SUPPORTING INFORMATION

Description of Drug-like Properties of Safranal and Its Chemistry behind Low Oral Exposure

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DETAILED METHODOLOGY

Determination of log *P*

log *P* of safranal was determined using octanol pre-saturated with water and water pre-saturated with octanol. A weighing amount of safranal was added to the ratio of 1:1, 1: 2, and 2:1 (v/v) followed by shaken in the water bath for 30 min at 37 ºC. After that separation of two phases was done by centrifugation, diluted necessarily with acetonitrile, filtered through 0.22 µm syringe filter, and transfer into vial to quantitate the compound concentration in both octanol ($C_{octanol}$) and water (C_{water}) layer by HPLC (Table 1). Experiment was performed using four replicates. Testosterone as standard was processed using methanol and analysed by LC-MS/MS (Table S1). Then, log *P* was calculated by using the following equation:

$$\text{Partition coefficient} = \log\left[\frac{C_{octanol}}{C_{water}}\right]$$

TABLE S1 LC-MS/MS conditions for quantitation of testosterone

LC-MS/MS system (Model & Make)	Dionex Ultimate 3000 & Thermo Scientific
Column	Purospher RP-18 (50 mm × 2.1mm, 3µ)
Mobile phase	0.1 % formic acid in water : acetonitrile (50: 50, v/v)
Elution	Isocratic
Flow rate	0.2 mL/min
Column temperature	45 °C
Run time	4 min
Retention time	~ 1.9 min
Stock solution	DMSO
Dilution	Methanol
Precursor/product transition pair	m/z 289.0 > 97.2
Sheath gas (Arb)	30
Auxiliary gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temperature	200 °C
Vaporizer temperature	300 °C
Positive ion voltage	3500 V
RF lens	197 V
Software	Chromeleon Xpress (HPLC) & LCQUAN (MS)

Determination of log *D*

For the evaluation of distribution coefficient (log *D*), experimentation was carried out in same manner as mentioned for log *P* experimentation except the use of PBS instead of water. Calculation was done by using following equation:

$$\text{Distribution coefficient} = \log\left[\frac{C_{octanol}}{C_{PBS}}\right]$$

Estimation of P-gp-dependent ATPase activity

P-gp-dependent ATPase activity of safranal was estimated according to manufacturers protocol (Sigma-Aldrich) using human P-gp membrane (Corning Gentest). Briefly, 25 µg of membrane (5 µL of 5 mg/mL) was pre-incubated with safranal (1.25-40 µM) in 96-wellplates at room temperature for 5 min. The reaction was initiated by the addition of 10 µL of 4 mM Mg ATP solution and incubated for 30 min. Then, 200 µL of detection reagent was added to each well and incubate for an additional 30 min at room temperature. Absorbance of sample was measured at 620 nm using multimode microplate reader (Make: Tecan; Model: Infinite M200PRO) to measure the amount of Pi. In parallel, the phosphate calibration standard was used for quantification of it in the sample. Reaction without enzyme sample was analysed in same way which served as control. Each reaction was performed in duplicate and elacridar (10 µM) was used as positive control. P-gp dependent ATPase activity was calculated using the following equation where Sa is the concentration of phosphate (µM) generated in unknown sample well, Rv is the reaction volume (µL), Sv is the sample volume (µL) added to the well, T is the reaction time (min).

$$Enzymatic\ activity = \frac{\mathrm{Sa} \times \mathrm{Rv}}{\mathrm{Sv} \times \mathrm{T}}$$

Estimation of BCRP-dependent ATPase activity

BCRP-dependent ATPase activity was also executed using human BCRP membrane (Corning Gentest) as per the manufacturer's protocol (Sigma-Aldrich). This study was performed in the same manner as described for P-gp dependent ATPase activity except concentration of safranal (1.25-20 µM) and cyclosporine (10 µM) was used as standard.

Determination of chemical stability in SGF and SIF

Chemical stability of safranal was determined in SGF and SIF which are prepared as mentioned in Indian Pharmacopoeia, 2014. SGF consisted of 0.1 (N) hydrochloric acid having pH 1.2 whereas SIF composed of phosphate buffer having pH 6.8. A known amount of safranal was dissolved in acetonitrile and spiked into the SGF and SIF separately at a concentration of 500 µM that incubated in the water bath at 37 °C for 2 h and 4 h, respectively. After that, sample (50 µL each) was taken out, added acetonitrile (200 µL) and centrifuged at 13145 rpm for 10 min. The supernatant was poured into vial and analyzed by HPLC (Table 1). Safranal spiked sample without incubation was considered as 0 h data. Study was performed using four replicates. Percentage stability at 2 h and 4 h was calculated based on 0 h data which considered as 100 %.

Determination of permeability

Permeability study was performed using PAMPA assembly where donor plate and acceptor plate were sandwiched and separation was done by a microfilter disc (Millipore). This filter disc was coated with 5% (v/v) hexadecane in hexane and dried overnight at room temperature. Acceptor plate contained PBS (250 µL) whereas doner plate (150 µL) contained safranal at concentration of 10, 25 and 50 µM. Then, sealed the assembly to prevent evaporation and kept for 5 h at room temperature. After that sample was taken from each well, diluted necessarily with acetonitrile, vortex mixed and analysed by HPLC (Table 1). Study was performed in triplicate and testosterone (25 µM) used as standard which was processed using methanol and estimated by HPLC (Table S2). Then, permeability (Pe, cm/s) was calculated using the below equation where, C_A is the compound concentration in acceptor compartment at time t; C_E is the equilibrium compound concentration in the total volume of donor and acceptor compartment at time t; V_D is the donor volume (0.15 mL); V_A is the acceptor volume (0.25 mL); S is the surface area of membrane considering porosity (0.048 cm2); t is the incubation time (18000 s).

$$P_e = -\ln\left[1 - \frac{C_A}{C_E}\right] \times \left[\frac{V_D \times V_A}{(V_D + V_A) \times S \times t}\right]$$

TABLE S2 HPLC conditions for quantitation of testosterone

HPLC system (Model & Make)	Prominence & Shimadzu
Column	LiChrospher C_8 (250 mm × 4.6 mm, 5 µ)
Mobile phase	Water : methanol:: 30: 70 (% v/v)
Elution	Isocratic
Flow rate	0.7 mL/min
Column temperature	Ambient
Wavelength for UV detection	254 nm
Run time	10 min
Retention time	~ 7.6 min
Stock solution	DMSO
Dilution	Methanol
Software	LabSolutions

Determination of plasma protein binding

Protein binding of safranal with rat plasma was determined by using Red Equilibrium Dialysis (RED) device (Pierce, Thermo Fisher Scientific). This device contains insert which was placed in base plate. Inserts contains two chambers such as buffer chamber (350 µL of PBS) and sample chamber (200 µL of blank rat plasma containing compound) which was separated via dialysis membrane. Then, plate was sealed with sealing tape and incubated in shaking water bath (Make: New Brunswich Scientific; Model: CLASSIC C76) with a speed

of 120 rpm at 37°C for 4 h. After that, 100 µL of each plasma and buffer was taken out and 100 µL of each blank buffer and plasma was added to it, respectively. Then, acetonitrile was added to sample, vortex mixed for 2 min, incubated for 30 min in ice, centrifuged at 14000 rpm for 10 min. Supernatant was transferred into vial and analysed by HPLC (Table 1). Study was carried out using four replicates and rifampicin (10 µM) was used as standard which was estimated by using HPLC (Table S3). Plasma protein binding was calculated using the following equation where $C_{buffer\ compartment}$ is the concentration of compound in the buffer and $C_{sample\ compartment}$ is the concentration of the compound in the plasma.

$$Protein\ bound\ fraction\ (\%) = 100 - [\frac{C_{buffer\ compartment}}{C_{sample\ compartment}} \times 100]$$

TABLE S3 HPLC conditions for quantitation of rifampicin

HPLC system (Model & Make)	Prominence & Shimadzu
Column	LiChrospher C_{18} (250 mm × 4.6 mm, 5 µ)
Mobile phase	Water : acetonitrile :: 30: 70 (% v/v)
Elution	Isocratic
Flow rate	0.7 mL/min
Column temperature	Ambient
Wavelength for UV detection	271 nm
Run time	12 min
Retention time	~ 8.6 min
Stock solution	DMSO
Dilution	Methanol
Software	LabSolutions

Determination of RBC partitioning

Partitioning of safranal between RBC and plasma was investigated using fresh blood from rat on the day of experimentation. Hematocrit value of blood was measured by automated hematology analyzers (XT1800i, Symsmex). Identical volume of blood and reference plasma was separately taken into microcentrifuge tube where safranal solution in DMSO was spiked at the concentration level of 1000 nM. Then, both the sample were mixed gently and incubated at 37 ºC where reference plasma (50 µL) and blood (200 µL) samples were taken out at the time point of 0, 10, 30 and 60 min. Blood sample was processed to obtain plasma followed by addition of acetonitrile (200 µL) separately to both this plasma and reference plasma of 50 µL each for plasma protein precipitation. Then, each sample was vortex mixed for 2 min and centrifuged at 14000 rpm for 10 min followed by supernatant was taken out and analysed by HPLC (Table 1). Study was carried out using three replicates. Propranolol used as standard where sample was processed using methanol and analysed by LC-MS/MS (Table S4). Blood to plasma ratio ($K_{RBC/plasma}$) was calculated by the following equation

where H is hematocrit value of blood, IRP is the intensity of the compound in reference plasma and IP is the intensity of the compound in plasma obtained from blood sample.

$$K_{RBC/plasma} = 1/H \times (I_{PL}^{REF}/I_{PL} - 1)) + 1$$

TABLE S4 LC-MS/MS conditions for quantitation of propanolol

LC-MS/MS system (Model & Make)	Dionex Ultimate 3000 & Thermo Scientific
Column	Purospher RP-18 (50 mm × 2.1mm, 3µ)
Mobile phase	0.1 % formic acid in water : acetonitrile (50: 50, v/v)
Elution	Isocratic
Flow rate	0.2 mL/min
Column temperature	45 °C
Run time	3 min
Retention time	~ 0.8 min
Stock solution	DMSO
Dilution	Methanol
Precursor/product transition pair	m/z 260.3 > 116.2
Sheath gas (Arb)	30
Auxiliary gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temperature	200 °C
Vaporizer temperature	300 °C
Positive ion voltage	3500 V
RF lens	181 V
Software	Chromeleon Xpress (HPLC) & LCQUAN (MS)

Determination of stability in mice and rat plasma

Plasma stability of safranal was carried out using both rat and mice plasma where blood was collected freshly before the experimentation. In short, a known amount of safranal was dissolved in acetonitrile and spiked into preheated plasma at a concentration level of 75 µg/mL. An aliquot of plasma (50 µL) was withdrawn at 0 h, 1 h, 2 h and 4 h. Then, ice-cold acetonitrile was added, vortex mixed for 2 min. and centrifuged at 13145 rpm for 10 min. supernatant was transferred into vial for injecting onto HPLC system (Table 1). Study was performed using four replicates.

Determination of metabolic stability

Metabolic stability of safranal was evaluated in MLM, RLM, DLM and HLM.[3,4] Briefly, reaction mixture composed of safranal (10 µM), microsomal protein (0.25 mg/mL), $MgCl_2$ (3.3 mM) and phosphate buffer (100 mM, pH 7.4). Reaction was initiated by addition of NADPH (1.2 mM), where total volume of the reaction mixture was 250 µL. Reaction was terminated using ice cold acetonitrile after 0, 15 and 30 min of incubation followed by vortex mixed for 2 min. and centrifuged at 3000 rpm for 15 min. Then, supernatant was taken out,

poured into vial and analysed by HPLC as described in Table 1 except HPLC system (Spectra system and Thermo Finnigan), run time (10 min) and retention time (~ 5.7 min) and software (ChromeQuest). Study was carried out in triplicate.

RESULTS

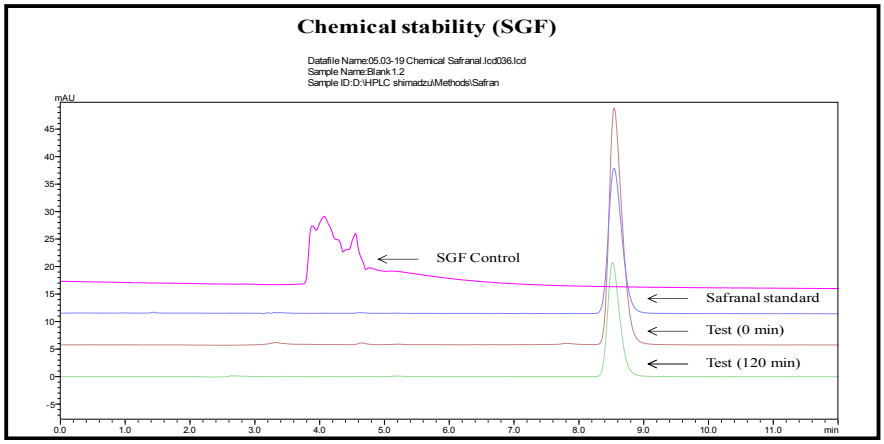

FIGURE S1 Representative HPLC chromatogram of control sample, safranal standard sample, test sample at 0 min and test sample at 120 min to assess chemical stability of safranal in SGF.

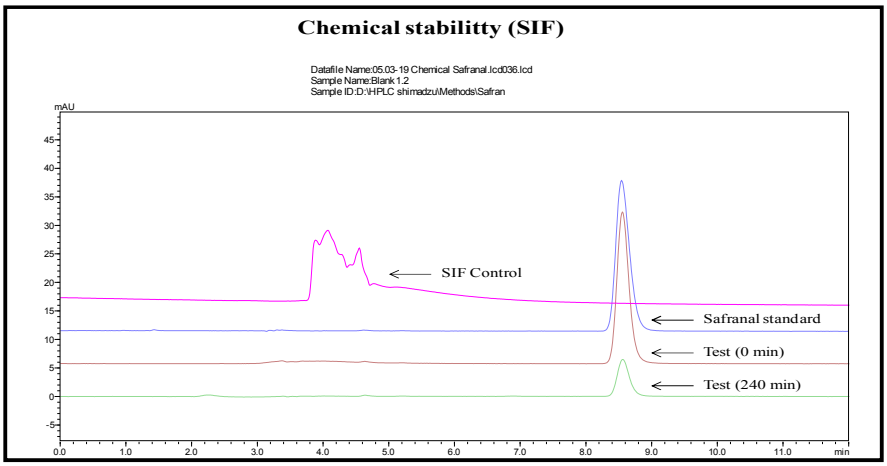

FIGURE S2 Representative HPLC chromatogram of control sample, safranal standard sample, test sample at 0 min and test sample at 240 min to assess chemical stability of safranal in SIF.

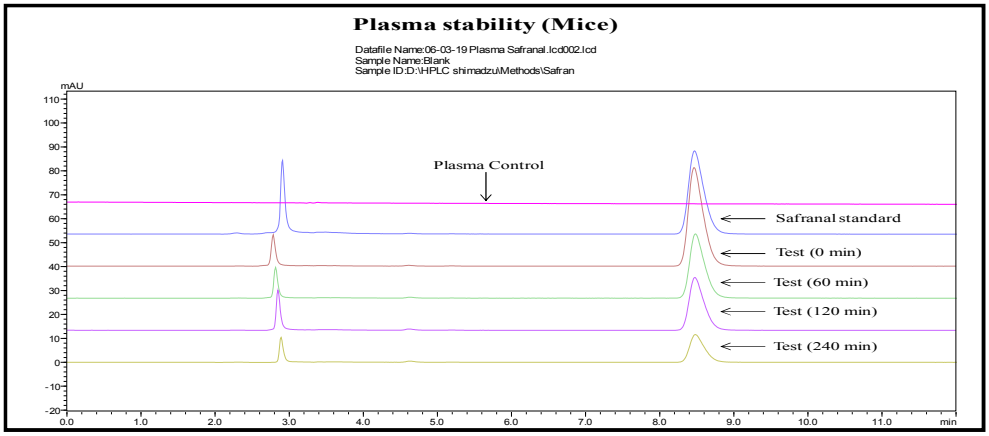

FIGURE S3 Representative HPLC chromatogram for control plasma sample, safranal standard sample, test sample at 0 min, test sample at 60 min, test sample at 120 min and test sample at 240 min to assess stability of safranal in mice plasma.

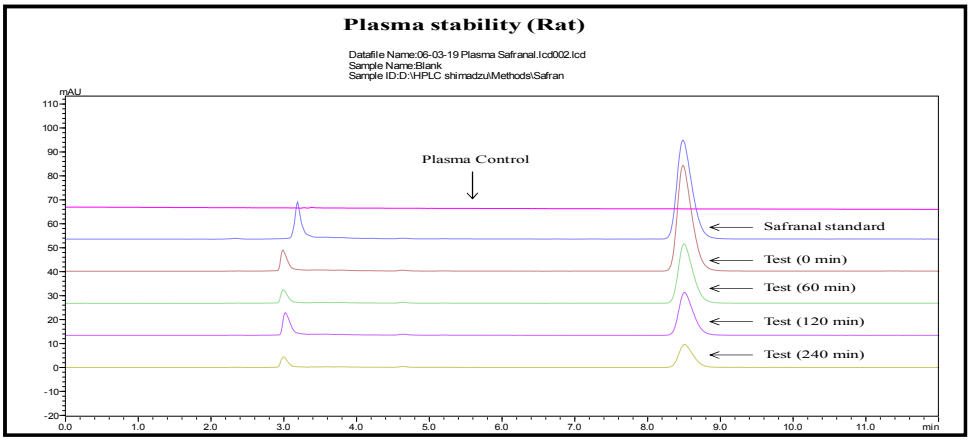

FIGURE S4 Representative HPLC chromatogram for control plasma sample, safranal standard sample, test sample at 0 min, test sample at 60 min, test sample at 120 min and test sample at 240 min to assess stability of safranal in rat plasma.

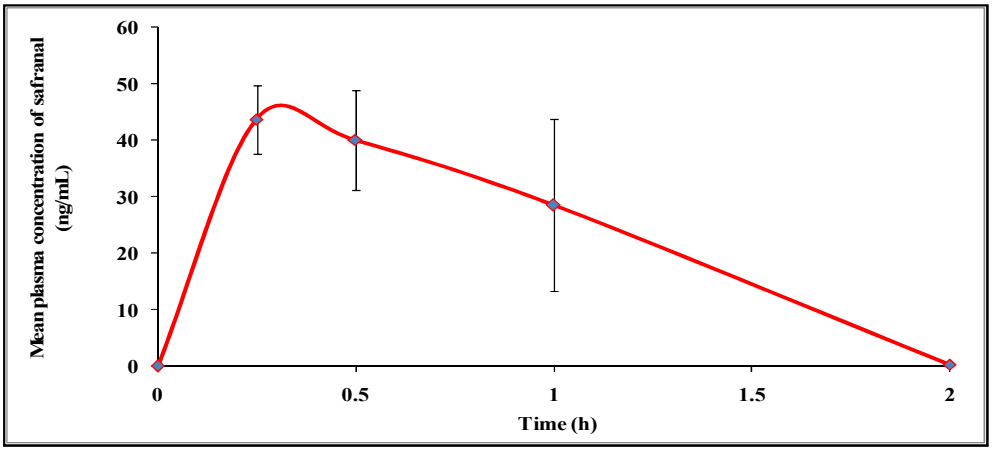

FIGURE S5 Mean plasma contration vs. time profile for oral pharmacokinetic study of safranal in Balb/C mice.