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Research Paper

Novel pepper extract for enhanced P-glycoprotein inhibition

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Abstract

Objectives Piperine is one of the most promising bioenhancers to date. Methods used for its extraction suffer from drawbacks such as use of organic solvents, poor extraction efficiency, tedious and expensive methodology. These methods are not encouraged with a view to reducing global warming. The objective was therefore to develop an alternative solvent-free extraction method.

Methods An aqueous extract of long pepper fruits was prepared using hydrophilic lipid Gelucire 44/14 as the extracting aid and this was compared with an alcoholic extract. Extracts were characterized using high-performance thin layer chromatography and differential scanning calorimetry. P-glycoprotein (P-gp) inhibitory activity of the aqueous and alcoholic extracts and pure piperine was compared using an in-vitro everted rat intestinal model using ornidazole as the model drug. The study was performed using two oral pretreatment dose levels (10 and 20 mg/kg) and durations (1 and 3 days). Exsorption of ornidazole from serosal to mucosal surface was monitored.

Key findings P-gp inhibitory activity of the aqueous extract was comparable with that of pure piperine ($P > 0.05$) and was significantly higher than the alcoholic extract ($P < 0.05$). Pure piperine and the aqueous extract exhibited significant P-gp inhibitory activity compared with control, which was irrespective of oral pretreatment dose and duration levels. No significant effect of oral pretreatment duration of the aqueous extract was observed. The observed enhancement in P-gp inhibitory activity of the aqueous extract may have been attributed to the P-gp inhibitory potential of Gelucire 44/14 and its efficient extraction and solubility enhancement ability.

Conclusions In the field of phytopharmaceuticals efficient and eco-friendly extraction processes are still a goal to be achieved. Extraction with Gelucire 44/14 could be a potential method of extraction for phytopharmaceuticals. Compared with conventional methods of extraction it is more efficient, easier to prepare, eco-friendly and scalable.

Keywords bioenhancer; Gelucire; pepper; piperine; P-glycoprotein

Introduction

The oral route is the most preferred and convenient route of administration. Most pharmaceutical research is focused on the development of oral dosage forms for known and new chemical entities. In the last three decades, drug discovery development has resulted in the launch of a higher number of lipophilic new chemical entities and similar numbers of lipophilic new chemical entities are in the pipeline. The major challenge for pharmaceutical development of these molecules is their poor solubility leading to poor or highly variable oral bioavailability.[1,2] Low and variable oral bioavailability cause difficulties in producing and controlling the desired pharmacological effect. The majority of research has contributed towards the development of the technology to enhance the bioavailability by virtue of improvement in the solubility of such molecules. However, the time and cost involved in these methodologies is huge. Secondly, for some molecules, though these methodologies have proved efficient *in vitro*, the in-vivo performance is not matching that of the in-vitro performance. The role of physiological parameters such as gastrointestinal transit of administered dose, site of absorption, fate of drug in the gastrointestinal tract (metabolism), the food effect and drug efflux transporter P-glycoprotein (P-gp) has been proved through many preclinical and clinical studies. Drug efflux transporters like P-gp not only affect therapeutic efficacy, but also have an effect on the absorption, distribution and elimination of a wide variety of drugs. Therefore, the drug discovery

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and development processes are giving increased effort to identifying the therapeutic agents which interact with these efflux transporters. Considering the difficulties in drug absorption by the body, the term 'bioenhancer' was coined in the arena of modern pharmaceutical research. Until now, many examples of bioenhancers have been explored, which act either by altering the permeation characteristics of the gastrointestinal tract (i.e. by inhibiting the drug efflux transporters such as P-gp) or inhibiting the metabolism of the drugs (e.g. verapamil, ciclosporin, erythromycin, different alkaloids).[3,4] Though it is observed that co-administration of one drug molecule can increase the bioavailability of another target drug molecule, such co-administration is not approved by regulatory agencies owing to pharmacological activity of the former.

Piperine, a major alkaloid obtained from *Piper nigrum* Linn. (black pepper) and *Piper longum* Linn. (long pepper), has been proved to be one of most promising bioenhancers to date. Pepper fruits contain volatile oil, crystalline alkaloids (principally piperine and piperettine) and a resin.[5] Pharmacological and clinical uses of piperine have been reviewed.[6–8] Piperine has been shown to inhibit human P-gp, several cytochrome P450 (CYP)-mediated pathways and phase-II reactions.[7,9–11] Piperine has been evaluated clinically for its ability to increase the serum response and lengthen the serum half-life of chemically diverse groups of drugs and nutrients in experimental animals and humans, many of which are established P-gp substrates.[9,12–14] Bioperine is a standardized extract from the fruits of black pepper or long pepper, which has enhanced the bioavailability of many drugs and nutrients.[15] In addition to this, a number of excipients have been found to disrupt the function of intestinal P-gp and were screened for P-gp inhibitory activity.[16–19]

Piperine is poorly water soluble, which limits its oral absorption. In everted sac studies, only 7–12% absorbed piperine was found in the serosal medium.[20] Conventional extraction methods available for piperine extraction from pepper do not improve aqueous solubility. On an industrial scale, pepper is comminuted into flakes or ground into coarse powder and then extracted repeatedly with an organic solvent such as acetone, ethanol or chlorinated hydrocarbons.[21] Apart from the poor extract quality, difficulties in handling large volumes of inflammable volatile organic solvents and residual solvent traces remaining in the final product limit the use of organic solvents for piperine extraction from pepper. The Environmental Protection Agency and other regulatory bodies have defined the limits for volatile organic compound emission from such pharmaceutical extraction operations.[22] Compliance with these regulations needs effluent reprocessing or reduction in production load accordingly. Additionally, limits for the residual solvent in the final product as per the International Conference on Harmonization and other regulatory guidelines are more difficult to achieve using these techniques.[23] Extensive use of organic solvents is also not encouraged with a view to reducing global warming.

One other method of extraction uses supercritical carbon dioxide.[24,25] The cost of the high-pressure equipment required to obtain supercritical extraction conditions is prohibitively high and limits the application of supercritical extraction to only high-value and low-volume materials. Recently, hydrotropes such as sodium alkyl benzene sulfonates and sodium butyl monoglycol sulfate were used for the selective extraction of piperine. However, the process is lengthy and the presence of inorganic phosphorus has to be monitored.[26] Therefore, it creates the need for the development of an alternative solvent-free green extraction technique for piperine which is simple, cost effective and can present piperine in solubilized form in water.

The objective of this study was to develop an extraction process for extracting piperine from pepper fruits using Gelucire 44/14 (G44) as the extracting aid. G44 is a hydrophilic lipid and has the ability to increase aqueous solubility of poorly water soluble drugs.[27,28] G44 has also exhibited P-gp inhibitory activity in the Caco-2 cell line model.[29] Therefore, use of G44 for extraction was expected to give an aqueous extract of piperine which may improve the bioenhancing potential of the extract through solubility enhancement of piperine along with synergistic augmentation of P-gp inhibitory activity. This extract was compared with the alcoholic extract of pepper. Extraction efficiency of other hydrophilic lipids (Pluronic F-68 (PF68) and Pluronic F-127 (PF127)) was studied to verify the effect of the hydrophilic lipophilic balance (HLB) on piperine extraction. All the extracts were characterized by high-performance thin layer chromatography (HPTLC) and differential scanning calorimetry (DSC). The P-gp inhibitory activity of different extracts and pure piperine were compared using the in-vitro rat intestinal everted sac model. Ornidazole was used as a model drug to evaluate P-gp inhibitory activity. Ornidazole is believed to be metabolized through CYP3A and it has considerable intestinal efflux through P-gp, which was observed from the in-vitro everted sac study.[30]

Materials and Methods

Materials

Dried fruits of *Piper longum* (long pepper) were purchased from Green Pharmacy, Pune, India. Identification and authentication of pepper fruits was carried out at the Department of Botany, Agarkar Research Institute, Pune, India. Long pepper fruit powder was prepared by grinding long pepper fruits, the resulting powder passed through 30 mesh screens and used for extraction purpose. Piperine (98.2% pure) was purchased from Natural Remedies Pvt. Ltd, Bangalore, India. Ornidazole was a generous gift from Emcure Pharmaceuticals Pvt. Ltd, Pune, India. Gelucire 44/14 (G44) and Gelucire 50/13 (G50) were generous gifts from Gattefosse, France, and supplied by Colorcon Asia Pvt. Ltd, Mumbai, India. Pluronic F-68 (PF68) and Pluronic F-127 (PF127) were received as gift samples from BASF India Ltd, Mumbai, India. Dulbecco's phosphate buffer solution (D-PBS) was procured from HiMedia, Mumbai, India. All other chemicals and solvents were purchased from Merck India, Mumbai, India.

Preliminary screening for selection of a suitable extracting aid and preparation of an aqueous extract

Hydrophilic lipids G44 (HLB 14) and G50 (HLB 13) were screened as extracting aids. Extraction efficiency of G44 and

Table 1 Piperine extracted with Gelucire 44/14 and Gelucire 50/13 at different ratios

Gelucire 44/14			Gelucire 50/13		
Ratio code	Pepper powder : G44	% Piperine extracted[a]	Ratio code	Pepper powder : G50	% Piperine extracted[a]
G44PL1	1 : 0.25	1.92 ± 0.06	G50PL1	1 : 0.25	1.68 ± 0.05
G44PL2[b]	1 : 0.50	2.38 ± 0.04	G50PL2	1 : 0.50	1.74 ± 0.06
G44PL3	1 : 0.75	2.41 ± 0.05	G50PL3	1 : 0.75	1.89 ± 0.05
G44PL4	1 : 1.00	2.49 ± 0.07	G50PL4	1 : 1.00	1.92 ± 0.08
G44PL5	1 : 1.50	2.53 ± 0.05	G50PL5	1 : 1.50	1.95 ± 0.07
G44PL6	1 : 2.00	2.52 ± 0.04	G50PL6	1 : 2.00	2.00 ± 0.04

G44PL; ratio code for pepper powder: Gelucire 44/14. G50PL; ratio code for pepper powder: Gelucire 50/13. [a]Mean ± SD (n = 5). [b]Optimized ratio for selected Gelucire grade which is referred to as aqueous extract.

G50 was evaluated at different pepper powder : Gelucire ratios as shown in Table 1 and processed by the following extraction method.

G44 and G50 were separately melted at 55–60°C in a water bath for 15 min. To each molten mass, accurately weighed pepper powder was added, mixed thoroughly to get a uniform mixture and allowed to cool at room temperature. A measured quantity of water was added, mixed well and centrifuged at 5000 rev/min for 5 min (Cryocentrifuge 2810R, Eppendorf, USA). Supernatant was filtered through a 0.45 μm membrane filter and subjected to HPTLC densitometric analysis at 343 nm.

The optimum concentration of Gelucire grade presenting high extraction efficiency was further referred to as the aqueous extract. PF68 and PF127 were also screened for extraction efficiency and processed by the above method.

Preparation of the alcoholic extract

The alcoholic extract was prepared by a cold maceration extraction process. Ground pepper fruits powder (10 g) was put in a 250-ml conical flask with 100 ml ethanol as the extracting solvent. It was shaken on a mechanical shaker at 180 rev/min for 8 h at 25°C. Supernatant was collected by decantation into a dark coloured bottle. The residue was re-extracted under the same conditions until the extraction solvent became colourless and the supernatant was collected. The total obtained supernatant was filtered and ethanol was evaporated from the filtrate using a rotary evaporator at 50°C. This resulted in a syrupy extract which was kept overnight in a vacuum oven at 50°C. The extract was quantified for piperine by HPTLC densitometric analysis at 343 nm. The extract was placed in a dark plastic bottle and stored in a desiccated environment until required for further study.

High-performance thin layer chromatography

Samples were spotted as bands (width, 8 mm) with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 × 10 cm, 250 μm thickness, E. Merck, Germany) using a Camag Linomat IV sampler (Switzerland). A constant application rate (0.1 μl/s) was used and the space between two bands was 6 mm. Slit dimension was kept at 6 × 0.45 mm and 10 mm/s scanning speed was employed. A model III TLC scanner with CATS 4.0 integration software was used for data acquisition and analysis. Mobile phase consisted of toluene : ethyl acetate (2 : 1, v/v). Linear ascending development was carried out in twin trough glass chambers saturated with the mobile phase. Optimized chamber saturation time for the mobile phase was 20 min at room temperature. The calibration curve for piperine was performed in the range 200–1400 ng/spot.

Differential scanning calorimetry

DSC analyses were performed using Mettler-Toledo DSC 821e instrument equipped with an intracooler (Mettler-Toledo, Switzerland). Indium and zinc standards were used to calibrate the DSC temperature and enthalpy scale. The samples were hermetically sealed in aluminium crucibles and heated at a constant rate of 10°C/min over a temperature range of 25–250°C. An inert atmosphere was maintained by purging nitrogen gas at flow rate of 50 ml/min. The data were collected in triplicate for each sample and were analysed using Mettler-Toledo Star[e] Version 8.10 software.

Animal study

The P-gp inhibitory activity of the aqueous and alcoholic extracts and pure piperine were compared using an in-vitro everted rat intestinal model. Male Wistar rats (200–250 g; Yash Enterprises, Pune, India) were maintained in a well-ventilated animal house in polypropylene cages. Animal handling routines were performed according to Good Laboratory Practices. Feed and water were provided freely. All studies were approved by the Institutional Animal Ethics Committee of Poona College of Pharmacy (Pune, India), and were conducted under the provisions of the approved protocol (CPCESA/6/2007). Ornidazole was used as a model drug to evaluate P-gp inhibitory activity. Effect of oral pretreatment with piperine (pure/aqueous extract/alcoholic extract) on ornidazole transport (efflux) across everted rat intestine was studied. P-gp inhibitory potential of the aqueous extract was studied at two dose levels (10 and 20 mg/kg) and two oral pretreatment durations (1 and 3 day). The study was carried out in seven sets with five rats in each set, as shown in Table 2. Doses were administered orally through a suspension of 0.25% carmellose sodium.

In-vitro transport across everted rat intestine

After oral pretreatment, rats were fasted overnight with free access to water. The next day the animals were anaesthetized with pentobarbital (30 mg/kg, i.p. injection) and the whole

Table 2 Experimental sets for animal study

Experimental set	Pretreatment with[a]	Pretreatment duration (days)
Control	–	–
Piperine 20/3	Pure piperine (20 mg/kg)	3
Aqueous extract 20/3	Aqueous extract (equivalent to piperine 20 mg/kg)	3
Aqueous extract 10/3	Aqueous extract(equivalent to piperine 10 mg/kg)	3
Aqueous extract 20/1	Aqueous extract (equivalent to piperine 20 mg/kg)	1
Alcoholic extract 20/1	Alcoholic extract (equivalent to piperine 20 mg/kg)	1
Blank	G44 (equivalent to amount of G44 used in aqueous extract 20/3)	3

[a]Dose calculated for body weight of Wistar rat in kg.

small intestine was flushed with 50 ml ice-cold saline. The rats were exsanguinated, the small intestine was isolated and two 12-cm segments were cut from the duodenum and from the lower ileum regions. Each segment was everted and one end was ligated tightly. From the other end, 1 ml ornidazole (10 mg/ml) was introduced (serosal side), which was previously dissolved in pH 7.4 isotonic D-PBS medium containing 25 mM glucose and 4% dimethyl sulfoxide (DMSO). The other end was also ligated tightly to form a 10 cm long everted sac. Each everted sac containing ornidazole solution was immersed into 40 ml D-PBS, containing 25 mM glucose and 4% DMSO, which was prewarmed at 37°C and pre-oxygenated for 15 min. Under aerated conditions, samples were taken from the mucosal medium periodically for 90 min. The samples were subjected to RP-HPLC analysis for quantification of ornidazole efflux.

Sample preparation for high-performance liquid chromatography analysis

Samples (0.5 ml) were withdrawn from the medium at 15, 30, 45, 60 and 90 min and replenished with blank medium kept at the same temperature. Samples were filtered through 0.45 μm membrane filters. Using a micropipette 200 μl samples were pipetted into 2 ml microcentrifuge tubes. To each tube 800 μl D-PBS was added, the tubes were capped and vortexed for 20 s. These solutions were subjected to RP-HPLC analysis for ornidazole determination.

High-performance liquid chromatography analysis

The HPLC system specifications were as follows: pump, PU-1580 (Jasco, Japan); injector, auto sampler (AS-1555; Jasco); column, RP C_{18}, 250 × 4.6 mm, 5 μm (Thermo Electron Corporation, USA); detector, UV/visible (UV-1575; Jasco). Data acquisition and analysis was carried out using Borwin/HSS 2000 software (LG 1580-04; Jasco). The chromatographic conditions were as follows: mobile phase 0.002 M acetate buffer (pH 4.8) : acetonitrile : methanol (60 : 30 : 10, v/v/v); flow rate, 1 ml/min. The mobile phase was filtered through a 0.45 μm membrane filter and degassed before use. The elution was monitored at 318 nm. The injection volume was 20 μl. The peak area of ornidazole was used for quantification of samples. The calibration curve for ornidazole was performed in the range 5–50 μg/ml in D-PBS.

Statistical analysis

Statistical analysis of the effects of different sets on cumulative efflux values of ornidazole were assessed by one-way analysis of variance and individual differences between various sets were examined using Tukey's post hoc test. A significance level of $P < 0.05$ was deemed to be statistically significant (GraphPad Prism, version 4.03.354, GraphPad Software, Inc., San Diego, CA, USA).

Results

High-performance thin layer chromatography analysis

HPTLC densitograms for pure piperine, the alcoholic and the aqueous extracts were comparable (Figure 1). The R_f value for piperine was 0.41. The calibration curve for piperine was found to be linear in the range 200–1400 ng/spot with a correlation coefficient of 0.9991 and regression equation as y = 5.4413x + 123.52.

Preliminary screening for selection of suitable extracting aid and preparation of aqueous extract

Piperine content in the different extracts with G44 or G50 at different ratios are shown in Table 1. G44 extracted more

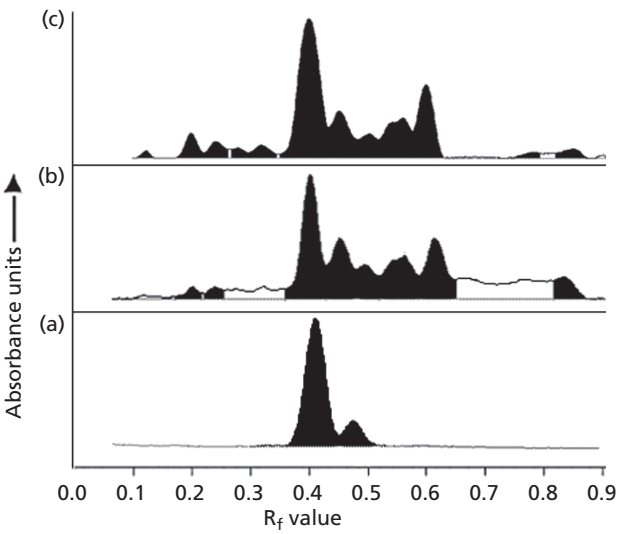

Figure 1 Overlay densitograms. (a) Pure piperine; (b) alcoholic extract of long pepper fruits; and (c) aqueous extract of long pepper fruits.

piperine in water than G50. The dispersibility property of G44 extract was found to be more than that of the G50 extract. The alcoholic extract was very difficult to disperse in water because of its sticky nature. Therefore, extraction with G44 was found to be more advantageous with respect to extraction efficiency and ease of dispersibility. Extraction efficiency was increased with an increase in G44 concentration up to a ratio of 1 : 0.5 (G44PL2). There was no considerable improvement in extraction efficiency with further increases in G44 concentration (Table 1). Therefore, G44PL2 was considered to be the optimum.

The alcoholic extract was found to be insoluble in water. Therefore, ethanol was used for its quantification and the amount of piperine was found to be 2.26 ± 0.07%. The aqueous extract extracted 2.38 ± 0.04% piperine in water. The extraction efficiency values for PF68 and PF127 were less than the Gelucires and alcoholic extracts (data not shown). PF127 extracted 1.69 ± 0.09% piperine from pepper powder at the ratio of pepper powder : PF127, 1 : 1. Also, PF68- and PF127-pretreated pepper powders were difficult to disperse in aqueous medium, therefore PF68 and PF127 were not considered for further study.

Differential scanning calorimetry

DSC study was used to evaluate any interaction between piperine and G44. DSC thermal profiles of pure piperine, G44, aqueous and alcoholic extracts are shown in Figure 2. Pure piperine was characterized by a single, sharp endothermic peak at T_{max} = 130.5 ± 1.3°C corresponding to its melting in DSC, which was in accordance with previous reports.[31] The DSC thermal profile of G44 showed an endothermic peak at T_{max} = 43.1 ± 0.9°C, which was attributed to the melting of G44.[32] The DSC thermal profile of the aqueous extract exhibited an endothermic peak at 42.4 ± 1.1°C corresponding to melting of G44 and another broad endothermic peak at 127.9 ± 1.5°C attributed to melting of piperine. The endothermic peaks observed in the DSC thermal profile of alcoholic extract were not distinguishable.

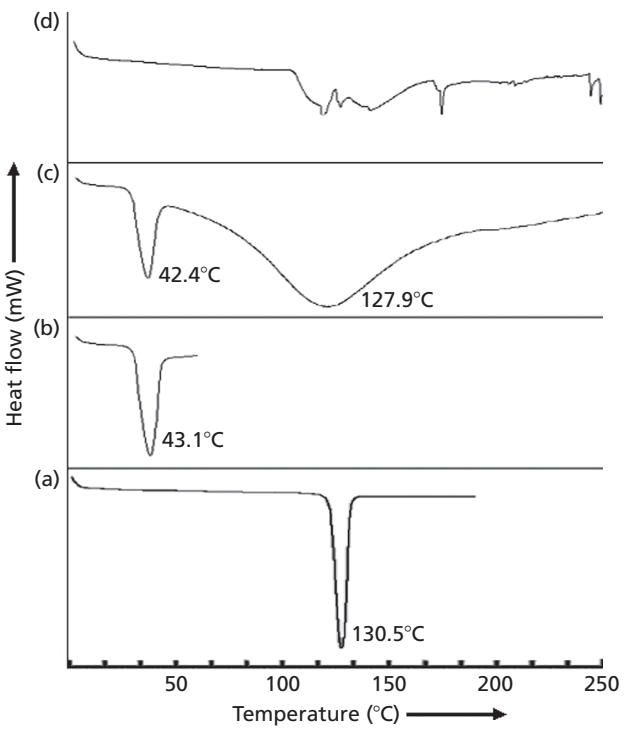

Figure 2 Differential scanning calorimetry thermal profiles. (a) Piperine; (b) Gelucire 44/14; (c) aqueous extract of long pepper fruits; and (d) alcoholic extract of long pepper fruits.

Animal studies

It has been suggested that the function of P-gp and its sensitivity to inhibitors may be similar in the everted rat intestine model and Caco-2 cells, and that these experimental models may be useful to study the function of P-gp in the human intestine.[33] A linear relationship was established between in-vitro everted sac and in-vivo studies, which suggested that the P-gp-related drug–drug interactions *in vivo* could be predicted by in-vitro everted sac studies. Therefore, use of the rat intestinal everted sac model in this study was justified.

In-vitro transport across everted rat intestine

The transport of ornidazole from serosal to mucosal surfaces across everted rat intestine before and after oral pretreatment of piperine (pure/aqueous extract/alcoholic extract) was determined at different anatomical regions of rat small intestine (duodenum and lower ileum). The time course of ornidazole transport for different sets across the everted duodenum and lower ileum are shown in Tables 3 and 4, respectively. More ornidazole was transported in the lower ileum than the duodenum. The transport of ornidazole increased with time in duodenal and lower ileal everted sacs for all sets.

In both segments of intestine, a significant decrease ($P < 0.05$) in ornidazole transport was observed due to oral pretreatment with pure piperine and the aqueous extract as compared with the control, which indicated P-gp inhibition. However, the alcoholic extract and G44 did not show a significant decrease ($P > 0.05$) in ornidazole transport as compared with the control. At the same dose, there was no significant difference in P-gp inhibitory activity of the aqueous extract and piperine (aqueous extract 20/3 and piperine 20/3). Therefore, it could be concluded that P-gp inhibitory activity of G44 extract was comparable with that of pure piperine. These results signified the efficiency of G44 extraction. Similar results were obtained when the aqueous extract was compared for effect of oral pretreatment durations (aqueous extract 20/3 and aqueous extract 20/1). Ornidazole transport was significantly reduced when the aqueous extract dose was reduced to half (aqueous extract 20/3 and aqueous extract 10/3). Effect of oral pretreatment with the alcoholic extract on ornidazole transport was also significantly less than the aqueous extract at the same dose level and for the same pretreatment duration (alcoholic extract 20/1 and aqueous extract 20/1). Also, when the alcoholic extract and blank were compared, no significant difference in P-gp activity was observed. This indicated comparable P-gp inhibition as evident from Tables 3 and 4.

Table 3 Difference in ornidazole transport from serosal to mucosal surface across the everted duodenum at different time intervals

Experimental set	Ornidazole concentration (μg/ml) at different time intervals (min)[a]				
	15	30	45	60	90
Control	47.93 ± 6.11	76.78 ± 6.98	108.43 ± 4.72	134.70 ± 0.70	170.30 ± 4.74
Piperine 20/3	31.30 ± 1.93	61.57 ± 0.72	78.62 ± 0.56	92.39 ± 0.21	131.17 ± 3.51*
Aqueous extract 20/3	34.75 ± 1.85	62.72 ± 1.73	82.67 ± 1.00	100.85 ± 4.58	135.06 ± 4.59*
Aqueous extract 10/3	42.05 ± 3.46	70.18 ± 4.72	95.88 ± 4.92	116.83 ± 5.25	152.65 ± 3.40*
Aqueous extract 20/1	37.56 ± 4.10	65.25 ± 2.04	87.48 ± 1.97	104.52 ± 1.03	136.68 ± 6.65*
Alcoholic extract 20/1	43.41 ± 1.83	72.69 ± 1.55	101.03 ± 2.65	121.45 ± 4.42	164.18 ± 7.74
Blank	46.99 ± 1.72	70.82 ± 4.02	103.07 ± 2.72	125.09 ± 3.66	165.71 ± 5.37

[a]Mean ± SD (n = 5). *Significantly different from control at a level of $P < 0.05$.

Table 4 Difference in ornidazole transport from serosal to mucosal surface across the everted ileum at different time intervals

Experimental set	Ornidazole concentration (μg/ml) at different time intervals (min)[a]				
	15	30	45	60	90
Control	72.24 ± 5.98	110.71 ± 7.76	160.78 ± 5.41	202.04 ± 4.28	233.14 ± 8.54
Piperine 20/3	39.72 ± 6.51	63.48 ± 4.82	92.38 ± 6.22	126.58 ± 8.40	171.77 ± 4.50*
Aqueous extract 20/3	42.14 ± 5.75	65.27 ± 5.01	96.88 ± 6.28	132.45 ± 9.28	176.53 ± 4.55*
Aqueous extract 10/3	40.12 ± 3.66	74.03 ± 5.88	103.31 ± 6.29	148.40 ± 5.54	191.75 ± 4.77*
Aqueous extract 20/1	44.15 ± 5.09	62.70 ± 5.63	97.17 ± 4.32	138.96 ± 7.87	180.73 ± 4.91*
Alcoholic extract 20/1	53.33 ± 5.28	87.79 ± 6.18	145.03 ± 9.43	174.48 ± 7.63	218.86 ± 6.26
Blank	55.70 ± 4.91	95.10 ± 3.60	140.22 ± 5.56	176.89 ± 8.11	226.81 ± 8.83

[a]Mean ± SD (n = 5). *Significantly different from control at a level of $P < 0.05$.

Discussion

Preliminary screening for selection of suitable extracting aid and preparation of aqueous extract

Piperine was extracted from pepper powder by both Gelucires (G44 and G50) and Pluronics (PF68 and PF127). The observed solubility enhancement of piperine with these amphiphilic excipients may be attributed to mechanisms such as wetting and solubilization through micelle formation.[34–39] From the data for extraction efficiency, most efficient extraction was observed with G44 compared with G50, PF68 and PF127. Differences in the chemical compositions of G50 and G44 clearly affected the extraction efficiency although they have closer HLB values. Gelucires belong to a family of materials which are made up of glycerides and fatty acid esters of polyethylene glycols. G44 is a hydrophilic lipid with a melting point of 44°C and HLB 14. G44 is composed of a well defined mixture of mono-, di- and triglycerides and mono- and di-fatty acid esters of polyethylene glycol and is generally recognized as safe (GRAS).[37,40] G50 includes predominantly long chain palmitic acid and stearic acid (C16–C18), whereas G44 comprises medium chain lauric acid and myristic acid (C12–C14). Also, G44 has a unique composition, which comprises surfactants (mono- and diesters of polyethylene glycols), co-surfactants (monoglycerides) and an oily phase (di- and triglycerides).[37] Due to this composition, G44 forms a fine emulsion when in contact with aqueous medium at body temperature. Also, among different grades of hydrophilic Gelucire only G44 has the unique property of self-emulsification or pseudosolubilization. It has been reported that the bioavailability of G44-treated drugs was enhanced due to this pseudosolubilization effect in gastrointestinal fluids.[36] Therefore, it could be concluded that higher extraction was achieved with G44 than G50, PF68 and PF127 due to its unique self-emulsifying property only and HLB was not a key factor in the extraction process.[41,42] Extraction of piperine was not increased linearly with increased amount of used lipid. This ambiguous behaviour may have been because of the unavailability of piperine in pepper powder for further extraction.

Differential scanning calorimetry

DSC thermal profile of aqueous extract presented a sharp melting peak for G44 and a broad melting peak for piperine. Presence of a broad peak indicated that piperine might have been partially solubilized in molten G44 in the DSC run. There was no other peak in the DSC thermal profile of the aqueous extract, which confirmed that piperine was the major extracted constituent. Also, there was no physical interaction observed between G44 and piperine.

In-vitro transport across everted rat intestine

In aqueous extract, piperine must have been presented in a solubilized form in gastrointestinal tract media by virtue of the pseudosolubilization ability of G44. Therefore, the P-gp inhibitory activity of the aqueous extract was comparable with that of piperine. In contrast, P-gp inhibitory activity of the alcoholic extract was much less. Aqueous solubility is important for in-vivo performance. The inability of the alcoholic extract to present piperine in solubilized form in gastrointestinal tract media may have been the responsible

factor for such poor P-gp inhibitory activity as compared with G44 extract. The sticky nature of the alcoholic extract may have been because of the presence of high residual moisture or presence of resinous material. Though G44 alone had decreased transport of ornidazole as compared with the control in this everted sac study, it was not statistically significant. Results were in accordance with previous reports.[19]

In-vitro everted sac studies showed that P-gp inhibitory activity of the aqueous extract was dose-dependent. P-gp inhibitory activity was decreased significantly when the dose of aqueous extract was reduced from 20 to 10 mg/kg body weight of the rat. However, no significant effect of oral pretreatment duration was observed. Therefore, P-gp inhibitory activity of the aqueous extract may have been attributed to increased solubility of piperine, which must have presented it at a site of action in the gastrointestinal tract, and to some extent the synergistic effect of P-gp inhibitory activity of piperine and G44 must have contributed.

The main advantage of using G44 was that it is GRAS listed.[37] This study has shown that G44 could be used to extract and solubilize active phytoconstituent from the crude natural source with the aid of a simple, economical and less time-consuming extraction process. In contrast, extraction with alcohol by maceration or any other technique involves high cost and time and therefore is least favoured for scale-up. Also, handling a large volume of inflammable solvent on a large scale is not advisable and not eco-friendly. G44 pretreated pepper powder could be directly used in various pharmaceutical and nutraceutical formulations. In this work, though the P-gp inhibitory activity of pure piperine and the aqueous extract were close, the work had the advantage of using the crude drug powder itself rather than using pure drug.

Conclusions

G44, a lauroyl macrogolglyceride, is a multifunctional lipid excipient and can be used to extract active phytoconstituents from the crude source with the aid of a simple process. The extraction process presented in this study has the potential to replace the conventional nonaqueous extraction method as it is a more efficient, economic and eco-friendly process. Selection of G44 for extraction of the P-gp inhibitor piperine is further functional considering the P-gp inhibitory activity of G44 itself. Use of G44 pretreated pepper powder could be directly used as a formulation in nutraceuticals to enhance the bioavailability of nutrients such as vitamins and others with low oral bioavailability having intestinal P-gp efflux and metabolism through the cytochrome enzyme system.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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