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Description of Druglike Properties of Safranal and Its Chemistry behind Low Oral Exposure

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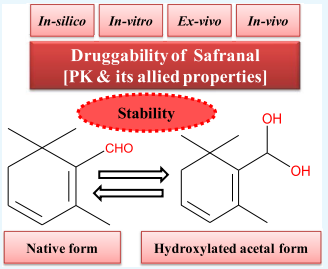
ABSTRACT: Safranal, a plant secondary metabolite isolated from saffron, has been reported for several promising pharmacological properties toward the management of Alzheimer's disease. In the present study, we observe and report for the first time about several druglike attributes of safranal, such as adherence to Lipinski's rule of five; optimum lipophilicity; high permeability; low blood-to-plasma ratio; less to moderate propensity to interact with P-glycoprotein (P-gp) or breast cancer-resistant protein (BCRP) transporters; and high plasma protein binding as common to most of the marketed drugs using *in vitro* and *ex vivo* models. In spite of the above attributes, *in vivo* oral absorption was found to be very poor, which is linked to the structural integrity of safranal in simulated gastric fluid, simulated intestinal fluid, plasma, and liver microsomes. Moreover, the presence of unsaturated aldehyde moiety in safranal remains in equilibrium with its hydroxylated acetal form. Further research work is required to find out the stable oral absorbable form of safranal by derivatization of its aldehyde group without losing its potency.



1. INTRODUCTION

Natural products have immense potential for the discovery of new therapeutics to treat a wide range of diseases. More than 40% of the marketed drugs have been derived directly or indirectly from natural origin.[1,2] Particularly, plant secondary metabolites have been used traditionally despite unprecedented advances in the modern system of medicine, which are predominantly associated with unwanted side effects. In this direction, safranal is one of the active constituents of *Crocus sativus*, commonly known as saffron. Safranal is present in the flower of this plant. It has been reported to possess numerous pharmacological activities for the treatment of several disease conditions.[3,4] This promising phytochemical has been relentlessly explored for Alzheimer's disease because of its beneficial action as neuroprotective and anti-inflammatory agent.[5−14] Though hundreds of reports are available in the literature for the biological activity of safranal, there is hardly any report on its pharmacokinetics as well as allied properties. Early evaluation of pharmacokinetic profile is necessary in the drug development process because unfavorable pharmacokinetics is the major hurdle for a new candidate to be a drug.[15] As the oral route is the most preferred and conventional route for drug administration, *in vitro*/*ex vivo* as well as *in vivo* investigations on the features of this molecule linked to oral exposure are needed. These examinations are crucial for any new therapeutics to establish a correlation between its *in vitro* and *in vivo* efficacy. With this background, it has become imperative to investigate the pharmacokinetics and allied properties of such a potent phytochemical like safranal (Figure 1).[5−14]

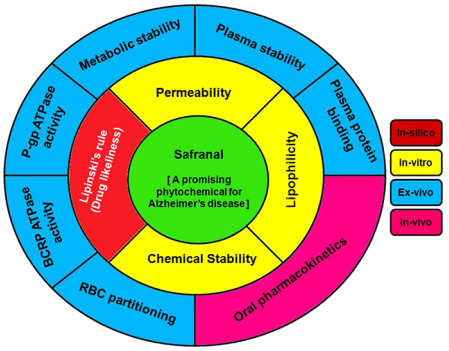


Figure 1. Summary of the present research work plan.

2. RESULTS AND DISCUSSION

Ongoing research on biological activities of safranal reveals that it has remarkable pharmacological actions toward the management of Alzheimer's disease.[5−14] Considering the success of plant-based natural products or their derivatives as marketed drugs, we aimed to investigate the drug-likeliness of

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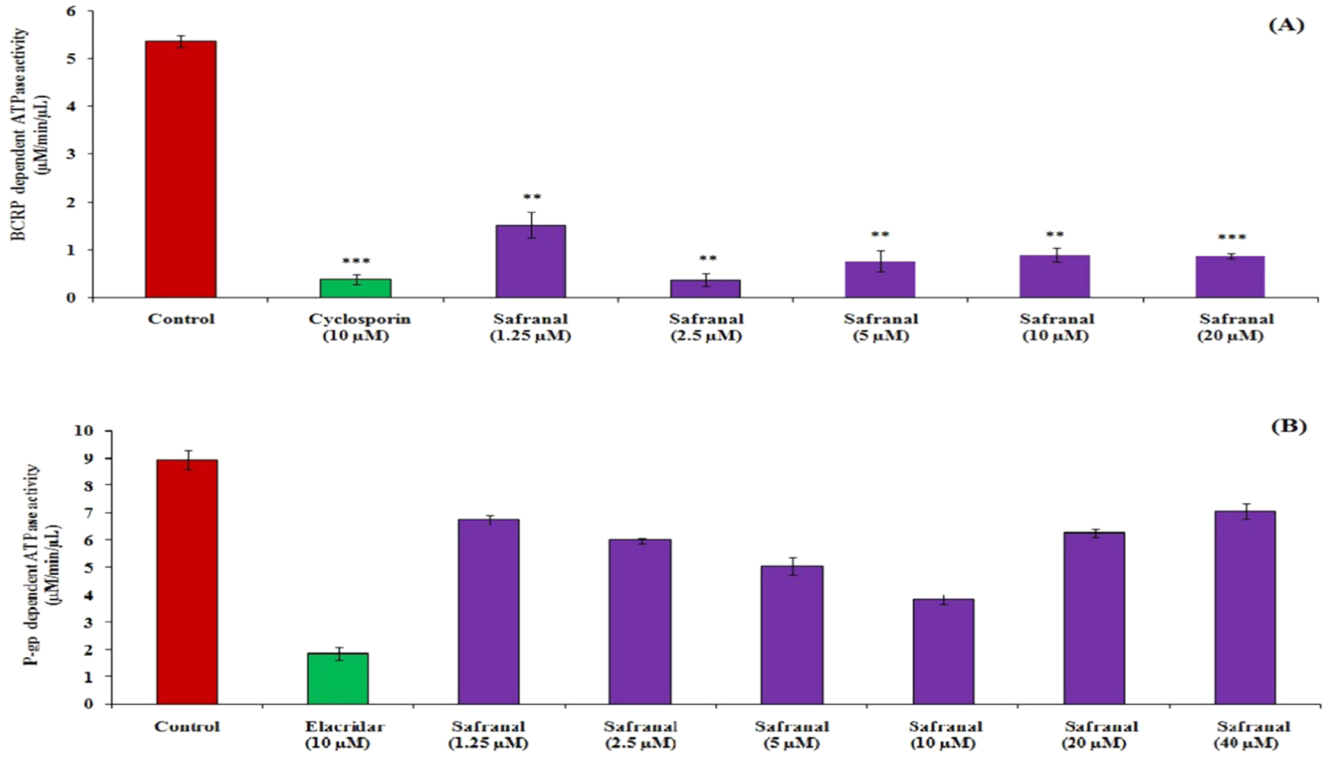

Figure 2. Effect of safranal on BCRP-dependent ATPase activity using cyclosporine as positive control (A) and P-gp-dependent ATPase activity using elacridar as positive control (B). Data are represented as mean ± standard error of the mean (SEM) (n = 2). Statistical significance level: *p < 0.05, **p < 0.01, and ***p < 0.001.

safranal through pharmacokinetic investigations, which is the crucial step for any candidate to become a drug.

First, chemical structure-based prediction of drug-likeliness demonstrates the following: molecular weight is less than 500 Da, calculated log P is less than 5, the number of hydrogen-bond donors is less than 5, and the number of hydrogen-bond acceptors is less than 10. Therefore, safranal does not violate any criteria for drug-likeliness as per Lipinski's rule of five, which helps to envisage drug-likeliness by acting as virtual filters.[16]

Lipophilicity is one such physicochemical property that influences the absorption characteristics of any molecule. We examined the partition coefficient (log P) as well as distribution coefficient (log D) of safranal, which is found to be in the range of 2.21 ± 0.14 to 2.47 ± 0.07 and 2.02 ± 0.28 to 2.38 ± 0.08, respectively, depending on the experimental ratios of octanol and water/phosphate-buffered saline, pH 7.4 (PBS). The obtained results for testosterone (3.09 ± 0.07) as standard have a good agreement with the reported value of around 3.28.[17,18] Results illustrate that safranal has optimum lipophilicity (0 < log P < 3), which is favorable for good oral absorption behavior through the gastrointestinal tract.

Investigation on the chemical stability of safranal in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) reveals that it was degraded up to 54 and 68%, respectively, in the experimental time frame of 2 and 4 h, respectively (Supporting Information). Therefore, this compound is found to be vulnerable to degradation and may be labile in the gastrointestinal tract that consequently can affect its oral exposure. This information will be beneficial to choose the proper route of administration as well as the need of formulation strategy during its further development.

The presence of transporters in the gastrointestinal tract restricts the entry of drugs through cell membranes. It occurs when the drug is a substrate of that particular transporter. Interaction with transporters is considered as a liability for a new chemical entity. This is because a co-administered drug (inhibitor of a particular transporter system) hinders absorption of a drug (substrate of same transporter system) that leads to change in oral bioavailability as well as its pharmacological action.[19] P-glycoprotein (P-gp) and breast cancer-resistant protein (BCRP) are the two important transporter systems that are involved in absorption for most of the marketed drugs. Therefore, the effect of safranal was evaluated on both the transporter systems through estimation of their ATPase activities using respective human protein. This assay is a simple, sensitive, rapid, and direct protocol to estimate the inorganic phosphate release linked to the activity of the transporters.[20] Investigating the effect of safranal on BCRP transporters demonstrates that it can significantly interfere with BCRP-dependent ATPase activity in a concentration-independent manner. Results also displayed that safranal can hinder this above transporter activity at a concentration of 2.5 μM, which is equivalent to cyclosporine activity at 10 μM (Figure 2A). On the other hand, results of the P-gp-dependent ATPase activity of safranal suggest that it can interfere with P-gp in a concentration-dependent manner through a very less effective way than elacridar, which served as a positive control (Figure 2B). Safranal is more likely to interact with BCRP than P-gp. As safranal is often taken as a dietary/food supplement, its intake should be avoided with allopathic drugs having the substrate of BCRP transporters.[21]

A panel of *in vitro*/*ex vivo* studies for pharmacokinetic profiling of safranal like permeability, plasma protein binding, red blood cells (RBC) partitioning, plasma stability, and

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metabolic stability in different microsomes like mice liver microsomes (MLM), rat liver microsomes (RLM), dog liver microsomes (DLM), and human liver microsomes (HLM) were also studied. *In vitro* permeability was accessed based on the parallel artificial membrane permeability assay (PAMPA) model, which is a straightforward and cost-effective method for the rapid determination of a molecule's passive permeability for oral absorption. The permeability ($\log P_e$) of safranal is found to be −3.88 ± 0.10, −3.93 ± 0.06, and −3.79 ± 0.11 in the concentration levels of 10, 25, and 50 μM, respectively. The experimental permeability of testosterone (−3.99 ± 0.18) is concordant with the reported results of −3.5 to −4.9 which may vary depending on parameters such as type of artificial membrane material, concentration of membrane material, incubation time, and lot-to-lot variation of plate.[22,23] Results suggest that safranal has high permeability as experimental $\log P_e$ value > −5. Moreover, the permeability of safranal is found to be concentration-independent in experimental conditions. Though low-permeability ($\log P_e$ value < −5) drugs are also available in the market, a new chemical entity is expected to be of high permeability to show that permeability is not a rate-limiting factor for absorption.[24]

The extent of plasma protein binding for safranal varied from 87 to 92% in the experimental concentration range of 5−20 μM (Figure 3). Plasma protein binding of rifampicin as

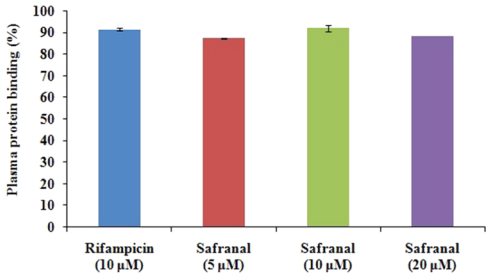

Figure 3. Plasma protein binding of safranal using rifampicin as standard. Data are represented as mean ± SEM (n = 4).

standard is found to be 91%, which matches with the reported results (82−92%).[25,26] Plasma protein binding can be classified as very high protein binding (>98%), high protein binding (85−98%), and medium-to-low protein binding (<85%).[27] Therefore, results indicate that plasma protein binding is high and concentration-independent. It is worth mentioning that the major proportion of marketed drugs is highly plasma protein-bound, and bioefficacy depends on free drug concentration but not on free drug fraction.[28]

The penetration of a drug into the RBC to a larger extent can affect its activity. Therefore, RBC partitioning of a compound is an integral part of the preclinical investigation. Moreover, such information will help to choose the appropriate matrix during the assessment of the pharmacokinetic behavior of that molecule. We evaluated the blood-to-plasma ratio for safranal and found that its value ($K_{RBC/Plasma}$) decreases from 0.88 ± 0.07 to 0.24 ± 0.03 at 0 and 60 min, respectively (Figure 4). The experimental result for propanolol ($K_{RBC/Plasma}$ from 1.07 ± 0.05 at 0 min to 0.90 ± 0.03 at 60 min) as standard is found to be well within the reported range in the literature (1.0 ± 0.2).[29] From the obtained results, it can be illustrated that safranal has a lower

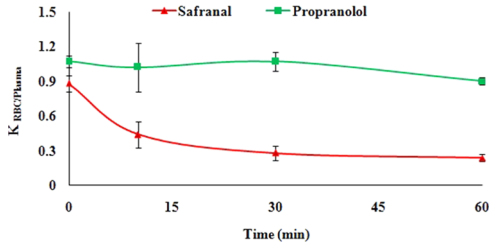

Figure 4. RBC partitioning of safranal using propanolol as standard. Data are represented as mean ± SEM (n = 3).

propensity to accumulate in RBCs unlike marketed drugs like rapamycin (3.5 ± 0.5), chloroquine (8.5 ± 0.1), and chlorthalidone (20.0 ± 0.3).[29]

The stability study of safranal in both mice and rat plasma showed a substantial decrease in compound concentration to 31 and 22%, respectively, under the experimental time period, *i.e.*, 0−4 h (Figure 5 and Supporting Information). Results

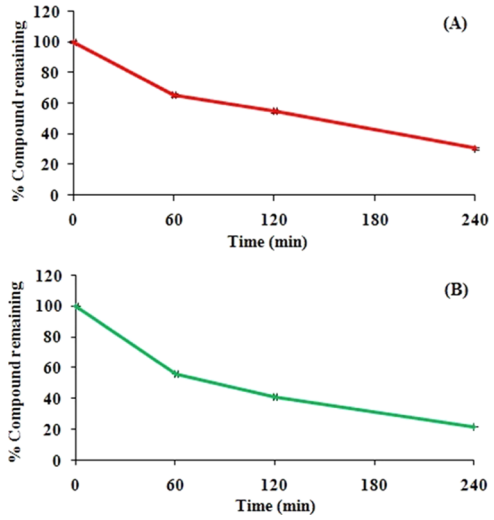

Figure 5. Stability of safranal in rat plasma (A) and mice plasma (B) at the initial concentration level of 75 μg/mL. Data are represented as mean ± SEM (n = 4).

indicate that the compound seems to be inadequately stable in plasma that may hamper with its oral exposure time. However, there are distinguished drugs in the market where the drug is very less stable in rat plasma but more stable in human plasma or vice verse like enalapril and procaine, respectively.[30]

The liver plays a major role in the elimination of drugs from the body, and therefore, *in vitro* metabolic stability can demonstrate the extent of oral exposure of the compound. In the present study, the metabolic stability of safranal was assessed by the substrate depletion approach using a range of microsomes like MLM, RLM, DLM, and HLM. The results are depicted in Figure 6, which illustrates that safranal degraded rapidly in all of the microsomes under the experimental conditions. Two marketed drugs, namely, atenolol and verapamil, remained intact in RLM under the same experimental conditions by 10 and 90%, respectively.[31]

The above-mentioned metabolic stability of safranal was assessed after quantitation of safranal remains in the reaction mixture by high-performance liquid chromatography (HPLC). It should be noteworthy to mention that the

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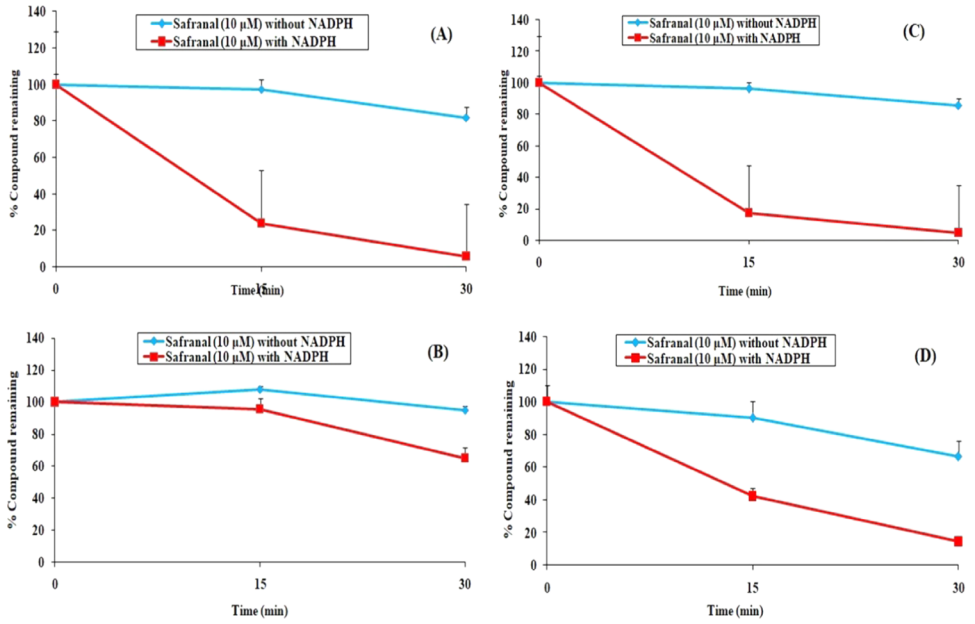

Figure 6. Metabolic stability of safranal in mice liver microsomes (A), rat liver microsomes (B), dog liver microsomes (C), and human liver microsomes (D). Data are represented as mean ± SEM (n = 3).

maximum wavelength for absorption (λ_{max}) for safranal is 310 nm, which shifted to 259 nm for a few study samples where retention times of both peaks in HPLC were found to be the same. This is probably due to the presence of rapid equilibrium between safranal and its corresponding hydroxylated acetal form (Figure 7). In fact, the calculation of λ_{max} for safranal and its acetal by empirical Woodward's rule showed a hypsochromic shift of the parent molecule. For simplicity, metabolic stability in different liver microsomes was assessed based on the higher response of safranal at λ_{max} of 310 nm in HPLC.

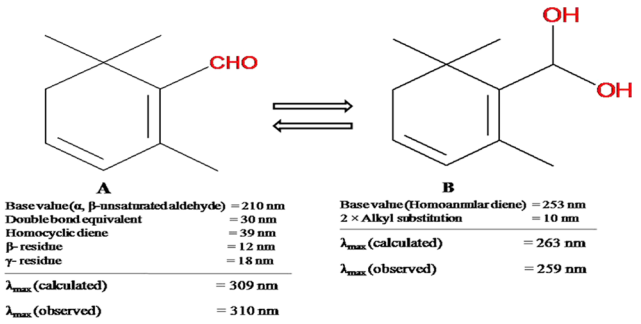

Figure 7. Calculated (309 and 263 nm) and observed (310 and 259 nm) λ_{max} values of safranal (A) and its hydroxylated acetal form (B), respectively.

A single-dose oral pharmacokinetic study of safranal was performed in Balb/C mice. The dose was administered at 100 mg/kg, considering the results mentioned above for chemical, plasma, and metabolic stability. Results showed that the plasma concentration of safranal at different time points is detectable up to 2 h, but most of the concentration data are below the lowest point of the calibration curve. If these plasma concentration data are used, it can be stated that safranal absorbs rapidly but have a very less half-life that leads to lower exposure of safranal after oral administration even at a high dose level (Supporting information). Inadequate oral exposure is also correlated to the *in vitro* results obtained in the chemical stability, plasma stability, and metabolic stability experimentations. Numbers of biological activities are reported in the literature for safranal after oral administration despite this unfavorable pharmacokinetic behavior, which is observed in the present investigation. This may be due to its excellent potency in native form and/or its degraded products and/or any of its metabolites. A similar phenomenon is also observed for phenolic compounds like curcumin.[32]

3. CONCLUSIONS

In spite of quite a few promising pharmacokinetic properties, poor oral exposure of safranal can be explained by its moderate chemical stability in SGF/SIF related to the unavailability of intact amount for absorption, low plasma stability linked to lesser extent of bioavailability, and petite metabolic stability connected to rapid clearance by liver. Therefore, this present investigation suggests the following key information for this useful phytoconstituent: (a) formulation approaches have to be applied for better oral exposure, (b) consideration for the presence of free aldehyde or hydroxylated acetal form during estimation, (c) contribution of degradation products/metabolites to the reported biological activities through orally, and (d) further research is required for stable form of safranal through derivatization of aldehyde group without losing its potency.

4. MATERIALS AND METHODS

4.1. Chemicals and Reagents. Safranal (purity ≥ 90%), testosterone (purity ≥ 98%), elacridar (purity ≥ 98%), cyclosporine (purity ≥ 98.5%), rifampicin (purity ≥ 98%), propanolol (purity ≥ 99%), reduced nicotinamide adenine dinucleotide phosphate (NADPH), ATP/GTP ATPase activity assay kit, formic acid (LC-MS grade), and olive oil were purchased from Sigma-Aldrich. BCRP human membrane (Lot #7304003) and P-gp human membrane (Lot #6314001) were purchased from Corning Gentest. Rapid equilibrium dialysis (RED) device was procured from Pierce,

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Thermo-Fisher Scientific. MLM (Lot #MS035-B), RLM (Lot #RT053-F), DLM (Lot #DG031-B), and HLM (Lot #PL050C-E) were purchased from Gibco. Magnesium chloride, acetonitrile, methanol, dimethyl sulfoxide (DMSO), hexane, and hydrochloric acid were purchased from Fischer Scientific. *n*-Octanol and ethylenediaminetetraacetic acid (EDTA) were procured from Loba Chemie and SD Fine Chemicals, respectively. All other chemicals/reagents used were of research grade. Ultrapure water from Direct-Q3 water purification system (Merck-Millipore) was used for all experimentations.

4.2. *In Silico* Evaluation of Drug-likeliness. Drug-likeliness based on Lipinski's rule was determined using online software (Schrodinger 2015-4, QikProp 4.6, New York, 2015), which is based on computational models. Drug-likeliness was evaluated in terms of violations in Lipinski's rule of five.[16]

4.3. *In Vitro*/*Ex Vivo* Biopharmaceutical Profiling. log *P* of safranal was evaluated by conventional shake-flask method in octanol and water system using 1:2, 1:1, and 2:1 (v/v) ratios. Safranal concentration in both octanol and water layer was analyzed by HPLC (Table 1). Testosterone (1:1 ratio) was used as standard where analysis of samples was carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Table S1; Supporting Information). log *D* of safranal was evaluated in the same manner using phosphate-buffered saline, pH 7.4 instead of water.[33]

Table 1. HPLC (Model: Prominence; Make: Shimadzu) Conditions for Quantitation of Safranal

parameter	condition
column	LiChrospher C_{18} (250 mm × 4.6 mm, 5 μm)
mobile phase	water:acetonitrile = 24:76 (% v/v)
elution	isocratic
flow rate	0.75 mL/min
column temperature	ambient
wavelength for UV detection	310 nm
run time	12 min
retention time	∼8.2 min
stock solution	acetonitrile
dilution	acetonitrile
software	LabSolutions

The P-gp-dependent ATPase activity for safranal (1.25−40 μM) was evaluated using human P-gp membrane where elacridar (10 μM) was used as standard.[34] This is a colorimetric assay to measure the release of inorganic phosphate in the reaction mixture composed of membrane protein, test compound, and ATP. BCRP-dependent ATPase activity for safranal (1.25−20 μM) was also performed using human BCRP membrane in the same manner as mentioned in the case of P-gp-dependent ATPase activity except cyclosporine (10 μM) was used as standard.[35] The chemical stability of safranal in SGF and SIF was determined by incubating safranal (500 μM) in the respective fluid for 2 and 4 h, respectively.[36] The sample spiked with safranal at the same concentration level but without incubation was considered as 0 h. Safranal concentration was determined by HPLC as described in Table 1. Permeability of safranal was determined by PAMPA,[33] where membrane material was 5% hexadecane in hexane (v/v) and safranal concentration was 10, 25, and 50 μM. Testosterone (25 μM) was used as a standard. The concentration of safranal and testosterone in both donor and acceptor plates was quantified by HPLC as mentioned in Tables 1 and S2 (Supporting Information), respectively. Plasma protein binding of safranal was measured by the rapid equilibrium dialysis (RED) method.[33] The study was performed using blank rat plasma and safranal concentrations of 5, 10, and 20 μM. The presence of safranal in both sample chamber and buffer chamber was determined by HPLC (Table 1). Rifampicin (10 μM) was used as a standard where rifampicin concentration was analyzed by HPLC (Table S3; Supporting Information). The blood-to-plasma ratio of safranal was assessed by incubating blank rat plasma as reference plasma and blood where safranal was spiked at the concentration level of 1000 nM, and incubation was done separately for 0, 10, 30, and 60 min. Propanolol was used as standard at the same concentration level.[34] Safranal was estimated by HPLC as mentioned above, and propanolol was determined by LC-MS/MS (Table S4; Supporting Information). The stability of safranal in mice plasma as well as rat plasma was investigated at 500 μM for 0, 1, 2, and 4 h.[37] Quantification of safranal was done by HPLC (Table 1). The metabolic stability of safranal was explored in MLM, RLM, DLM, and HLM using substrate depletion approach where substrate concentration was 10 μM, protein concentration was 250 μg/mL, NADPH concentration was 1.2 mM, and incubation time was 0, 15, and 30 min. The reaction mixture without NADPH served as a negative control.[34,36] The concentration of safranal was analyzed by HPLC (Table 1). All of the above-mentioned parameters were evaluated, considering that the experimental solvents/buffers have no effect on safranal. Detail experimental procedures are provided in the Supporting Information.

4.4. *In Vivo* Pharmacokinetic Study. The pharmacokinetic study of safranal was performed in healthy Balb/C mice with prior approval from Institutional Animal Ethics Committee, CSIR-IIIM, Jammu. Prior to experimentation, the animals were housed under standard laboratory conditions for acclimatization. Overnight fasted animals were divided into groups (five animals/group) for sparse sampling. Dose formulation of safranal was prepared in olive oil (100%, v/v) with a dose volume of 10 mL/kg. Safranal was administered through the oral route at 100 mg/kg single dose followed by the collection of the blood sample at time points of 0, 0.25, 0.5, 1, and 2 h from retro-orbital plexus into a microcentrifuge tube containing aqueous EDTA solution. Plasma was obtained through centrifugation, and then, the sample was processed by the protein precipitation technique using acetonitrile. Safranal concentration in the sample was determined by HPLC (Table 1) using a matrix match calibration curve where standards were prepared by spiking a known amount of safranal into blank plasma. After that, the obtained plasma concentration data at respective time points were analyzed to determine the pharmacokinetic parameters by PK Solutions software (Summit Research Services) using the noncompartmental method.[35,36]

4.5. Statistical Analysis. Statistical significance was assessed using online Student's *t*-test (QuickCalcs, GraphPad software) between alone group and combined treatment group for *in vivo* experimentations or control *vs* treatment data for *in vitro* experimentations. Data are represented as mean ± SEM. *p*-value of less than 0.05, 0.01, and 0.001 was

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considered statistical significant, highly statistical significant, extremely statistical significant, respectively.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00160.

Detailed methodology for the assessment of log *P* and log *D*, ATPase activity, chemical stability, permeability, plasma protein binding, plasma stability, RBC partitioning, metabolic stability, and pharmacokinetic study; HPLC chromatograms for chemical stability and plasma stability; and plasma concentration *vs* time profile for pharmacokinetic study of safranal (PDF)

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Author Contributions

A.D., P.K., S.B., and A.G. performed all of the *in vitro and in vivo* profiling of safranal including the HPLC or LC-MS/MS method development. Acquisition of consumables and proof reading were carried out by G.S. Chemical data interpretation was performed by D.M. Overall study design, statistical data evaluation, and MS preparation were performed by U.N.

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Notes

The authors declare no competing financial interest.

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