Indian Journal of Traditional Knowledge
Vol 21(04), October 2022, pp 789-796
DOI: 10.56042/ijtk.v21i4.55946

Pharmacokinetic interaction potential assessment of cladrin, a potent bioactive constituent of *Butea monosperma,* and raloxifene, a prescription anti-osteoporotic by *in vitro* ADME approach

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Received 07 October 2021; revised 17 November 2021; accepted 02 December 2021

Raloxifene is a well-known modulator of estrogen receptors which is structurally similar to tamoxifen. As flavonoids can interact with the estrogen modulator raloxifene *in vitro*, we performed an *in vitro* stability study and *in situ* permeability assay of raloxifene and cladrin in female Sprague-Dawley rats when administered alone and when co-administered. The *in vitro* study samples were analyzed by HPLC; raloxifene administered individually and in combination with cladrin was compared. In this study, we investigated the absorption, metabolic stability, plasma stability, determination of permeability and plasma protein binding of both drugs in SD rats using an established *in situ* single pass intestinal perfusion model. Increase in the bioavailability of raloxifene and cladrin alone or in co-administration also could be because of the activation of P-glycoprotein in the rat intestine. Further the present report concludes on the basis of ATPase assay of both raloxifene and cladrin alone and in combination showed that both drugs are P-gp substrate. In *in situ* permeability assay showed that the both drugs competitively lower the permeability of each other but still the predicted human permeability value lied in the range of high permeability drug.

Keywords: Cladrin, Flavonoids, Herb-Drug interaction, Pharmacokinetics, Raloxifene, SPIP

IPC Code: Int Cl.[22]: A61K 36/00, A61K 39/00, A61K 45/06

The herbal medicine *Butea monosperma* belongs to the family Fabaceae. It is traditionally utilized for medicinal use since ancient times in our traditional Ayurveda and Unani medicines as an anti-fungal, anti-hyperglycemic, hepato-protective and osteo-protective[1], antitumor and antimicrobial compound[2]. It is commonly known as 'fire of forest' or Palas, commonly found in the hilly regions of Sri Lanka, Burma, Java and a few parts of India[3,4]. Crude bark extract of *Butea monosperma* consists of different types of methoxylated isoflavones like cajanin, pruretin, formononetin, iso-formononetin and cladrin (CLD)[5,6]. The recent past studies showed that methoxy isoflavones formononetin, iso-formononetin and cajanin etc increase the peak bone density, bone biomechanical strength as well as bone formation rate in growing rats as compared to control, they also increase the formation of osteoblasts *in vitro* models[5,7,8]. The other isoflavone CLD, (3, 4 - dimethoxy daidzein), one of the important isoflavones of *Butea monosperma,* is structurally similar to the methoxylated daidzein. A recent study has clearly demonstrated that CLD has a strong protective role against post-menopausal bone loss. CLD also increases the formation of bone density in *in vitro* assays by the modulation of extracellular signal regulated kinase (Erk) pathway[1,9]. In addition, CLD also stimulates the differentiation and proliferation of osteoblasts by activating the MEK-Erk signaling pathway[9]. The presence of O-methylation increases the lipophilicity of CLD and leads to oral absorption in the body and results in excellent systemic bioavailability as compared to other isoflavones. Various other pharmacokinetic studies have also reported that CLD is an excellent inducer of

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osteoblast proliferation and differentiation as compared to other flavonoids such as daidzein[9]. In spite of above CLD has been also found to improve hyperlipidemia, obesity, adipogenesis and induced bone formation[10], increased lumbar vertebral strength, enhanced bone formation rate, coupled remodeling, microarchitecture of trabecular bones, cortical thickness and also stimulates expression of dose-dependent osteogenic genes in bone[1].

Another drug such as raloxifene (RLX) belongs to the family of benzothiophene and it demonstrates selective estrogen receptor modulators (SERMs) profiles. Selective estrogen receptor modulators function through the binding with estrogen receptor and induce the effect of estrogen agonists in different cells but in similar conditions it antagonizes the estrogen receptors in other cells also. The binding affinity of RLX to estrogenic receptors is comparable to 17 β-estradiol. Post oral administration, RLX undergoes first pass through glucuronidation via rapid absorption and enterohepatic cycle. Till date no study has shown the metabolism of RLX by cytochrome P-450. The absorption rate of RLX has nearly 60% but the poor bioavailability (2%) was reported. In spite of poor bioavailability, RLX has shown better potential to prevent bone loss, increased bone strength and decrease callous bone reabsorption in the ovariectomized condition[11]. Due to poor bioavailability of RLX, there is a tendency to consume huge amounts of conventional medications as a supplement for health benefits even while being on prescription medicines poses a risk of herb-drug interaction(s). Herb-drug interactions may have both pharmacokinetic and pharmacodynamic components, for example, ginseng reduced warfarin and alcohol levels in the blood as well as induced mania when taken concurrently with phenelzine, a non-selective and irreversible monoamine oxidase inhibitor used as an antidepressant and anxiolytic agent.

Similarly, case studies show that ginkgo might worsen bleeding when used with warfarin or aspirin raise blood pressure when taken with thiazide diuretics, and even cause coma when taken with trazodone, a serotonin antagonist and reuptake inhibitor used to treat depression[12].

To the best of our knowledge, there is no study reported which has addressed herb-drug interaction potential of promising anti-osteoporotic agent CLD or CLD containing multicomponent herbal medicament(s) co-administration while being on anti-osteoporotic RLX prescription therapy. The objective of presented study was to assess the pharmacokinetic interaction potential assessment of CLD, a potent bioactive constituent of *Butea monosperma* and RLX, a prescription anti-osteoporotic by *in vitro* ADME Approach and predict the safety of *in vivo* co-administration compatibility of the same.

Methodology

Materials and Methods

Chemicals and reagents

CLD was procured from Aritech chemazone Pvt Ltd. Urban Estate Kurukshetra, Haryana, India and RLX was gifted from Cadila Pharmaceuticals Laboratories Ltd. Mumbai, India. Potassium chloride (KCL), calcium chloride ($CaCl_2$), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydrogen carbonate ($NaHCO_3$), high-pressure liquid chromatography (HPLC)-grade acetonitrile and methanol were procured from Sisco Research Laboratories Private Ltd. Mumbai, India. Formic acid, phenol red, urethane and charcoal, magnesium chloride ($MgCl_2$), dimethyl sulfoxide (DMSO) and sodium chloride (NaCl) were procured from Sigma-Aldrich Ltd. A P-glycoprotein ATPase determination kit (human) was procured from BD Gentest (Woburn, MA). Triple distilled water was acquired from the water purification system Millipore Elix, procured from Millipore India Private Ltd. New Delhi, India. Ethanol was procured from Merck, Germany. All other utilized chemicals and reagents were of analytical grade.

Animals and legal prerequisites

Female Sprague-Dawley (SD) rats weighing 180-225 g were acquired from the National Laboratory Animal Center, CSIR-Central Drug Research Institute, Lucknow (India) and harbored at the relative humidity of 40-60% and temperature of 25±2°C in well-ventilated environment. SD rats were kept on a light-dark cycle for 12 h along with liberated access to water and fasted at least 12-18 h before experiments. Prior to experiment, animals were acclimatized for at least 7 days. Consent from the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Drug Research Institute, Lucknow (India) was taken. Experiment protocols were confirmed prior to the initiation of study.

Instrument and chromatographic conditions

The compounds RLX and CLD were analyzed by using sophisticated analytical methods HPLC Nexera

(Ultra Pressure Liquid Chromatography) Shimadzu. The UPLC Nexera instrument is well equipped with the SIL-30 AC auto-sampler, LC-30 AD pumps, DGU-20A degasser, SPD- M20A photodiode array (PDA) detector. All the HPLC parameters were monitored through LabSolution software. The compounds RLX and CLD were detected by PDA at 285 and 249 nm, after separation through C-18 Phenomenex® column with a mobile phase consisting of acetonitrile and 0.1% (v/v) formic acid in water in the proportion of 35:65 (v/v) and flow rate was kept 1 mL/min. The mobile phase was properly strained through the 0.22 µm Millipore filter and ultrasonically degassed for 10 min earlier to use.

In vitro preclinical pharmacokinetics study

In vitro SGF and SIF stability study

To prepare a simulated gastric fluid buffer (SGF), 37.2 mg of KCl and 234 mg NaCl in 100 mL triple distilled water (TDW) was mixed and hydrochloric acid was used to set the pH 1.2. To prepare simulated intestinal fluid (SIF) buffer 90 mg of NaOH and 680 mg of KH_2PO_4 in 100 mL TDW was dissolved and orthophosphoric acid was used to set pH to 6.8. The studies were carried out at least in triplicates. SGF and SIF (990 µL each) were withdrawn in separate glass test tubes. The buffers were pre-incubated at a temperature of 37±2°C in a shaking water bath for 10-15 min. From stock solution of 1 mg/mL, 10 µL RLX and CLD was spiked in pre-incubated SGF and SIF buffer to make the 10 µg/mL concentration respectively and instantly subjected to incubation. Incubation mixture (50 µL) was sampled at 0, 5, 15, 30, 45, 60, 90 and 120 min followed by termination with 100 µL of acetonitrile 1:2 (v/v) and followed by 10 min centrifugation at 10,000 rpm. The 30 µL supernatant was subjected for analysis by HPLC Nexera[13,14].

In vitro plasma stability study

Plasma from female SD rat was utilized to perform the *in vitro* plasma stability study. Plasma (990 µL) sample in a test tube was incubated in a shaking water bath at 37±1°C for 5-10 min before spiking the drug. To yield a concentration 10 µg/mL, 10 µL aliquot of RLX and CLD (1 mg/mL) was spiked in plasma before incubation. Quickly after post incubation, 50 µL incubated solution was sampled at the function of time (0, 15, 30, 60, 90, 120 min). The withdrawn plasma sample was mixed with acetonitrile (ACN) in the proportion of 1:2 (v/v) to precipitate the plasma protein. Subsequently, the plasma mixture was vortexed at 2500 rpm for 10 min in a bench-top mixer, and centrifuged at 10,000 rpm for 10 min. 30 µL of the supernatant obtained was directly run into HPLC for analysis. Decrement of the parent compound in the plasma sample was estimated to evaluate plasma stability of drugs[13,14].

Microsomal stability study

The stability of RLX and CLD in microsome was measured in rat liver microsome (RLM). To prepare the incubation mixture 3 mM $MgCl_2$ was mixed with 100 mM PBS (pH 7.4) and to this added microsomes (0.5 mg/mL) and testing drug RLX and CLD (10 µg/mL) in glass test tubes. The mixture was incubated at 37±1°C for 10 min on a shaking water bath. Post incubation, 1.2 mM NADPH was added to initiate the reaction, and 50 µL of the incubation mixture was picked up at a prescribed time point up to 60 min. Precipitation of microsomal protein was done by mixing withdrawn samples with chilled ACN followed by vortexing, centrifugation and collection of supernatant for immediate injection into HPLC for analysis.

RLM was isolated by the previously described method with some modification. Briefly, SD rats were fasted with liberated access to water for 12-18 h prior to sacrifice. 1.15% w/v KCL solution was used for liver perfusion to eliminate the residual blood and rat livers were collected. Further rat livers were cleaned, weighed and homogenized with a homogenization buffer at different rpm when subjected to different required centrifugation as per previously described methods. Post centrifugations, microsomal pellets collected were resuspended in a homogenization buffer. Concentration of protein content in microsomal samples was estimated by the Bradford method wherein the standard taken was bovine serum albumin. The developed and quantified microsome samples were further stored in 20 mg/mL aliquots at -80°C until use[15,16].

In vitro plasma protein binding study

The charcoal-dextran adsorption method was used to evaluate plasma protein binding (PPB) of RLX and CLD with little modification. Briefly, potassium chloride (0.05 g), potassium dihydrogen phosphate (0.05 g), hydrate disodium hydrogen phosphate (0.36 g) and sodium chloride (2.0 g) were mixed in 250 mL volume of triple distilled water to prepare Dulbecco phosphate buffered saline (DPBS) and pH was adjusted to 7.4. 100 mL. DPBS was used to

suspend 0.66 g of dextran-coated charcoal and continuously stirred on the magnetic stirrer at room temperature for at least 24 h. In a glass tube, 3 mL of charcoal mixture was centrifuged for 15 min and the supernatant was carefully decanted to get the charcoal pellets. RLX and CLD were spiked in fresh rat plasma samples and equilibrated at 37°C for 30 min prior to mixing with charcoal pellets. Above mixture was incubated for 2 h under continuous stirring at 37±2°C. 300 µL of the incubated mixture was collected at 0, 0.2, 0.5, 5, 10, 15, 30, 45, 60, 90 and 120 min and centrifuged for 1 min at 12,000 g. Then, 100 µL of supernatant was further mixed with 200 µL acetonitrile and centrifuged at 10,000 g for 10 min; the collected supernatant was injected directly into UPLC for investigation[16,17].

In situ SPIP studies

In situ single-pass intestinal perfusion studies (SPIP) studies were conducted using female SD rats (n=4). Rats were fasted at least overnight (12-16 h) with free access to water prior to study. 1.5 g/kg of body weight urethane was injected intraperitoneally to anesthetize animals and they were kept on the heating pad to maintain the normal body temperature. After achieving normal body temperature, a midline longitudinal abdominal slit was made in SD rats, and approximately 10 cm jejunum lumen was cleaned with pre-warmed (37°C) saline. In brief, catheterization of both the proximal and distal end of the rat jejunum lumen was done with an inlet and outlet polypropylene tube, respectively. The inlet tube was coupled with a perfusion pump and outlet tube was used to receive intestinal effluent. Cotton pad was soaked with normal saline and the whole excised area was wrapped with it. Perfusion buffers consisting of RLX and CLD (10 µM) and phenol red (20 µM) were infused at a 0.2 mL/min flow rate. To estimate the change in water flux across the incised intestinal section, a non-absorbable marker, phenol red, was used. Steady-state outlet concentrations were maintained for 30 min and then outlet samples were collected for 120 min at intervals of 15 min. Post-sample collection catheterized rat lumen was measured without stretching and the animal was sacrificed. Samples were spun at 2000 g for 10 min and the supernatant was immediately injected into the HPLC Nexera system[18-21].

Permeation data analysis

The basis of the SPIP experiment is to reach the steady state across the intestinal lumen which was confirmed by plotting the graph of outlet vs. inlet drug concentration ratio (corrected concentration) against time. To obtain corrected outlet concentrations, it was multiplied by inlet concentration of phenol red: outlet concentration of phenol red ratio.

The effective permeability coefficient $P_{eff(rat)}$ and drug absorption rate constant $K_{(a)}$ were calculated using Eq. (1) and (2), respectively;

$$P_{eff(rat)} = Q_{in} \text{ In } (C_{out}/C_{in})/2\pi rl \quad \text{... (1)}$$

$$K_a = [1-C_{out}/C_{in}]*Q_{in}/\pi r2l \quad \text{... (2)}$$

Where, C_{out} is the permeant drug corrected concentration; C_{in} is the permeant drug concentration; Q_{in} is the entering perfusate flow rate (0.2 mL/min); r is the intestinal inner radius (0.18 cm) and l is the intestine length.

The value of human permeability $P_{eff(human),}$ is predicted using Fagerholm Eq. (3) given below:

$$P_{eff(human),} = 3.6* P_{eff(rat)}+0.03*10^{-4} \quad \text{... (3)}$$

All the results are presented as mean standard deviation [18–21].

P-gp ATPase assay

P-gp ATPase activity of both drugs carried out by using human P-glycoprotein ATPase assay kit. P-glycoprotein ATPase activity was evaluated by estimating the released inorganic phosphate from ATP as per protocol of manufacturer. In brief, 20 µL of 1 mg/mL of human P-gp membrane (20 g) was incubated at 37°C for 5 min in a 96-well plate. To this 40 µL reaction mixture was added along with the test drug in the presence or absence of sodium orthovanadate (300 M). Further, 20 µL of 12 mM Mg ATP solution was added to start the reaction and incubated for 20 min at 37°C with 200 µL of detection reagent (8% ascorbic acid, 0.8% ammonium molybdate, 3 mM zinc acetate). The reaction was ended after 20 min by the addition of 30 µL of stop solution (10% sodium dodecyl sulphate solution). The released inorganic phosphate from ATP was identified by its absorbance measured at 800 nm. Propranolol and verapamil were used as negative and positive controls, respectively, to estimate the effectiveness of the study[19].

Results

For the analysis of RLX and CLD, the HPLC method was developed. The chromatographic separations were carried out with a mobile phase comprising acetonitrile (35:65) and formic acid 0.1%

(v/v), which was strained through a 0.22 µm Millipore filter and ultrasonically degassed for 15 min before being used. RLX and CLD had retention durations of 8.1 and 10.2 min, respectively as in Fig. 1. The total chromatographic run time was 12 min and Table 1 represents the chromatographic condition of both drugs.

SGF, SIF stability studies

To estimate the stability of RLX and CLD under different states in SGF and SIF (*in vitro*) stability studies were done. These studies demonstrated the stability of the drug molecule in the gastrointestinal tract prior to absorption. As shown in Fig. 2, RLX and CLD alone and in combination were found to be more than 60.43±11.79, 41.86±18.38, 70.76±5.99, 44.76±8.28% stable in acidic (SGF) conditions, respectively. RLX and CLD alone and in combination were found to be more than 33.68±9.30, 9.19±3.53, 12.97±0.62, 13.68±0.91% stable in basic (SIF) conditions respectively.

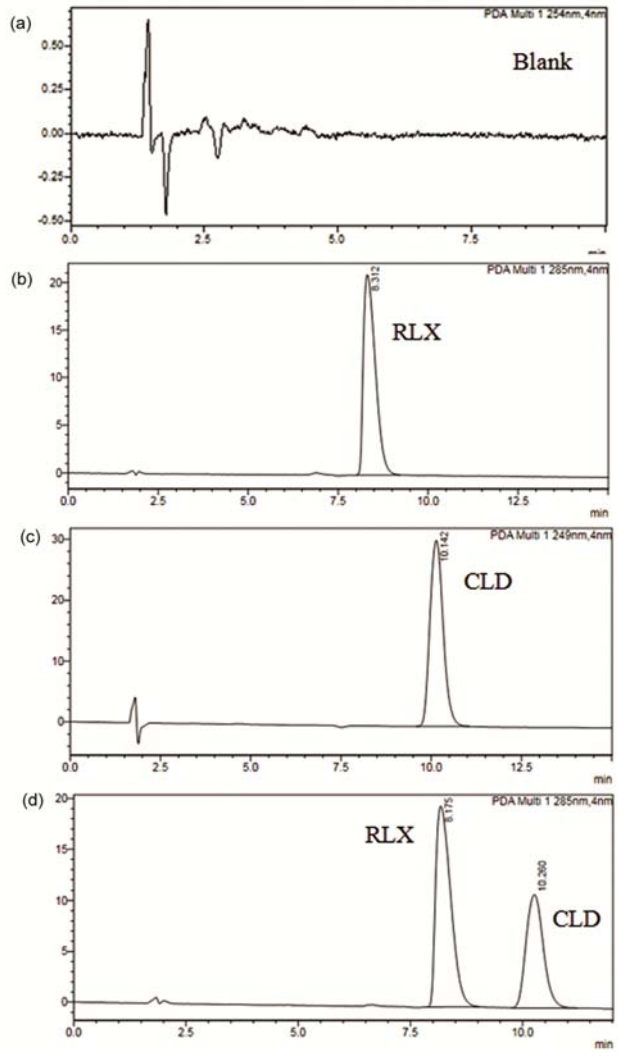

Fig. 1 — Chromatogram of a. Blank b. Raloxifene c. Cladrin d. Raloxifene + Cladrin at concentration 10 µg/mL

Plasma stability

To estimate the stability of RLX and CLD alone and together in rat plasma, an *in vitro* plasma stability study was carried out. The plasma stability study demonstrated the stability of the drugs in rat plasma. As shown in Fig. 3, RLX and CLD alone and in combination were found to be more than 17.88±9.38, 9.97±7.25, 27.34±3.33, 27.41±0.86%, respectively, in plasma. The assessment of stability of drugs in plasma is essential to estimate the analyte stability in the female SD rat plasma whereby it indicates the pharmacological response for a prolonged duration and reaches unchanged at the active site.

Microsomal stability study

The metabolic stability of RLX and CLD were evaluated using female RLMs in an *in vitro* condition. As shown in Fig. 4, approximately, 76.19±3.57, 67.78±18.79, 78.15±1.65, 79.87±0.34% of the intact RLX and CLD alone and in combination respectively, endured in the incubation solution up to 60 min. The result shows that the drug RLX and CLD alone and in combination are stable in RLM at 37±1°C up to 60 min.

Plasma protein binding

The plasma protein binding (PPB) of RLX and CLD was observed to be 95.70±0.52, 92.71±7.69, 96.73±0.49 and 97.39±3.08% alone and in combination, respectively. The traditional method for the evaluation of PPB could not be utilized for antiosteoporotic such as RLX and CLD. Hence, the charcoal adsorption method was significantly employed in the current study. The technique is fully based on the kinetics of charcoal adsorption. It includes measurement of the time required for the decrement of bound drug concentration after the removal of free drugs by charcoal adsorption. Thereafter, the percentage PPB is evaluated from the decrement of the

Table 1 — Chromatographic conditions for analysis of tested drugs

Drugs	Mobile phase	Wavelength
Raloxifene	Acetonitrile: Formic acid 0.1%(v/v) in milli Q (35: 65)	285 nm
Cladrin	Acetonitrile : Formic acid 0.1%(v/v) in milli Q (35: 65)	249 nm

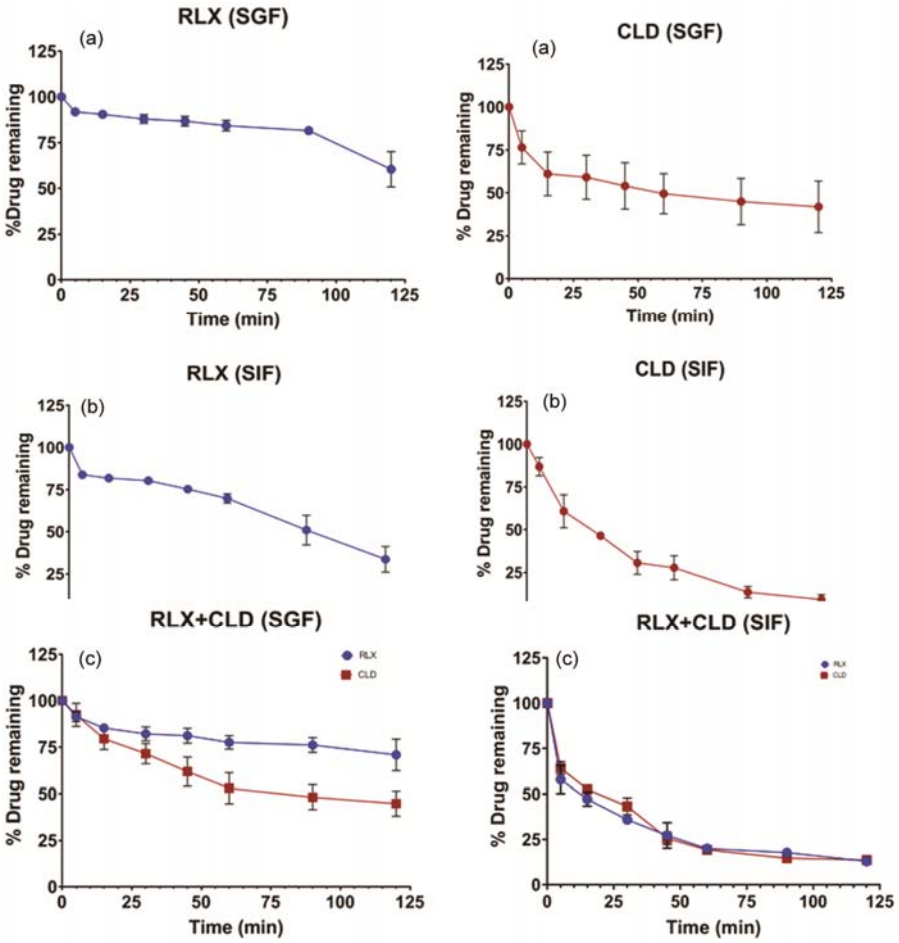

Fig. 2 — % Drug remaining vs. time profile of a. Raloxifene b. Cladrin c. Raloxifene + Cladrin in Simulated Gastric Fluid (Mean ± SEM) and in Simulated Intestinal Fluid (Mean ± SEM)

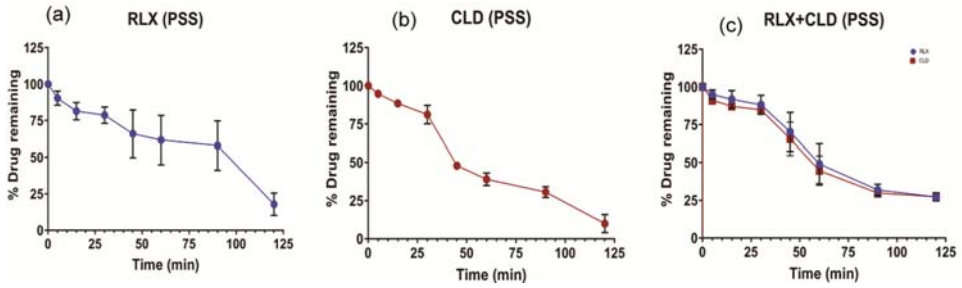

Fig. 3 — % Drug remaining vs. time profile for plasma stability of a. Raloxifene b. Cladrin c. Raloxifene + Cladrin (Mean ± SEM)

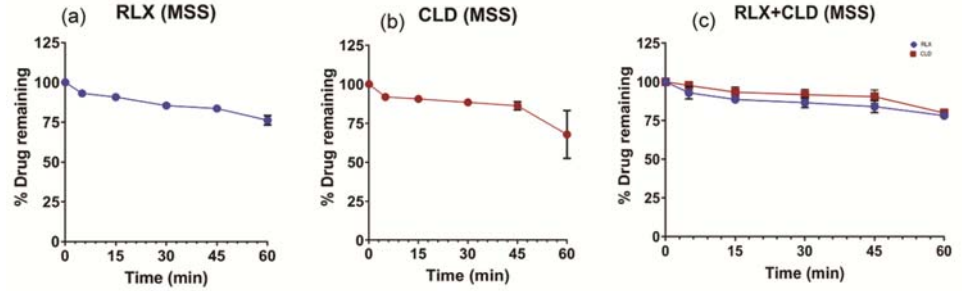

Fig. 4 — % Drug remaining vs. time profile for microsomes stability of a. Raloxifene b. Cladrin c. Raloxifene + Cladrin (Mean ± SEM)

Table 2 — The molecular weight, mean P_{eff} and $K_{(a)}$ values of tested drug in rats

Drugs	Molecular wt. (g/mol)	Mean P_{eff} (rat) ($*10^{-5}$ cm/s)	$K_{(a)}$ ($*10^{-3}$ min^{-1})	Mean predicted P_{eff} (human) ($*10^{-4}$ cm/s)
Raloxifene	473.584	19.99±8.96	16.42±5.82	7.18±3.22
Cladrin	298.29	23.60±11.03	17.66±3.07	8.50±3.97
Raloxifene + Cladrin	473.584, 298.29	10.93±0.93, 10.76±6.65	12.05±2.83, 11.67±6.28	3.97±0.33, 3.87±2.39

percentage drug remaining in supernatant after the mixing of charcoal in incubated plasma. Since the coating of dextran on charcoal effectively prevents the absorption of protein by charcoal, therefore it will lead to the correctness in results.

In situ SPIP studies

Table 2 shows the values of P_{eff} and $K_{(a)}$, values of the RLX and CLD. The P_{eff} value of RLX and CLD was found to be 19.99±8.96, 23.60±11.03 and 10.93±0.93, 10.76±6.65 cm/s alone and in combination, respectively. The rat P_{eff} value was used to predict human P_{eff} value which was found to be 7.18±3.22, 8.50±3.97, 3.97±0.33, 3.87±2.39 cm/s alone and in combination, respectively.

P-gp ATPase assay

Affinity of RLX and CLD and verapamil for human P-glycoprotein were estimated through ATPase assay. Fig. 5 shows the ATPase activities of these molecules with and without verapamil at different concentrations: verapamil at concentration of 20 µM and RLX and CLD at 10 and 20 µM with and without verapamil in different concentrations alone and in combination, respectively.

Discussion

In the present study, we have developed an HPLC method for concurrent determination of RLX and CLD. The method was effectively used to determine the SGF/SIF/Plasma stability study, microsomal stability study, plasma protein binding, *in situ* SPIP permeability and ATPase assay of RLX and CLD for the first time. Both drugs RLX and CLD were found to be more stable in SGF when compared to SIF with no sign of any significant herb-drug interaction. The RLX and CLD were observed to be stable up to 2 h in plasma at concentration of 10 µg/mL. The metabolic stability of RLX and CLD was determined using female SD RLM in an *in vitro* condition. About 75% of the entire RLX and CLD remained in the incubation mixture till 60 min. This indicates that both drugs RLX and CLD were stable in RLM and had showed no significant herb-drug interaction. The plasma protein binding of RLX and CLD were done using a charcoal adsorption method as this technique is simple and cost-effective and restrains non-specific binding of drugs.

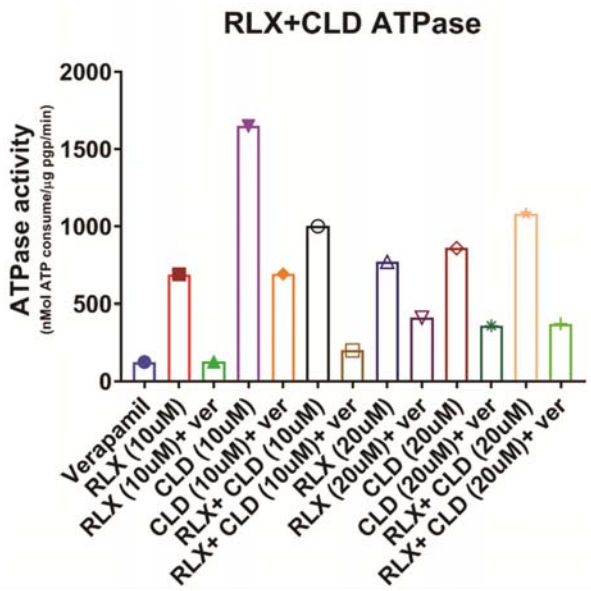

Fig. 5 — Human P-gp ATPase activities of verapamil, Raloxifene, Cladrin, Raloxifene + Cladrin and combinations of data

Both the drugs RLX and CLD showed high binding with plasma protein in both the conditions when given alone and in combination indicating no herb-drug interaction. The human ATPase assay of RLX and CLD was assessed with and without verapamil (a potent P-gp inhibitor) at two different concentrations (10 and 20 µM) to investigate the affinity of both the drugs to human P-gp. A significant change was observed, suggesting that both drugs RLX and CLD might act as substrates of human P-gp. The rat P_{eff} value was used to predict human P_{eff} value by eq. (3). As per the Biopharmaceutical classification system (BCS), human P_{eff} value of highly permeable drug is > 1.5 * 10–4 cm/s (30). Therefore, RLX and CLD both can be categorized as highly permeable drugs.

Conclusions

Overall, this study demonstrated the stability of the drug molecule in the gastrointestinal tract with more stability in SIF compared to SGF of RLX and CLD alone and in combination, respectively. Both drugs were stable in plasma as well as RLMs for 120 and 60 min, respectively and plasma protein binding of both drugs was found to be very high. The permeability in *in situ* SPIP study and the predicted human value is in the

range of the high permeable drugs as per BCS classification. It was also observed that both the drugs were stable with no significant herb-drug interaction. Furthermore, human P-gp ATPase assay showed the significant affinity towards both drugs for human P-gp indicating their P-gp substrate nature.

Acknowledgement

Authors acknowledge the Department of Science and Technology (DST), New Delhi for providing INSPIRE research fellowship to Divya Chauhan. CSIR-CDRI communication number 10327.

Conflict of Interest

No conflict of interest among the authors declared.

Authors' Contributions

DC and NS conducted most of the experiment, data analysis, data mining and draft the manuscript. PKY and AH contributing animal studies and HPLC data analysis. MR edited the manuscript. SS, SC and AA assisted the data analysis work. JRG and MKC guided the experimental and Wahajuddin conceptualized, supervised and edited the manuscript.

References

1 Khan K, Sharan K, Swarnkar G, Chakravarti B, Mittal M, *et al.*, Positive skeletal effects of cladrin, a naturally occurring dimethoxydaidzein, in osteopenic rats that were maintained after treatment discontinuation, *Osteoporos Int*, 24 (4) 2013 1455-1470.

2 Confavreux B C, Pialat B J, Bellière A, Brevet M, Decroisette C, *et al.*, Bone metastases from lung cancer: A paradigm for multidisciplinary onco-rheumatology management, *Joint Bone Spine*, 86 (2) 2019 185-194.

3 Ahmed F, Siddaraju N, Harish M & Urooj A, Effect of *Butea monosperma* Lam. leaves and bark extracts on blood glucose in streptozotocin-induced severely diabetic rats, *Pharmacogn Res*, 4 (1) 2012 3336.

4 Bavarva H J & Narasimhacharya A, Preliminary study on antihyperglycemic and antihyperlipaemic effects of Butea monosperma in NIDDM rats, *Fitoterapia*, 79 (5) 2008 328-331.

5 Pandey R, Gautam A K, Bhargavan B, Trivedi R, Swarnkar G, *et al.*, Total extract and standardized fraction from the stem bark of Butea monosperma have osteoprotective action: evidence for the nonestrogenic osteogenic effect of the standardized fraction, *Menopause*, 17 (3) 2010 602-610.

6 Maurya R, Yadav D K, Singh G, Bhargavan B, *et al.*, Osteogenic activity of constituents from Butea monosperma, *Bioorg Med Chem Lett*, 19 (3) 2009 610-613.

7 Bhargavan B, Gautam A K, Singh D, Kumar A, Chaurasia S, *et al.*, Methoxylated isoflavones, cajanin and isoformononetin, have non-estrogenic bone forming effect via differential mitogen activated protein kinase (MAPK) signaling, *J Cell Biochem*, 108 (2) 2009 388-399.

8 Srivastava K, Khan K, Tyagi A M, Khan M P, Yadav D K, *et al.*, Greater skeletal gains in ovary intact rats at maturity are achieved by supplementing a standardized extract of *Butea monosperma* Stem Bark that confers better bone conserving effect following ovariectomy and concurrent treatment withdrawal, *Evid Based Complement Alternat Med*, 2013.

9 Gautam A K, Bhargavan B, Tyagi A M, Srivastava K, Yadav D K, *et al*., Differential effects of formononetin and cladrin on osteoblast function, peak bone mass achievement and bioavailability in rats, *J Nutr Biochem*, 22 (4) 2011 318-327.

10 Gautam J, Khedgikar V, Choudhary D, Kushwaha P, Dixit P, *et al*., An isoflavone cladrin prevents high-fat diet-induced bone loss and inhibits the expression of adipogenic gene regulators in 3T3-L1 adipocyte, *J Pharm Pharmacol*, 68 (8) 2016 1051-1063.

11 Goldstein S R, Siddhanti S, Angelina C V & Leo P, A pharmacological review of selective oestrogen receptor modulators, *Hum Reprod Update*, 6 (3) 2000 212-224.

12 Chen X W, Serag E S, Sneed K B, Liang J, Chew H, *et al*., Clinical herbal interactions with conventional drugs: From molecules to maladies, *Curr Med Chem*, 18 (31) 2011 4836-4850.

13 Raju K S R, Rashid M, Gundeti M, Taneja I, Malik M Y, *et al*., LC-ESI-MS/MS method for the simultaneous determination of isoformononetin, daidzein and equol in rat plasma: Application to a preclinical pharmacokinetic study, *J Chromatogr B Analyt Technol Biomed Life Sci*,1129 2019.

14 Taneja I, Raju K S, Challagundla M, Raghuvanshi A, Goel A, *et al*., LC-ESI-MS/MS method for bioanalytical determination of osteogenic phytoalexin, medicarpin and its application to preliminary pharmacokinetic studies in rats, *J Chromatogr B Analyt Technol Biomed Life Sci*, 1001 2015 9-16.

15 Saxena A, Valicherla G R, Jain G K, Bhatta R S, Saxena A K, *et al*., Metabolic profiling of a novel antithrombotic compound, S002-333 and enantiomers: metabolic stability, species comparison and *in vitro-in vivo* extrapolation, *Biopharm Drug Dispos*, 37 (4) 2016 185-199.

16 Singh S K, Valicherla G R, Bikkasani A K, Cheruvu S H, Hossain Z, *et al*., Elucidation of plasma protein binding, blood partitioning, permeability, CYP phenotyping and CYP inhibition studies of Withanone using validated UPLC method: An active constituent of neuroprotective herb Ashwagandha, *J Ethnopharmacol*, 270 2021.

17 Yuan J, Yang D C, Birkmeier J & Stolzenbach J, Determination of protein binding by *in vitro* charcoal adsorption, *J Pharmacokinet Biopharm*, 23 (1) 1995 41-55.

18 Zakeri-Milani P, Valizadeh H, Tajerzadeh H, Azarmi Y, Islambolchilar Z, *et al*., Predicting human intestinal permeability using single-pass intestinal perfusion in rat, *J Pharm Pharm Sci*, 10 (3) 2007 368-379.

19 Wahajuddin, Raju K S, Singh S P & Taneja I, Investigation of the functional role of P-glycoprotein in limiting the oral bioavailability of lumefantrine, *Antimicrob Agents Chemother*, 58 (1) 2014 489-494.

20 Dahlgren D, Roos C, Peters K, Lundqvist A, Tannergren C, *et al*., Evaluation of drug permeability calculation based on luminal disappearance and plasma appearance in the rat single-pass intestinal perfusion model, *Eur J Pharm Biopharm*, 142 2019 31-37.

21 Lennernäs H, Intestinal permeability and its relevance for absorption and elimination, *Xenobiotica*, 37 (10-11) 2007 1015-1051.