clearance from the liver.

2.14. CYP Inhibitory Action of Crocetin. CYP is a superfamily of enzymes that play a pivotal role in drug metabolism. Assessment of its potential to inhibit the CYP enzymes is critical as simultaneous administration with the drug of the same CYP substrate can affect the metabolic clearance of the drug.[72,73] This may cause augmentation of the plasma concentration of the drug, leading to the precipitation of potential dose-dependent adverse effects.[74] Therefore, we determined the inhibitory action of crocetin for the USFDA-recommended panel of CYP isoforms using respective index reactions in HLM.[75] The results of experimental positive controls, i.e., a standard inhibitor for each CYP isoform, have
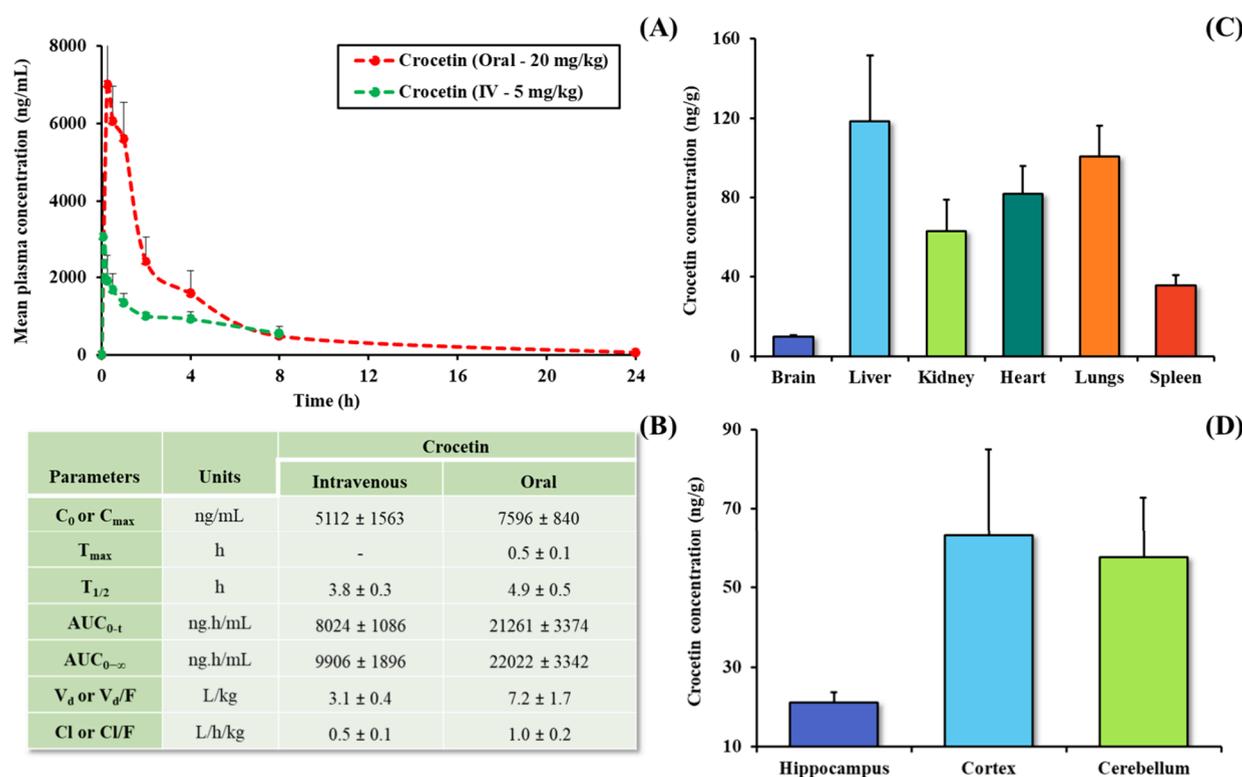
Figure 7. Mean plasma concentration vs time profile (A) and pharmacokinetic parameters (B) of crocetin after oral and IV administration in rats. Organ distribution of crocetin at 0.083 h after IV administration in rats (C). Brain distribution of crocetin at 0.083 h after IV administration (D). C_0 represents the initial plasma concentration; C_{max} represents the maximum plasma concentration after oral administration; T_{max} represents the time to reach the highest plasma concentration; $T_{1/2}$ represents elimination half-life; AUC_{0-t} represents the area under the curve for plasma concentration from zero to the last measurable plasma sample time; $AUC_{0-\infty}$ represents the area under the curve for plasma concentration from zero to time infinity; V_d represents the volume of distribution; V_d/F represents the volume of distribution after oral administration; Cl represents clearance; Cl/F represents clearance after oral administration. Data are represented as the mean $\pm$ SEM ($n = 5$).

good agreement with their reported values (Figure 6C).[76] The observed IC$_{50}$ value of crocetin for CYP isoforms is mentioned in Figure 6C. CYP inhibitor is typically categorized as potent (IC$_{50} \leq 1$ μM), moderate (1 μM < IC$_{50}$ < 10 μM), and weak or no (IC$_{50} \geq 10$ μM) inhibitor. Crocetin weakly inhibits CYP2D6 (IC$_{50}$ −20.4 μM) and CYP2C8 (IC$_{50}$ −35.3 μM) while negligibly inhibiting other CYP isoforms (CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4) even at a higher concentration (i.e., 50 μM). These in vitro findings closely resemble the in silico outcomes for the low CYP inhibitory promiscuity of crocetin (Table 3). For a compound intended to be a drug, it is desirable to lack any CYP inhibition capability so that drug interaction can be avoided upon concurrent intake of coprescribed drug(s). The present results demonstrate that crocetin is less likely to cause pharmacokinetic interaction with the drug (substrate of investigated CYP enzyme) and thereby safely coadministered. Hence, the absence of CYP inhibitory action could be advantageous for using crocetin as a drug.

2.15. Brain Permeability of Crocetin. The BBB mainly controls drug permeability into the brain and is the main bottleneck for developing CNS-acting drugs. Therefore, we adopted an extended PAMPA model by using PBL as the lipid type of the artificial membrane to estimate the permeability of crocetin in the brain.[77] The present study was carried out using carbamazepine (BBB+) and sulfasalazine (BBB−) as standards, where results are correlated well with the literature values (Figure 6D).[77] The observed log P_{app} value of crocetin was

found to be 0.10 $\pm$ 0.00 (Figure 6D). The log P_{app} values of <2 and <2 are considered BBB+ and BBB−, respectively. Thus, crocetin can be categorized as a BBB− compound. However, a mixed opinion (BBB+/BBB−) on crocetin's brain penetration is reflected in the in silico findings (BBB permeability/BBB score/CNS permeability) from various software (Table 3). We used the PAMPA-BBB model, which only examines the drug's transcellular passive diffusion process. Considering data on brain permeability at the in vivo level, caffeine (BBB+) and quinidine (BBB−) are reported to display negative results in this assay system due to their carrier-mediated influx/P-gp-mediated efflux.[77] Therefore, further in vivo studies should be done to explore the brain penetration ability of crocetin.

2.16. Oral Bioavailability of Crocetin. The oral route is the most convenient route of drug administration in prolonged therapy for better patient compliance, especially for geriatrics. Alternatively, a large number of marketed drugs have serious concerns about oral bioavailability.[78] Moreover, several molecules fail to show in vivo efficacy despite strong in vitro action due to poor oral bioavailability.[79] Although bioavailability-related information on crocetin is described earlier, but lack adequate experimental/characterized compound information to be reproduced.[7,19] Therefore, we performed the pharmacokinetic study of crocetin using a rat model after oral and IV administration to determine the oral bioavailability of crocetin. After being given orally, crocetin took a relatively short time (∼30 min) to reach a maximum plasma concentration of 23 μM (Figure 7A,B). This rapid absorption
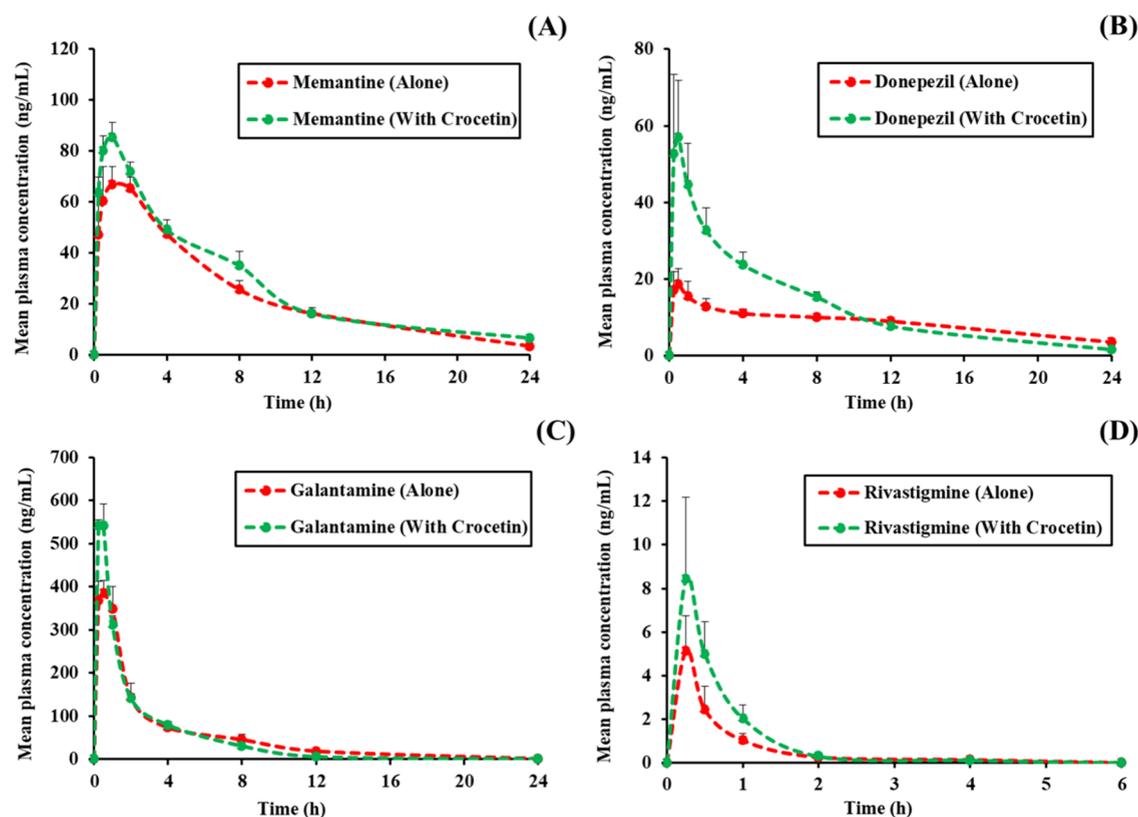
Figure 8. Mean plasma concentration vs time profile of memantine (A), donepezil (B), galantamine (C), and rivastigmine (D) after oral administration alone and in combination with crocetin. Data are represented as mean ± SEM ($n = 5$).

behavior of the crocetin in the in vivo system is inconsistent with in vitro PAMPA-based data demonstrating the limitation of the in vitro model as well as absorption mechanisms of crocetin other than transcellular diffusion. However, it closely matches the reported data based on the Caco-2 permeability model. Crocetin displayed an excellent half-life (>2h), which correlates quite well with the metabolic stability data in RLM (Figure 6B) as well as in silico data under the long $T_{1/2}$ category (>3 h) (Table 3). The volume of distribution of crocetin was not too high (>10 L/kg) and clearance from the body occurred at a fairly slower rate (≤ 1 L/h/kg). The utterance of the software data resembles these parameters at the in vivo level (Table 3). The experimental oral bioavailability of crocetin was calculated to be 55−66% (Figure 7B). Although an equivocal dose-dependent report on oral bioavailability is available,[7] our results correspond to the in silico projections (Table 3). All the marketed anti-Alzheimer drugs, except galantamine, have lower oral bioavailability than crocetin in the preclinical model.[80−82] Hence, crocetin's considerably high oral bioavailability is encouraging for further development as a drug.

2.17. Brain Penetration of Crocetin. In pursuance of assessing brain penetration of crocetin, we monitored crocetin concentration in various major organs, including the brain. We administered crocetin via the IV route to rats and measured the organ concentration after 5 min of dosing as the highest concentration of crocetin in plasma was observed within 5 min. The estimated crocetin concentrations in the experimental organs are found in the following order: liver > lungs > heart > kidney > spleen > brain (Figure 7C). The highest liver concentration indicates the involvement of the liver as a major metabolic site of crocetin. The achieved concentration of

crocetin in the brain was found to be 31 nM. The software-based predictions also describe a similar BBB penetrable behavior of crocetin (Table 3). Therefore, results reveal that crocetin has a brain penetration ability, which ascertains the limitation of the in vitro BBB assay system.

AD typically destroys neurons and their connections in the various brain regions. The hippocampus is the primary site for memory function. Later on, AD impacts the parts of the cerebral cortex that control language, reasoning, and social behavior.[83] The cerebellum's role in AD has been neglected for a long time. Still, recent studies have demonstrated that Aβ gets deposited in the cerebellum and affects synaptic transmission and plasticity.[84] Thus, we investigated the ability of crocetin to reach active sites after 5 min of crocetin administration to rats. The estimated crocetin concentration in the experimental brain parts is in the following order: cortex > cerebellum > hippocampus (Figure 7D). Comparing the concentrations attained in the whole brain and distinct brain parts reveals the accumulation of crocetin in particular brain regions instead of the entire brain. Therefore, the results illustrate that crocetin could reach the target sites of the brain, which may be helpful in enabling proper memory function that gets impaired during AD. Moreover, crocetin concentration in the target site should further be monitored in order to correlate with the bioefficacy.

2.18. Pharmacokinetic Interaction of Crocetin with Anti-Alzheimer Drugs. Concomitant administration of one drug with another often causes boosting or lowering of plasma concentration, leading to the precipitation of adverse effects or therapeutic failure.[85,86] Devoid of any such drug−drug interaction ascertains safe coadministration of two prescribed drugs. Therefore, elucidating the likelihood of pharmacokinetic

Table 4. Pharmacokinetic Parameters of Various Anti-Alzheimer Drugs after Oral Administration Alone and in Combination with Crocetin[a]

parameter	unit	memantine		donepezil		galantamine		rivastigmine	
		alone	with crocetin	alone	with crocetin	alone	with crocetin	alone	with crocetin
C_{max}	ng/mL	77 ± 10	86 ± 6	19 ± 4	61 ± 19	420 ± 49	583 ± 36	5.1 ± 1.6	8.6 ± 3.6
T_{max}	h	1.3 ± 0.3	0.8 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
AUC_{0-t}	ng h/mL	578 ± 37	673 ± 49	196 ± 8	320 ± 37*	1265 ± 41	1187 ± 65	3.3 ± 1.1	5.8 ± 2.0
$AUC_{0-\infty}$	ng h/mL	606 ± 43	784 ± 58	261 ± 32	336 ± 41	1269 ± 41	1189 ± 65	3.8 ± 1.2	6.1 ± 2.0
$T_{1/2}$	h	5.2 ± 0.6	10.2 ± 2.3	9.3 ± 3.0	5.5 ± 1.3	2.9 ± 0.4	3.1 ± 0.1	2.3 ± 0.8	1.2 ± 0.4
V_d/F	L/kg	24.9 ± 1.9	37.4 ± 7.8	108.4 ± 22.5	54.0 ± 8.8	8.0 ± 1.1	9.3 ± 0.7	1224 ± 348	473 ± 165
Cl/F	L/h/kg	3.4 ± 0.3	2.6 ± 0.2	9.3 ± 1.0	7.2 ± 0.8	1.9 ± 0.1	2.0 ± 0.1	512 ± 168	366 ± 170

[a]Data compared: alone vs combination with crocetin. Statistical significance: $p < 0.05$(*)/0.01(**)/0.001(***). Data are represented as mean ± SEM ($n = 5$).
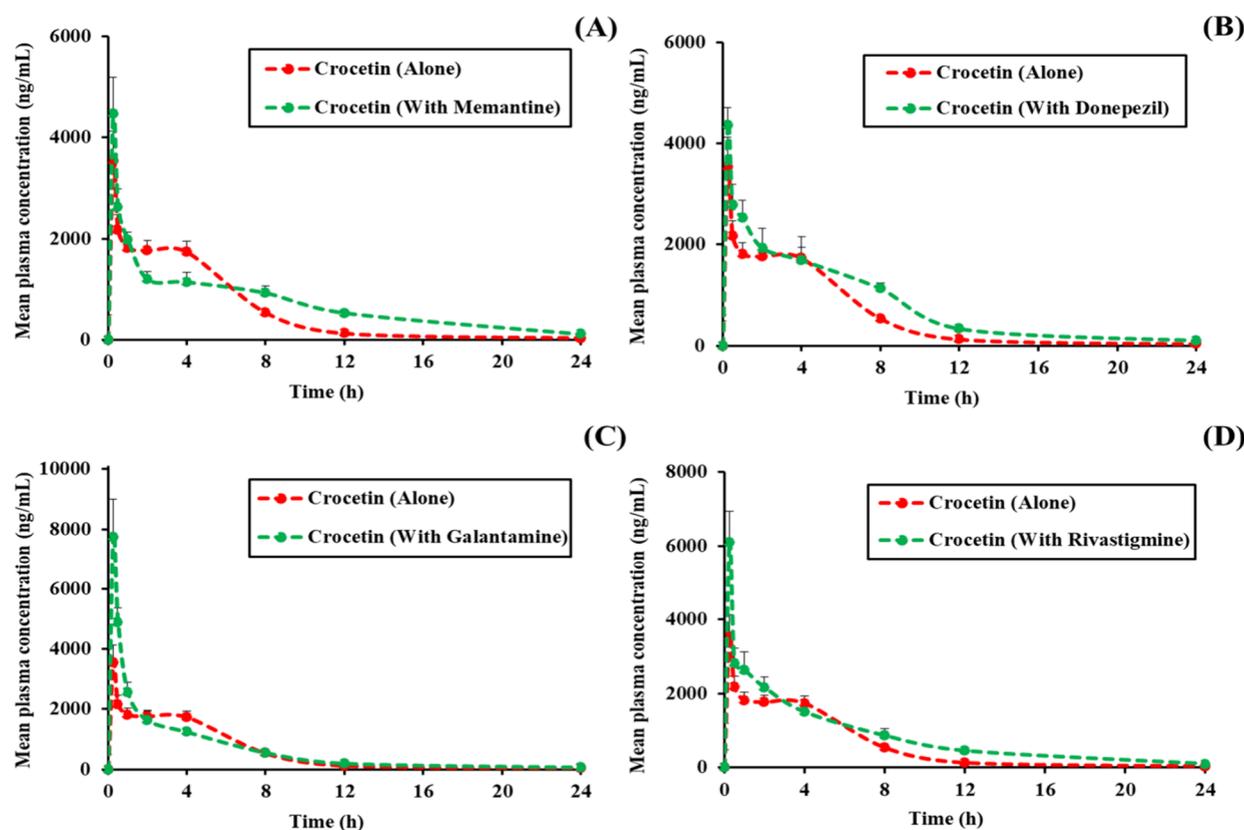
Figure 9. (A−D) Mean plasma concentration vs time profiles of crocetin in rats after oral administration as alone and in combination with various anti-Alzheimer drugs. Data are represented as mean ± SEM ($n = 5$).

interaction between two simultaneously prescribed drugs is vital to avoid drug−drug interaction. The same can also happen in the case of concurrent administration of any phytotherapy with prescribed medication in the form of a phytochemical-drug interaction. Currently, cholinesterase inhibitors such as donepezil, galantamine, rivastigmine, and NMDA receptor antagonist memantine are clinically used drugs for treating AD.[87] Therefore, using the rat model, we explored the pharmacokinetic interactions of these anti-Alzheimer drugs in the presence of crocetin. In order to obtain a sustained effect, pretreatment of crocetin was given to rats consecutively for 7 days prior to the pharmacokinetic interaction study with any anti-Alzheimer drug.

Memantine was given orally alone and in combination with crocetin to rats. The pharmacokinetic interaction study data of memantine are represented in Figure 8A and Table 4. The

experimental pharmacokinetic parameters of memantine upon alone treatment corroborate well with the reported literature.[88] Although crocetin treatment increased the $T_{1/2}$ of memantine, it lacks statistical significance. Moreover, no marked alteration was observed in any other pharmacokinetic parameters of memantine due to coadministration of crocetin.

Donepezil was given orally alone and in combination with crocetin to rats. The pharmacokinetic interaction study data of donepezil are represented in Figure 8B and Table 4. The experimental pharmacokinetic parameters of donepezil in the absence of crocetin treatment are in line with the reported literature.[89] Crocetin treatment enhanced the C_{max} and reduced the volume of distribution of donepezil in a statistically insignificant manner. Although AUC under the experimental time frame augmented substantially upon crocetin treatment, AUC under the extended time frame

Table 5. Pharmacokinetic Parameters of Crocetin after Oral Administration Alone and in Combination with Various Anti-Alzheimer Drugs[a]

parameters	units	crocetin				
		alone	with memantine	with donepezil	with galantamine	with rivastigmine
C_{max}	ng/mL	3537 ± 487	4465 ± 1490	4353 ± 606	7740 ± 2299	6330 ± 1023*
T_{max}	h	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
AUC_{0-t}	ng h/mL	14021 ± 1755	16925 ± 1365	19200 ± 2598	16364 ± 1115	20012 ± 3378
$AUC_{0-\infty}$	ng h/mL	14343 ± 1795	18070 ± 1406	20338 ± 2659	17195 ± 1091	21102 ± 3356
$T_{1/2}$	h	6.0 ± 0.6	5.5 ± 1.2	7.1 ± 1.6	7.8 ± 0.9	7.2 ± 1.4
V_d/F	L/kg	12.7 ± 1.8	9.4 ± 2.6	12.0 ± 4.1	13.3 ± 1.4	11.1 ± 2.7
Cl/F	L/h/kg	1.5 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.0 ± 0.2

[a]Data compared: alone vs combination with galantamine/donepezil/memantine/rivastigmine. Statistical significance: $p < 0.05(*)/0.01(**)/0.001(***)$. Data are represented as mean ± SEM ($n = 5$).

suggests a lack of statistically significant alteration. Due to crocetin treatment, negligible modifications were observed in any other pharmacokinetic parameters of donepezil.

Galantamine was given orally alone and in combination with crocetin to rats. The pharmacokinetic interaction study data of galantamine are represented in Figure 8C and Table 4. The experimental pharmacokinetic parameters of galantamine upon alone treatment are correlated well with the reported literature.[90] Crocetin treatment did not notably affect any pharmacokinetic parameters of galantamine compared with only galantamine treatment.

Rivastigmine was given orally alone and in combination with crocetin to rats. The pharmacokinetic interaction study data of rivastigmine are represented in Figure 8D and Table 4. The experimental pharmacokinetic parameters of rivastigmine in the absence of crocetin treatment corroborate well with the reported literature.[81] Although crocetin treatment elevated the C_{max} and lowered the volume of distribution of rivastigmine, it lacks statistical significance. Additionally, no noteworthy alteration was observed in the pharmacokinetic parameters of rivastigmine due to the coadministration of crocetin.

Donepezil and galantamine are mainly metabolized by CYP2D6 and CYP3A4 among the experimental anti-Alzheimer drugs. In order to check any changes in the CYP expression in the liver, Western blot analysis data reveal that concomitant treatment of crocetin with donepezil could cause down-regulation of CYP2D6 protein expression (0.73-fold) and simultaneous treatment of crocetin with galantamine caused down-regulation of CYP3A4 protein expression (0.61-fold) compared to only donepezil or galantamine treatment, respectively (Figure S1). The overall observed effect on the pharmacokinetics of donepezil and galantamine is possibly linked to weak and noninhibitory action by crocetin on CYP2D6 and CYP3A4, respectively (Figure 6C).

In parallel to assess the pharmacokinetics of individual anti-Alzheimer drugs, we also monitored the pharmacokinetic profile of crocetin (Figure 9A−D and Table 5). In the present segment, animals were pretreated for 7 days with crocetin to assess the pharmacokinetic profile in the case of both alone and combination treatments. When we compared the pharmacokinetic parameters of crocetin compared to single-dose (Figure 7B), a lowering of C_{max} was only observed (Table 5). However, other pharmacokinetic parameters are alike, as observed after a single-dose crocetin treatment. Concomitant treatment of crocetin with individual anti-Alzheimer drugs, an elevation of the C_{max} and AUC of crocetin was observed, but it lacks statistical significance. All other pharmacokinetic parameters are identical irrespective of the alone or combination therapy. Therefore, overall results reveal that minimal pharmacokinetic alterations are unlikely to cause any severe impact on the bioefficacy of anti-Alzheimer drugs upon combination treatment.

3. CONCLUSIONS

Considering the chemical structure of crocetin in the frame of ADME/PK characteristics, it is incongruent with a CNS class of drugs. In the quest to explore the druggability of crocetin using in silico, in vitro, and in vivo approaches, results demonstrate it has several acceptable drug-like attributes to the following aspects: rules of drug-likeliness, lipophilicity, pK_a, P-gp inhibition ability, plasma stability, RBC partitioning, metabolic stability, CYP inhibition profile, oral bioavailability, BBB permeability, penetration to target site of brain, drug interaction with anti-Alzheimer drugs. In vivo studies ascertain its GI and BBB permeability characteristics by highlighting the limitations of the in vitro models. On the other hand, aqueous solubility, chemical stability, plasma protein binding, and P-gp induction are some concerns associated with this molecule. In these contexts, formulation/delivery strategies are evident to provide an amicable solution for marketed drugs with low aqueous solubility and inadequate chemical stability. High plasma protein binding of crocetin should be taken into account for selecting doses during bioefficacy studies. The ability of crocetin to induce P-gp can certainly be a drawback of a druggable candidate, but it can be helpful for $A\beta$ clearance in treating AD. The current experimental substantiation suggests favorable ADME/PK behavior and potential drug-gable candidature of crocetin. Considering the literature evidence of promising efficacy (in vitro/ex vivo) against AD and safety/toxicity data, as well as current ADME/PK findings of crocetin, warrant further investigations of it to be a potential anti-Alzheimer drug.

4. EXPERIMENTAL SECTION

4.1. Materials. Galantamine hydrobromide (purity ≥98%), donepezil hydrochloride (purity ≥98%), memantine hydrochloride (purity ≥98%), and chlorambucil (purity ≥98%) were purchased from TCI. Rivastigmine tartrate (purity ≥95%), (S)-mephenytoin (purity ≥98%), ketoconazole (purity ≥98%), paracetamol (purity ≥98%), 6β-hydroxy testosterone (purity ≥97%), sulfasalazine (purity ≥98%), astemizole (purity ≥98%), zosuquidar hydrochloride (purity ≥98%), and chloroquine phosphate (purity ≥98%) were procured from Cayman. Propranolol hydrochloride (purity ≥99%), verapamil hydrochloride (purity ≥99%), ranitidine hydrochloride (purity ≥98%), omeprazole (purity ≥98%), fluvoxamine maleate

(purity ≥97%), sulfaphenazole (purity ≥98%), phenacetin (purity ≥98%), diclofenac sodium (purity ≥98%), tranylcypromine sulfate (purity ≥97%), quercetin hydrate (purity ≥95%), quinidine anhydrous (purity ≥80%), enalapril maleate (purity ≥98%), dextromethorphan hydrobromide (purity ≥98%), bupropion hydrochloride (purity ≥98%), (2S,3S)-hydroxybupropion hydrochloride (purity ≥98%), sertraline hydrochloride (purity ≥98%), amodiaquine dihydrochloride dihydrate (purity ≥97%), testosterone (purity ≥98%), N-desethylamodiaquine dihydrochloride (purity ≥95%), 4′-hydroxydiclofenac (purity ≥98%), 4-hydroxymephenytoin (purity ≥98%), dextrorphan tartrate (purity ≥97%), NADPH tetrasodium salt (purity ≥98%), diazepam (purity ≥98%), carbamazepine (purity ≥98%), isoniazid (purity ≥99%), budesonide (purity ≥98%), rifampicin (purity ≥97%), Rh123 (purity ≥85%), PBL extract (Product no: 141101P), N-methyl-2-pyrrolidone (NMP), Tween-80, and ammonium formate (MS-grade) were procured from Sigma-Aldrich. HLM (lot no. #PL050F-C), MkLM (lot no. #MK065-B), DLM (lot no. #DG035-C), RLM (lot no. #RT064-B), and MLM (lot no. #2513990-A) were obtained from Gibco. CYP3A4 pAb (lot no. WB3191725) and CYP2D6 pAb (lot no. WB3191065) were purchased from Invitrogen. Acetonitrile, methanol, and MS-grade formic acid were purchased from Thermo Fisher Scientific. All other experimental materials were of bioreagent grade or above. Distilled water and ultrapure water from the water purification system (Make: Merck-Millipore; Model: Direct-Q3) were used for animal and analytical experimentations, respectively.

4.2. Test Article. Crocetin was synthesized from commercially available crocin (Product no. C1527, TCI) using an alkaline hydrolysis protocol and purified as per the previously reported method.[91] The characterization data of crocetin (Figures S2−S5) are as follows: ^{1}H NMR (400 MHz, MeOD:DMSO) δ 7.32 (dd, J = 10.30 Hz, 1.28 Hz, 2H), 6.89−6.86 (m, 1H), 6.78−6.65 (m, 5H), 6.54−6.53 (m, 2H), 2.05 (s, 6H), 2.01 (s, 6H), ppm; ^{13}C NMR (100 MHz, MeOD:DMSO) δ 171.6, 144.9, 140.0, 138.0, 136.8, 132.8, 128.0, 125.1, 13.2, 13.0 ppm; HRMS (ESI): calcd for $C_{20}H_{25}O_4$ $[M + H]^+$: 329.1759, found: 329.1753. The purity of crocetin (>96%) with a Trans:Cis ratio of 9:1 was determined by HPLC (Figure S6).

4.3. In Silico Investigations. To predict drug-likeliness, physicochemical, pharmacokinetic, and allied properties of crocetin, we used several online computational tools, which were as follows: SwissADME (Swiss Institute of Bioinformatics),[92] molsoft (Molsoft L.L.C.),[93] ADMETlab (Central South University, National University of Defense Technology, and Zhejiang University),[94] pkCSM (Bio21 Institute, University of Melbourne),[95] admetSAR (East China University of Science and Technology),[96] CYPrules (National Taiwan University),[97] and CYPlebrity (University of Vienna).[98]

4.4. In Vitro Investigations. To evaluate pharmacokinetic and allied properties, a battery of in vitro studies for crocetin was performed using respective standards (positive/negative control). Detailed methodology for each study is described in the Supporting Information. The key information on in vitro studies of crocetin is as follows: (a) solubility of crocetin was determined in various media, viz. water, PBS, SGF (pH 1.2), FaSSGF (pH 1.6), FeSSGF (pH 5.0), SIF (pH 6.8), FaSSIF (pH 6.5), and FeSSIF (pH 5.8) using the shake-flask method.[30] (b) log P and log D of crocetin were evaluated in n-octanol/water and n-octanol/PBS, respectively, using the

shake-flask method.[40] (c) pKₐ of crocetin was estimated by the UV-metric method using Britton-Robinson buffer in the pH range from 1.81 to 12.21.[41] (d) Chemical stability of crocetin was assessed in SGF and SIF for 2 and 4 h, respectively. SGF and SIF were prepared as per Indian Pharmacopoeia, 2018.[39] (e) GI permeability of crocetin was investigated using the PAMPA assay, where the membrane composed of 5% hexadecane in hexane (v/v).[47] (f) P-gp induction and inhibition of crocetin was performed in LS180 cells based on intracellular accumulation of Rh123 as a P-gp substrate.[51] (g) Plasma stability of crocetin was carried out for 2 h using rat plasma from control rats.[40] (h) Protein binding of crocetin with rat plasma from control rats was determined using the rapid equilibrium dialysis method.[61] (i) RBC partitioning of crocetin was estimated using rat blood and plasma from control rats for 1 h.[39] (j) Brain permeability of crocetin was assessed using the PAMPA assay, where a membrane composed of 2% PBL in n-dodecane (w/v).[77] (k) Metabolic stability of crocetin was tested in HLM, MkLM, DLM, RLM, and MLM using a protein concentration of 1 mg/mL and an incubation time of 30 min.[68] (l) CYP inhibitory actions of crocetin were studied in HLM using USFDA recommended marker reactions viz. phenacetin O-deethylation for CYP1A2, bupropion hydroxylation for CYP2B6, amodiaquine N-deethylation for CYP2C8, diclofenac 4′-hydroxylation for CYP2C9, S-mephenytoin 4′-hydroxylation for CYP2C19, dextromethorphan O-demethylation for CYP2D6, and testosterone 6β-hydroxylation for CYP3A4.[76]

4.5. In Vivo Investigations. To explore the pharmacokinetics of crocetin, several in vivo studies were executed using animal models.[99,100] Animal experiments were conducted as per the "Committee for Control and Supervision of Experiments on Animals (CCSEA)" (Government of India, New Delhi, India) guidelines with prior approval of study protocol from our "Institutional Animal Ethics Committee" (Approval number: IAEC/79/255/8/2021 and IAEC/80/278/2/2022). Detailed methodology for each study is described in the Supporting Information. The key information on the in vivo studies for crocetin is as follows: (a) oral bioavailability of crocetin was assessed using a rat model upon oral (20 mg/kg) and intravenous (5 mg/kg) administrations. Blood samples were collected, processed, and analyzed by LC−MS/MS to quantify the amount of crocetin in plasma. Finally, plasma concentration data were used to calculate the pharmacokinetic parameters of crocetin using PK solution software. (b) Organ distribution of crocetin was evaluated using a rat model after 5 min of its dose administration (10 mg/kg) via the IV route. Organ samples like brain, heart, lungs, liver, kidneys, and spleen were collected, homogenized individually using PBS (350 mg/mL), processed, and estimated the specific tissue concentrations of crocetin by LC−MS/MS. (c) Intrabrain distribution of crocetin was elucidated using a rat model after 5 min of its dose administration (10 mg/kg) via the IV route. Various brain parts, viz. hippocampus, cortex, and cerebellum, were collected, homogenized using PBS (350 mg/mL), processed, and quantitated the crocetin concentration by LC−MS/MS. (d) Pharmacokinetic interaction studies of crocetin were carried out using a rat model with anti-Alzheimer drugs, viz. memantine (2 mg/kg), donepezil (2.3 mg/kg), galantamine (2.4 mg/kg), and rivastigmine (1.2 mg/kg). Animals were pretreated with crocetin (20 mg/kg), and all the experimental anti-Alzheimer drugs were given orally. Blood samples were collected and processed, and the concentrations

of crocetin and all the experimental anti-Alzheimer's drugs in plasma by LC−MS/MS were determined. Plasma concentration data were used to calculate pharmacokinetic parameters of individual anti-Alzheimer drugs using PK solution software.[72] The liver was also collected at the end of the pharmacokinetic study and was snap-frozen. Western blotting was performed using liver homogenate in RIPA buffer to monitor the protein expression of CYP2D6 and CYP3A4. Statistical significance ($p < 0.05/0.01/0.001$) was evaluated by an unpaired Student's t test using GraphPad Prism software.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02116.

> Characterization data of crocetin by NMR, HR-MS, and HPLC; protocol for CYP inhibition studies; and detailed methodology for in vitro and in vivo studies (PDF)

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Author Contributions

D.M.: all in silico, in vitro, and in vivo experimentations, sample processing, data analysis unless otherwise mentioned, and writing—original draft; S.D.: sampling for in vivo studies, data evaluation; H.K.: synthesis of crocetin; D.K.: P-gp induction and inhibition assays, Western blot analysis; K.S.: dose administration via IV route; P.W.: LC−MS/MS analysis; B.V.: HPLC analysis; A.K.: supervision of P-gp induction and inhibition assays, Western blot analysis; S.D.S.: supervision of synthesis of crocetin; Z.A.: resources; U.N.: conceptualization, planning and supervision of experimentations, writing—review and editing.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AD, Alzheimer's disease; ADME, absorption distribution metabolism and excretion; CNS, central nervous system; USFDA, United States Food and Drug Administration; BBB, blood-brain barrier; log P, partition coefficient; log D, distribution coefficient; pK_a, ionization constant; SGF, simulated gastric fluid; FaSSGF, fasted-state simulated gastric fluid; FeSSGF, fed-state simulated gastric fluid; SIF, simulated intestinal fluid; FaSSIF, fasted-state simulated intestinal fluid; FeSSIF, fed-state simulated intestinal fluid; PBS, phosphate-buffered saline; BCS, biopharmaceutical classification system; PAMPA, parallel artificial membrane permeability assay; Rh123, rhodamine 123; CYP, cytochrome P450; MLM, mouse liver microsomes; RLM, rat liver microsomes; DLM, dog liver microsomes; MkLM, monkey liver microsomes; HLM, human liver microsomes; NADPH, nicotinamide adenine dinucleotide phosphate; IC_{50}, half maximal inhibitory concentration; IV, intravenous; ChEIs, cholinesterase inhibitors; NMDA, N-methyl-D-aspartate; C_0, initial plasma concentration; C_{max}, maximum plasma concentration; T_{max}, time to reach the highest plasma concentration; AUC_{0-t}, area under the curve for plasma concentration from zero to the last measurable plasma sample time; $AUC_{0-\infty}$, area under the curve for plasma concentration from zero to time infinity; V_d, volume of distribution; V_d/F, volume of distribution after oral administration; Cl, clearance; Cl/F, clearance after oral administration; PBL, polar brain lipid; NMP, N-methyl-2-pyrrolidone; IS, internal standard; CCSEA, Committee for

Control and Supervision of Experiments on Animals; IAEC, Institutional Animal Ethics Committee

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