Research Article

Biomedical Chromatography

Received 30 April 2010, Accepted 18 June 2010 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/bmc.1494

Influence of *Moringa oleifera* on pharmacokinetic disposition of rifampicin using HPLC-PDA method: a pre-clinical study

Anirban Pal,[a]* Dnyaneshwar Umrao Bawankule,[a] Mahendra Pandurang Darokar,[a] Subhash Chandra Gupta,[a] Jai Shankar Arya,[a] Karuna Shanker,[b] Madan MohanGupta,[b] Narayan Prasad Yadav[a] and Suman Preet Singh Khanuja[a]

ABSTRACT: The influence of active fraction isolated from pods of an indigenous plant, *Moringa oleifera* (*MoAF*) was studied on the pharmacokinetic profile of the orally administered frontline anti-tuberculosis drug rifampicin (20 mg/kg b.w.) in Swiss albino mice. The antibiotic rifampicin alone and in combination with *MoAF* (0.1 mg/kg b.w.) was administered orally and heparanized blood samples were collected from the orbital plexus of mice for plasma separation at 0, 1, 2, 3, 4 and 5 h, post treatment. Plasma rifampicin concentration, pharmacokinetic parameters and drug metabolizing enzyme (cytochrome P-450) activity were determined. The pharmacokinetic data revealed that *MoAF*-treated animals had significantly increased rifampicin plasma concentration, C_{max}, K_{el}, $t_{½(a)}$, $t_{½(el)}$, K_a and AUC as well as inhibited rifampicin-induced cytochrome P-450 activity. In conclusion, the result of this study suggested that the bioavailability-enhancing property of *MoAF* may help to lower the dosage level and shorten the treatment course of rifampicin. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: bioenhancer; rifampicin; *Moringa oleifera*; pharmacokinetics; mice; bioavailability

Introduction

Bioenhancers are molecules, which do not possess drug activity of their own but promote and augment the biological activity or bioavailability or the uptake of drugs in combination therapy. Isolated plant biomolecules or their semisynthetic derivatives have provided useful clues in the production of medicines (Khanuja *et al*., 2005). An interesting observation is that the combination of piperine isolated from *Piper nigrum* with essential drugs, such as antibiotics, antihypertensive and antiepileptics as well as nutrient supplements, led to dose economy due to enhanced uptake, higher blood concentration and the drug being available for a longer duration in the body (Atal *et al*., 1985; Hiwale *et al*., 2002; Khajuria *et al*., 1998; Lambert *et al*., 2004; Singh *et al*., 2005).

Moringa oleifera exhibited the antispasmodic, anti inflammatory, diuretic (Caceres *et al*., 1992), anti-obesity (Mehta *et al*., 2003; Krishnaraju *et al*., 2010) and anti-inflammatory activity in ovalbumin-induced airway inflammation in a guinea pig model of asthma (Mahajan *et al*., 2009) and reduced the arsenic-induced toxicity in rats (Gupta *et al*., 2005). Aqueous extracts of *M. oleifera* seeds lowered plasma fluoride concentrations in rabbits receiving fluorinated drinking water (Ranjan *et al*., 2009) and its flacculent mechanism is due to its content of low-molecular-mass protein (Kwaambwa *et al*., 2010). An *in-vitro* study of active fraction of *M. oleifera* pods (*MoAF*) against *Mycobacterium tuberculosis* (H37Ra) exhibited no anti-tuberculosis activity at the concentration at which it enhanced the anti-tubercular activity of rifampicin (Arya, 2003). A methodology for estimating the nitrile glycoside in *MoAF* has been reported by our group (Shanker *et al*., 2007).

Rifampicin acts by binding to, and inhibiting, DNA-dependent RNA polymerase in prokaryotic but not eukaryotic cells. It is one of the most active anti-tuberculosis agents; it enters the phagocytic cells and can kill intracellular microorganisms including the tubercle bacillus. Resistance can develop rapidly in a one-step process and is thought to be caused by chemical modification of microbial DNA-dependent RNA polymerase, resulting from a chromosomal mutation. Unwanted effects of rifampicin are relatively infrequent, but the commonest of them are skin eruption, fever and gastrointestinal disturbance. Liver damage with jaundice has been reported and sometimes this may be fatal. Rifampicin causes induction of hepatic metabolizing enzymes, resulting in an increase in the degradation of warfarin and glucocorticoid narcotic analgesics and failure of oral contraceptives (Rang *et al*., 2003). Under such situations, bioenhancers can be employed to reduce the drug-associated toxicity. The objective of the present study was to test the bioavailability enhancement of rifampicin when used in conjunction with *MoAF* as a bioenhancer as well as

* Correspondence to: A. Pal, Central Institute of Medicinal and Aromatic Plants, CSIR, Lucknow-226015, India. E-mail: a.pal@cimap.res.in

[a] Division of Molecular Bioprospection, Central Institute of Medicinal and Aromatic Plants, CSIR, Lucknow-226015, India

[b] Analytical Chemistry, Central Institute of Medicinal and Aromatic Plants, CSIR, Lucknow-226015, India

Contract/grant sponsor: Department of Biotechnology, Government of India

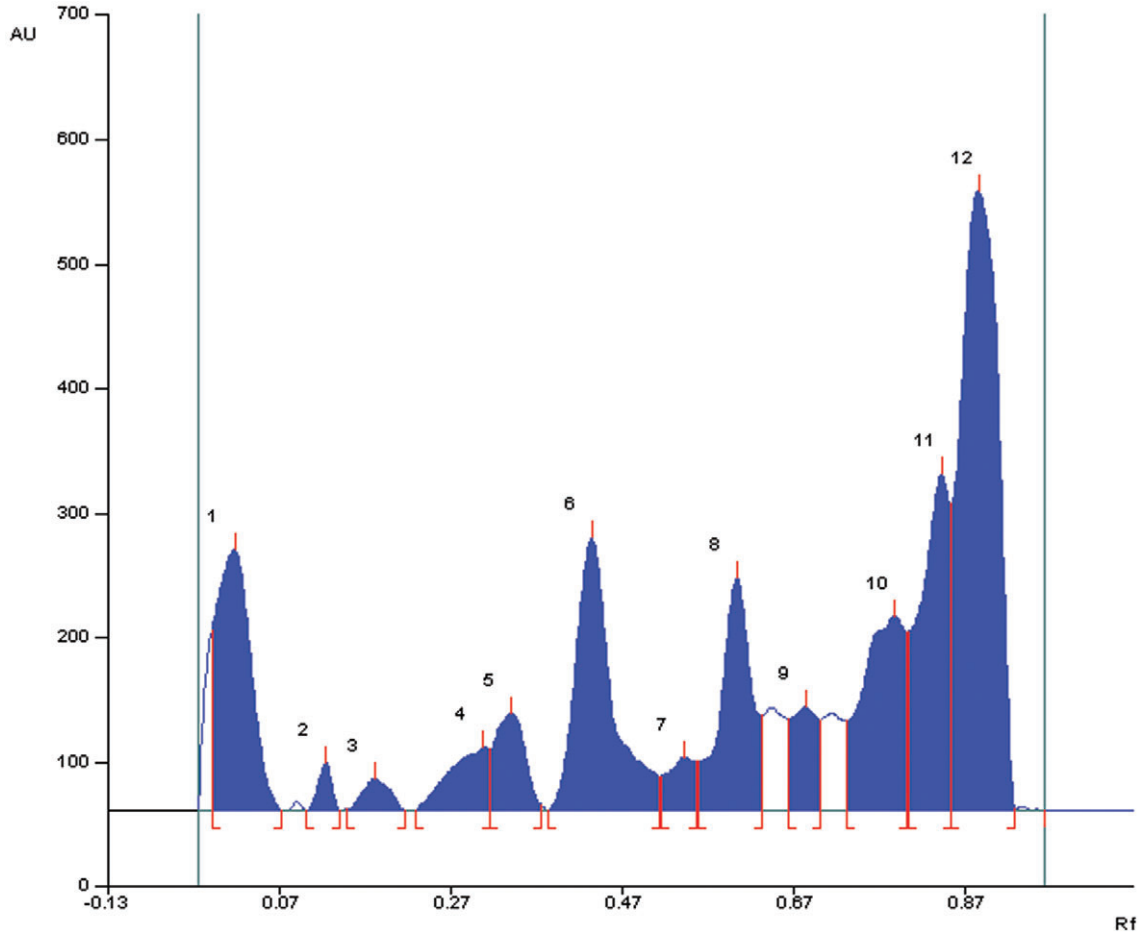

Figure 1. Chemical fingerprint of standardized active fraction of *Moringa oleifera* pods (*MoAF*).

its effect on the rifampicin-induced drug-metabolizing enzyme cytochrome P-450 in Swiss albino mice.

Materials and Methods

Isolation of Active Fraction from Pods of *Moringa oleifera* (*MoAF*)

A 20 kg sample of the *M. oleifera* pods was collected and air-dried at room temperature. The material was extracted with ethanol, filtered and concentrated at 40°C under reduced pressure. Concentrated extract was then lyophilized, which resulted in 208 g of crude extract. A 200 g sample of lyophilized extract was suspended in 400 mL of distilled water and stirred at room temperature until the maximum level of dissolution and filtered. The insoluble fraction was dissolved in methanol. The water-soluble fraction was fractionated by liquid–liquid extraction using various organic solvents such as hexane, chloroform, ethyl acetate and *n*-butanol. Each fraction was dried by adding $NaSO_4$ (anhydrous). The solvents were removed under reduced pressure and tested for its bioenhancing activity. The results indicated that the ethyl acetate fraction obtained from the crude pod extract possessed the activity enhancement property. This fraction was considered as the active fraction of *Moringa oleifera* pods (*MoAF*). The process was repeated in replicate batches and the quality of the *MoAF* was ensured by means of chemical fingerprinting. *MoAF* dissolved in methanol (70 mg/mL) and 5 µL of test solution were spotted onto silica gel $60F_{254}$ (Merck) with Linomat-IV. The TLC was developed with MeOH–$CHCl_3$ (1.8:8.2) and spots were scanned with a CAMAG TLC Scanner-3, at 220 nm. The HPTLC profile of standardized *MoAF* is depicted in Fig. 1.

Pharmacokinetic Study

Animals. The *in-vivo* pharmacokinetic study was carried out using Swiss albino mice of both sexes (20–25 g), bred and maintained under standard conditions (temperature of 22 ± 3°C with 50–70% relative humidity and 12:12 h of light and dark cycles) at the animal house of the institute. The animals were fed with pellet feed containing 22–24% protein, 4–5% fat, 4–5% crude fiber, 45–55% nitrogen free extracts, 15% Bengal gram, 0.4–0.6% phosphorus, 1–1.5% calcium and 8% insoluble ash) and acclimatized in the experimental animal room for one week prior to use in experiments. Experiments were conducted as per the protocols approved by the institutional animal ethics committee for the purpose of control and supervision on experiments on animals (CPCSEA), Government of India.

***MoAF* and rifampicin administration in Swiss albino mice.** Sixty Swiss albino mice were divided into two groups. These groups were further divided into six sub-groups (0, 1, 2, 3, 4 and 5 h time interval) with five mice in each group. The mice in group

I were administered rifampicin (Sigma-Aldrich, USA) orally at 20 mg/kg.The mice in group II were co-administered rifampicin along with the active fraction of *M. oleifera* (0.1 mg/kg b.w.) in a single dose. Blood samples of mice from both the groups were collected at time intervals of 0, 1, 2, 3, 4 and 5 h post rifampicin administration. The blood samples from each animal were centrifuged at 2500 rpm for 10 min for the separation of plasma. To 200 µl samples of plasma solutions, we added 500 µl of dichloromethane. The mixture was vigorously vortex-mixed for 5 min and then the organic layer separated. The process was repeated three times to ensure complete extraction. The organic layer pooled and evaporated by flushing dry nitrogen gas gently and the same was reconstituted with 100 µL methanol prior to HPLC analysis.

High-performance Liquid Chromatography Analysis

The rifampicin concentration in plasma was estimated using RP-HPLC. Rifampicin (Sigma-Aldrich, St Louis, MO, USA; 1 mg/mL) was used as the standard solution. All solvents used for extraction and analyses were of HPLC grade (Merck, India). The HPLC system was composed of a 1525-binary solvent delivery pump, a 2996-photo diode array (PDA) detector 2996 and a 717 plus autosampler; data acquisition and processing were by Empower® software and all components were from Waters (USA). The column was a Symmetry C_{18} (250 × 4.6 mm) from Waters (USA). The isocratic mobile phase was a mixture of phosphate buffer saline (Sigma) (0.01 M, pH 7.4) and acetonitrile (40:60, v/v). The elution was held at a constant temperature of 30°C with an injection volume of 10 µL and a flow-rate of 1.0 mL/min. Data acquisition was performed in the range of 200–400 nm; further analysis and quantitation were executed at 220 nm. The representative HPLC chromatograms of plasma samples obtained from rifampicin-treated and untreated animals are depicted in Fig. 2.

Estimation of Cytochrome P-450

Normal healthy mice (18–22 g) were used for experiment. Before the start of the experiment the experimental animals were fasted for 4 h with *ad libitum* water. Experimental animals were divided into groups of vehicle control, rifampicin (20 mg/kg b.w.) and rifampicin (20 mg/kg b.w.) plus active fraction of *M. oleifera* (0.1 mg/kg b.w.). Animals were euthanised by cervical dislocation after 2 h. Liver were collected immediately after euthanasia, and microsomes were isolated by differential centrifugation. The total cytochrome P-450 was estimated from microsomes and expressed as nanomoles per milligram protein as per the published method by Leblond *et al*. (2001).

Statistical Analysis

Results were expressed as means ± SEM and statistical significance was assessed using unpaired Student's *t*-test using Graph Pad Prism statistical software.

Results

Co-administration of *MoAF* and Rifampicin: Effect on Bioavailability of Rifampicin

The comparison of the plasma concentration (mean ± SEM) of rifampicin alone and in combination with the active fraction of *M. oleifera* is depicted in Fig. 3. The mean plasma concentrations of rifampicin at 2, 3, 4 and 5 h were significantly higher in mice treated with the active fraction of *M. oleifera* as compared with those treated with rifampicin alone. Table 1 depicts the pharmacokinetic parameters (means ± SEM) of the same experiment; C_{max}, K_{el}, $t_{1/2(el)}$, K_a, $t_{1/2(a)}$, $AUC_{(0-5)}$, $AUC_{0-\alpha}$ and C_{max} in the the active fraction of *M. oleifera* plus rifampicin treated group were significantly higher ($p < 0.05$), while other pharmacokinetic parameters were unaltered.

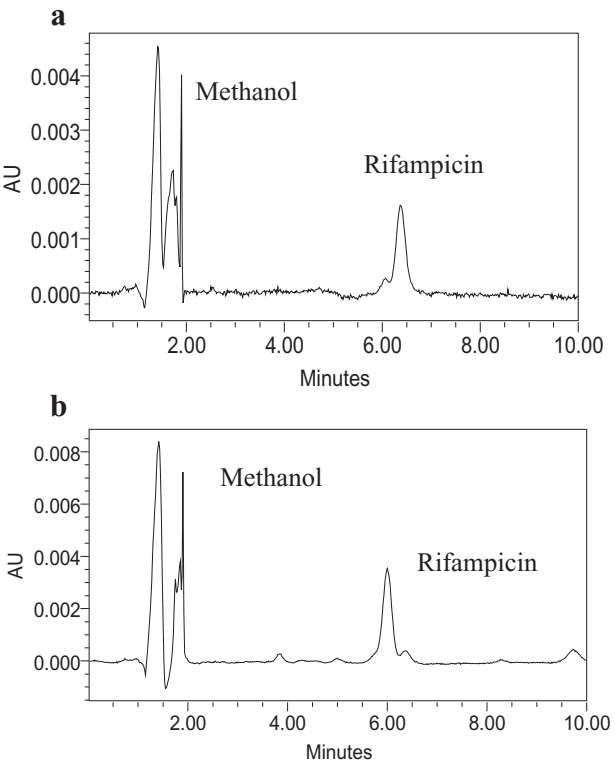

Figure 2. Representative HPLC chromatogram of (a) plasma sample of Rifampicin treated animal and (b) plasma sample of Rifampicin + *MoAF* treated animal.

Co-administration of *MoAF* and Rifampicin: Effect on Rifampicin-induced Cytochrome P-450.

Table 2 compares the activity of the drug metabolizing enzyme cytochrome P-450 in mice treated with vehicle, rifampicin and *MoAF* in combination with rifampicin. Cytochrome P-450 activity was significantly increased (($p < 0.05$), in the group treated with rifampicin when compared with the vehicle treated mice. Cytochrome P-450 activity was significantly decreased ($p < 0.05$), in the group administered the active fraction of *M. oleifera pods* in combination with rifampicin, when compared with rifampicin alone.

Discussion

Rifampicin is being used as an anti-tuberculosis drug in humans as well as animals; absorption of rifampicin has been found to be inadequate and irregular. It is an established fact that the concentration of drug in blood and target organ directly affects the efficacy of those particular drugs (Rang *et al*., 2003). Piperine, an

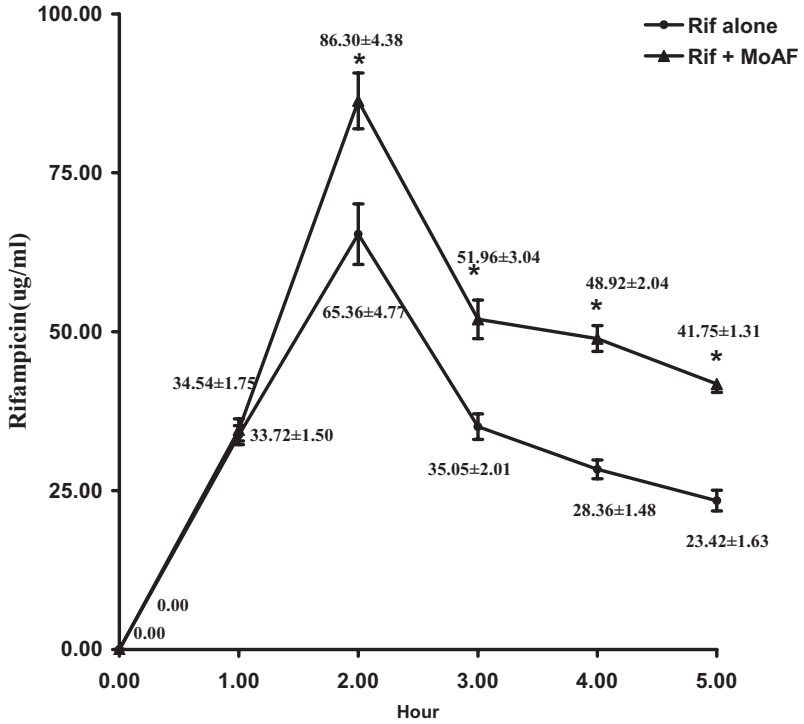

Figure 3. Plasma concentration of Rifampicin alone and in combination with MoAF treated swiss anbino mice.

Table 1. Comparative pharmacokinetics of mice treated Rifampicin alone and in combination with *MoAF*

Pharmacokinetic parameters	Rifampicin	Rifampicin + *MoAF*
C_{max} (µg/mL) [observed]	12.19 ± 0.28	17.43 ± 0.25*
T_{max} (h) [observed]	2.00	2.00
K_{el} (h^{-1})	0.14 ± 0.17	0.03 ± 0.02*
$t_{1/2\ (el)}$ (h)	4.83 ± 0.57	24.40 ± 17.01*
K_a (h^{-1})	0.09 ± 0.02	0.03 ± 0.02*
$t_{1/2(a)}$ (h)	7.79 ± 1.78	20.15 ± 11.89*
AUC_{0-5} (µg/mL/h)	33.118 ± 0.355	49.442 ± 0.2738*
$AUC_{0-\alpha}$ (µg/mL/h)	66.91 ± 15.47	343.44 ± 13.46*
C_{max} (µg/mL) [calculated]	7.12 ± 0.11	10.90 ± 0.34*
T_{max} (h) [calculated]	1.64 ± 0.39	2.14 ± 0.34*

* $p < 0.05$ (rifampicin vs Rifampicin plus MoAF), $n = 6$.
C_{max}, maximum serum concentration; T_{max}, time to reach maximal serum concentration; $t_{1/2(a)}$, absorption half-life, absorption constant; $t_{1/2(el)}$, elimination constant, elimination half-life; $AUC_{(0-5)}$, area under the concentration–time curve.

amide alkaloid isolated from different *Piper* species, has been reported to enhance the bioavailability of co-administered drugs (Zutshi *et al*., 1985; Bano *et al*., 1987; Bano *et al*., 1991; Velpandian *et al*., 2001) resulting in increased plasma concentration, due to inhibition of the drug metabolizing enzymes and P-glycoprotein (Bhardwaj *et al*., 2002; Bano *et al*., 1991). The present study was undertaken to observe the effect of active fraction of *M. oleifera* pods (*MoAF*) as a bioenhancer on the pharmacokinetic profile of rifampicin. The finding of the present study revealed that the mean plasma concentration of rifampicin was higher in *MoAF*-treated mice. The increased plasma concentration of rifampicin could be due to the inhibition of drug metabolizing enzyme cytochrome P-450, which was observed in this experiment. Another reason for the increased bioavailability could be the enhanced blood supply in enteric vessels as a result of vasodilatation induced by niazirine isolated from *M. oleifera* (Faizi *et al*., 1994). The higher values of AUC, C_{max}, and $t_{1/2}$ in *MoAF*-treated mice were indicative of enhanced systemic availability of rifampicin and suppression of the drug metabolizing enzyme cytochrome P-450.

Conclusion

This study suggests that the bioavailability-enhancing property of *MoAF* may help to lower the dosage level and shorten the treatment course of rifampicin, which may help to reduce the side effects of rifampicin as an anti-tuberculosis drug.

Table 2. Effect of active fraction of *M. oleifera* pods on rifampicin-induced drug-metabolizing enzyme (cytochrome P-450)

Treatment	Cytochrome P-450 (nmol/mg protein)
Control	2.177 ± 0.43
Rifampicin	5.34 ± 0.35*
Rifampicin + MoAF	4.27 ± 0.08[a]

*$p < 0.05$ (vehicle control vs rifampicin). [a]$p < 0.05$ (rifampicin vs rifampicin plus the MoAF); $n = 6$.

Acknowledgement

The authors are thankful to the Department of Biotechnology, Government of India, for financial assistance in carrying out this study.

References

Arya JS. *In-vitro* screening of phytochemicals for improving efficacy of antibiotics. Thesis, Barkatullah Vishwavidyalaya, Department of Biotechnology, Bhopal, India. 2003.

Atal CK, Dubey RK and Singh J. Biochemical basis of enhanced drug bioavailability by piperine: evidence that piperine is a potent inhibitor of drug metabolism. *Journal of Pharmacology and Experimental Therapeutics* 1985; **232**: 258–262.

Bano G, Amla V, Raina RK, Zutshi U and Chopra CL. The effect of piperine on pharmacokinetics of phenytoin in healthy volunteers. *Planta Medica* 1987; **17**: 568–569.

Bano G, Raina RK, Zutshi U, Bedi KL, Johri RK and Sharma SC. Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers. *European Journal of Clinical Pharmacology* 1991; **41**: 615–617.

Bhardwaj RJ, Glasser H, Becquemount L, Klotz U, Gupta SK and Fromm MF. Piperine, a major constituent of black pepper, inhibits human P-glycoprotein and CYP3A4. *Journal of Pharmacology and Experimental Therapeutics* 2002; **302**: 645–650.

Caceres A, Saravia A, Rizzo S, Zabala L, Leon ED and Nave F. Pharmacological properties of Moringa oleifera: Screening for antispasmodic, anti inflammatory and diuretic activity. *Journal of Ethnopharmacology*. 1992; **36**: 233–237.

Faizi S, Siddiqui BS, Saleem R, Siddiqui S, Aftab K and Gilani AH. Isolation and structure elucidation of new nitrile and mustard oil glycosides from *Moringa oleifera* and their effect on blood pressure. *Journal of Natural Products* 1994; **57**: 1256–1261.

Gupta R, Kannan GM, Sharma MS and Flora SJ. Therapeutic effects of *Moringa oleifera* on arsenic-induced toxicity in rats. *Environmental Toxicology and Pharmacology*. 2005; **20**: 456–464.

Hiwale AR, Dhuley JN and Naik SR. Effect of co-administration of piperine on pharmacokinetics of beta-lactam antibiotics in rats. *Indian Journal of Experimental Biology* 2002; **40**: 277–281.

Khajuria A, Zutshi U and Bedi KL. Permeability characteristics of piperine on oral absorption—an active alkaloid from peppers and a bioavailability enhancer. *Indian Journal of Experimental Biology* 1998; **36**: 46–50.

Khanuja SPS, Arya JS, Santha Kumar TR, Saikia D, Kaur H, Singh M, Gupta SC, Shasany AK, Darokar MP, Srivastava SK, Gupta MM, Verma SC and Pal A. Nitrile glycoside useful as a bioenhancer of drugs and nutrients, process of its isolation from *Moringa oleifera*. United States Patent no. 6,858,588, 2005.

Krishnaraju AV, Sundararaju D, Srinivas P, Rao CV, Sengupta K and Trimurtulu G. Safety and toxicological evaluation of a novel anti-obesity formulation LI85008F in animals. *Toxicology Mechanisms and Methods* 2010; **20**: 59–68.

Kwaambwa HM, Hellsing M and Rennie AR. Adsorption of a water treatment protein from *Moringa oleifera* seeds to a silicon oxide surface studied by neutron reflection. *Langmuir* 2010; **26**: 3902–3910.

Lambert JD, Hong J, Kim DH, Mishin VM and Yang CS. Piperine enhances the bioavailability of the tea polyphenol (–)-epigallocatechin-3-gallate in mice. *Journal of Nutrition Research* 2004; **134**: 1948–1952.

Leblond F, Guevin C, Demers C, Pellerin I, Gascon-Barre M and Pichette V. Downregulation of hepatic cytochrome P450 in chronic renal failure. *Journal of American Society of Nephrology* 2001; **12**: 326–332.

Mahajan SG, Banerjee A, Chauhan BF, Padh H, Nivsarkar M and Mehta AA. Inhibitory effect of *n*-butanol fraction of *Moringa oleifera* Lam. seeds on ovalbumin-induced airway inflammation in a guinea pig model of asthma. *International Journal of Toxicology* 2009; **28**: 519–527.

Mehta K, Balaraman R, Amin A H, Bafna PA and Gulati OD. Effect of fruits of *Moringa oleifera* on the lipid profile of normal and hypercholesterolaemic rabbits. *Journal of Ethnopharmacology* 2003; **86**: 191–195.

Rang HP, Dale MM, Ritter JM and Moore PK. *Pharmacology*, 5th edn. Churchill Livingstone: Edinburgh, 2003; 650–651.

Ranjan R, Swarup D, Patra RC and Chandra V. *Tamarindus indica* L. and *Moringa oleifera* M. extract administration ameliorates fluoride toxicity in rabbits. *Indian Journal of Experimental Biology* 2009; **47**: 900–905.

Shanker K, Gupta MM, Srivastava SK, Bawankule DU, Pal A and Khanuja SPS. Determination of bioactive nitrile glycoside(s) in drumstick (*Moringa oleifera*) by reverse phase HPLC. *Food Chemistry*. 2007; **105**: 376–382.

Singh M, Varshneya C, Telang RS and Srivastava AK. Alteration of pharmacokinetics of oxytetracycline following oral administration of piper longum in hens. *Journal of Veterinary Science*. 2005; **6**: 197–200.

Velpandian T, Jasuja R, Bhardwaj RK, Jaiswal J and Gupta SK. Piperine in food: interference in the pharmacokinetics of phenytoin. *European Journal of Drug Metabolism and Pharmacokinetics* 2001; **26**: 241–247.

Zutshi RK, Singh R, Zutshi U, Johri RK and Atal CK. Influence of piperine on rifampicin blood levels in patients of pulmonary tuberculosis. *Journal of Association of Physicians, India* 1985; **33**: 223–224.