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Research Article
Lignans of *Phyllanthus niruri* Solid Dispersion: A Potential Alternative Gout Therapy

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Abstract

Background: Phyltetralin (1) and phyllanthin (2) are two potent anti-hyperuricemic lignans in the standardized *Phyllanthus niruri* extract (PNF5) yet displayed low oral bioavailability in rats. **Objective:** The purposes of the current study were to investigate the contributing factors towards the poor bioavailabilities of the lignans in PNF5 and increase the bioavailability by developing a formulation method, which can specifically overcome these contributing factors. **Materials and Methods:** Aqueous solubility, pH stability, P-glycoproteins efflux and first-pass metabolism of 1 and 2 in PNF5 were evaluated accordingly using *in vitro* methods. The PNF5 was subsequently formulated as Gelucire®44/14-based solid dispersion capsules (PNF5-SDC) and the oral bioavailabilities of 1 and 2 in PNF5-SDC were estimated compared to that of the conventional PNF5 powder filled capsules (PNF5-PFC). **Results:** The study shown that the solubility of PNF5 was 0.12 ± 0.02 mg mL^{-1}, pH value of GIT lumen were not significant affected the lignans absorption ($p>0.05$). The *in vitro* absorption of lignans significantly improved by combination with P-glycoprotein inhibitors: Verapamil ($p<0.001$) and quinidine ($p<0.05$). In addition, there were no significant variation for the amount of 1 ($p = 0.4363$) and 2 ($p = 0.5396$) and no metabolites were observed when PNF5 incubated with homogenized liver. Moreover, the relative oral bioavailability of the lignans in PNF5-SDC was increased by 3 fold ($p<0.05$), higher than those in PNF5-PFC when orally administered in rats. **Conclusion:** The results suggest that the poor bioavailability of 1 and 2 was due mainly to its poor aqueous solubility and P-glycoprotein (P-gp) efflux. Gelucire®44/14-based solid dispersion could significant improve the bioavailability of lignans.

Key words: Lignans, *Phyllanthus niruri*, gout, hyperuricemia, solid dispersion, Gelucire®44/14, P-glycoprotein efflux, first-pass metabolism, bioavailability

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gout has been increasing for few decades in men over 40 years of age and reported as the most common form of the clinical manifestation in inflammatory arthritis[1]. Gout is caused by high levels of uric acid in the blood (chronic hyperuricemia) following purine catabolism[2]. Thus, lowering uric acid level is the key factor for gout prevention[3]. The current therapeutic agents for treating hyperuricemia however, are highly limited due to their adverse reactions[4]. Thus, researches to identify an alternative treatment for gout using herbal medicine have been extensively carried out. Recently, a lignan-rich extract, PNF5 containing phyltetralin (1) and phyllanthin (2) of *Phyllanthus niruri* L. (Euphorbiaceae) which had reduced the plasma uric acid level in hyperuricemic rats through the uricosuric action, comparable to that of clinically used allopurinol and benzbromarone was developed[5]. However, this bioactive lignans in PNF5 possessed very low oral bioavailability[5], yet the contributing factors towards its low bioavailability were remained scanty.

Many biomolecules possess low oral bioavailability, due to multiple factors including their poor aqueous solubility that produced low dissolution rates in the gastrointestinal tract (GIT)[6] and poor pH stability in the GIT tract causes the compounds degradation. In addition, the P-glycoproteins (P-gp) as an adenosine triphosphate (ATP) dependent multidrug resistance associated protein (MRP1), located at the apical membranes of enterocytes, involves in xenobiotics efflux into the gut lumen, thereby reducing absorption and oral bioavailability of bioactive compounds[7]. Moreover, the first-pass metabolism of the bioactive molecules in the liver was another important factor which may also contribute to poor oral bioavailability of biomolecules[8].

Formulation development is a useful strategy for bioavailability improvement. As a semi-solid commercially used surfactant with amphiphilic characteristic, Gelucire®44/14 could facilitate dissolution by inhibiting the precipitation of drugs in the GI tract[9] and increase the extent of drug absorption from the GIT lumen into enterocytes by inhibiting the enterocytic efflux transporter, P-gp[10]. Thus, the objectives of the present study were to investigate the potential contributing factors towards the poor bioavailability of the lignans in PNF5, to prepare the PNF5-Gelucire®44/14 solid dispersion capsules (PNF5-SDC) and to compare the bioavailability of lignans by oral administer the PNF5-SDC and the conventional PNF5 powder filled capsules (PNF5-PFC) in rats.

MATERIALS AND METHODS

Plant material, bioactive fraction and chemicals: The leaves of *P. niruri* were collected from Pulau Langkawi, Malaysia. A voucher specimen was identified by the curator and deposited at the herbarium of the School of Biological Sciences, Universiti Sains Malaysia (Reference No. 11040). The extraction and fractionation was conducted following the method as previously described[11]. The external standard lignans of 1 and 2 were isolated from our laboratory[11]. The purity of the standards (>95% w/w) were determined by a Waters Delta analytical HPLC system, equipped with a Waters 2996 Photodiode Array Detector (PDA). Quinidine and anhydrous D-glucose were purchased from BDH (Poole, England). The n-octanol, verapamil, sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid and HPLC grade acetonitrile were purchased from Merck (Germany). The NADPH regeneration kit was purchased from Dromega (USA).

Animals: Male Sprague-Dawley (SD) rats weighing 280±20 g were purchased from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Penang, Malaysia. The rats were fed with standard food pellets (Gold Coin, Penang, Malaysia) and maintained under 12/12 h light/dark cycle at 27. Water was provided *ad libitum*. The study protocol was approved by the Animal Ethics Committee, Universiti Sains Malaysia, Penang, Malaysia [USM/Animal Ethics Approval/2012/(79)(386)] and [USM/Animal Ethics Approval/2011/(73)(338)].

High Performance Liquid Chromatography (HPLC) method: The HPLC method was performed following the methodology described[11]. The standard calibration curves, precision and accuracy of within-day and between day analyses, recovery evaluation, the limit of detection (LOD) and the limit of quantification (LOQ) were evaluated.

Water solubility of PNF5: A saturated water solution was obtained by agitating the excess PNF5 powder in deionised water for 3 h at 25°C. The amount of solute in the saturated solution was determined by HPLC quantification. The solubility of PNF5 was calculated using the following equation:

$$\text{Solubility} = \frac{\text{Weight of PNF5 dissolved}}{\text{Volume of water}}$$

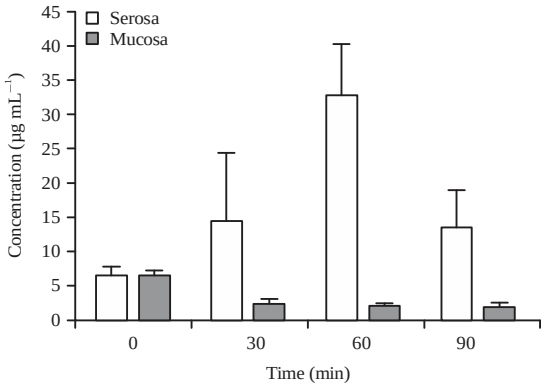

Fig. 1: D-glucose absorption by using everted rat gut sacs. Values are presented as Mean±SD (n = 4)

Octanol/water partition coefficient values (log p) of phyltetralin (1) and phyllanthin (2): The log p-values of 1 and 2 were determined using the shake flask method[12]. The aqueous phase was separated and quantified using HPLC. The log p-values of 1 and 2 were calculated from the ratio of lignan concentration in n-octanol phase over that in the aqueous phase for each pH studied.

Effect of P-glycoprotein efflux on the oral absorption of phyltetralin (1) and phyllanthin (2) in PNF5-*in vitro* everted gut sac method: The everted gut sac method[13,14] was applied in the current study. Briefly, the rat small intestine of six male SD rats of similar weight and age were excised and sectioned into three segments of 3-4 cm length, washed with saline (0.9% NaCl solution) and then immediately kept at 37°C in medium 199 (M 199) (M 3769-1 L, SIGMA, USA), bubbled with a gas mixture of O_2/CO_2 (95:5) at pH 7.5. The serosa surface at one end of the small intestine was then progressively invaginated into the lumen until the mucosa surface was completely everted inside out. One end of the everted gut sac was tied closed with a cotton thread, whereas the other end was tied onto a 5 cm cannula. The lumen was filled with oxygenated M 199 and the gut sac was next placed inside a 25 mL universal bottle containing 20 mL of oxygenated M 199 medium at 37°C.

The integrity and viability of the gut sacs was validated[15] involving the measurement of the D-glucose concentration in both serosa and mucosa surfaces, using a glucose assay kit (SIGMA, GAGO 20 kit, USA). Briefly, the similar concentration of D-glucose solution was added in both the serosa and mucosa sides and incubated for 0, 30, 60 and 90 min (Fig. 1). Thereafter, the glucose concentration of the serosa side increased as the glucose on the mucosa side would pass through the gut wall into the serosa side. After 60 min, the glucose concentration level at the serosa side declined, possibly due to the reduced viability of the gut sac tissue. The incubation time was therefore confirmed at 60 min.

To investigate the effect of P-gp efflux on the oral bioavailability of 1 and 2, the test samples, comprising 0.1 mg mL^{-1} of PNF5 only (Group 1), 0.1 mg mL^{-1} of PNF5 combined with the P-gp inhibitors verapamil at 100 μM (Group 2) and 0.1 mg mL^{-1} of PNF5 plus P-gp inhibitors quinidine at 1.3 mM (Group 3) were added separately into the medium in contact with the mucosal surface incubated for 60 min. After the incubation period, the medium at the serosa surface was removed and the amount of lignans in the medium was quantified by HPLC. The absorption of 1 and 2 was determined by the amount of active compound in the serosa side over the initial amount of active compound added in the mucosa side per unit surface area of gut sac.

Role of first pass metabolism in the oral absorption of phyltetralin (1) and phyllanthin (2)-*in vitro* study: In order to evaluate the contribution of first pass metabolism to the low oral bioavailability of 1 and 2 in PNF5, rat livers were collected after euthanized 3 SD rats by carbon dioxide asphyxiation. The liver tissue was homogenized[16] in M 199 and the supernatants were taken by centrifuged the homogenizer at 13,000×g for 15 min for the *in vitro* incubation study. In detail, each incubation tube contained 0.12 mL of supernatants, 80 μL of PNF5 (200 ppm) and 10x concentrate of NADPH regeneration kit (comprises 26 mM of $NADP^+$, 66 mM glucose-6-phosphate, 66 mM $MgCl_2$ and glucose-6-phosphate dehydrogenase in 5 mM sodium citrate at pH 5.5, Dromega, USA). These tubes incubated in the water bath at 37°C for 50 min and the reaction was terminated by the addition of 0.06 mL of 3% trichloroacetic acid in all tubes. The tubes were then vortexed and centrifuged at 13,000×g for 15 min. The supernatants were taken and deproteinized using acetone (AR) and filtered using 0.45 μm syringe filter (Whatman Schleicher and Schuell) prior to HPLC analysis. In order to identify metabolites, the samples incubated in the absence of the NADPH regeneration system were also carried out for comparison, since the generated NADPH will serves as the source of electrons for the P450 oxidative reactions. For this *in vitro* method, positive control were also carried out using two well-known P450 3A4 (the main enzyme involved in the metabolism of xenobiotics in the human body) substrates verapamil (70 μg mL^{-1}) and quinidine (147 μg mL^{-1})[17,18]. In the quantification of verapamil, isocratic elution was carried with the mobile phase consisted of H_2O and ACN (60:40, v/v) at the flow rate of 0.9 mL min^{-1}. The chromatographic separation was monitored at 210 nm

using UV detector. Whilst, isocratic elution for quinidine determination was carried with the mobile phase consisted of H_2O and ACN (93:7, v/v) at the flow rate of 0.9 mL min^{-1}. The chromatographic separation was monitored using fluorescence detection with $\lambda_{excitation}$ at 340 nm and $\lambda_{emission}$ at 425 nm.

Preparation and optimization of PNF5 Gelucire® solid dispersion: The PNF5-Gelucire® solid dispersion was formulated by mixing G44/14 and PNF5 using fusion method in different proportions. However, the PNF5 solubility in the molten G44/14 should be first determined in order to estimate the maximum amount of PNF5 could be solubilised in G44/14. Thus, a known amount of PNF5 was gradually dispersed in the liquefied G44/14, which had been heated to 50°C under stirring in order to melt it. The weighed amount of PNF5 was added to G44/14 until excess where PNF5 could not be further solubilized. This value was estimated as the solubility of PNF5 in G44/14. As a result, when >49.6±1.9% of PNF5 was added in G44/14, excess PNF5 could be monitored. Thus, different ratio of PNF5 in G44/14 (<50% of PNF5) at 10:90, 20:80, 30:70 and 40:60 were prepared and filled into hard gelatin size No. 4 capsules (PNF5-SDC), separately. The dissolution profiles of lignans for each PNF5-SDC were compared. In addition, the conventional PNF5 powder filled capsules (PNF5-PFC) (size No. 4) were also prepared by mixing standardised PNF5 powder and lactose in the proportion of 5:21. The capsule content uniformities of the above two formulations were 96.86-103.13, 98.14-102.16% for PNF5-PFC and PNF5-SDC, respectively.

The dissolution were tested using basket method at a rotation speed of 100 rpm[19] . Samples were taken by an automated programmable auto-sampler (PREMIERE 5100 Dissolution Test System, DISTEK, USA). The concentration of the bioactive compounds was determined using the HPLC-UV method. The percentage of the lignans that dissolved in the medium was calculated and plotted as percentage of bioactives lignans released vs time profiles and compared by identifying the difference factor (f_1) and the similarity factor (f_2) between the PNF5-PFC and PNF5-SDC as following:

$$f_1 = \left\{ \left[\sum_{i=1}^{P} |R_t - T_t| \right] / \left[\sum_{i=1}^{P} R_t \right] \right\}$$

$$f_2 = 50 \log \left\{ \left[1 + (1/P) \sum_{i=1}^{P} (R_t - T_t)^2 \right]^{-1/2} \times 100 \right\}$$

where, f_1 is the difference factor, f_2 is the similarity factor, P is over all time points, R is reference products (i.e., conventional capsules) and T is test products (i.e., solid dispersion capsules).

Differential Scanning Calorimetry (DSC) of PNF5 solid dispersion: The samples of G44/14(A), PNF5(B), G44/14 and PNF5 physical mixture (C), G44/14 and PNF5 solid dispersion (D) were prepared for the evaluation. The DSC was performed using a Perkin-Elmer differential scanning calorimeter Pyris 6 DSC (Perkin Elmer Ltd., UK) equipped with an intracooler. Pyris 6 software was used to control the machine, monitor the processing and calculate and analyse the results. Helium gas was used to purge the instrument at a flow rate of 20 mL min^{-1}. Temperature and heat flow calibrations were conducted using indium and zinc standards, whereas the baseline was automatically calibrated by placing two empty sample pans in the sample and reference discs in the furnace. Afterwards, 9-11 mg of fresh samples, that prepared 24 h prior to the experiment, were transferred into the aluminium pans and smashed uniformly to cover the pan bottom. Lids were placed onto the sample pans and crimped to form a non-hermetic seal. A heating rate of 10°C min^{-1} was used to cover the range of 0-320°C.

Relative oral bioavailability: The bioavailability and pharmacokinetics study were conducted following two-way crossover study design. Eight SD rats were randomly separated into two groups. The rats were fasted overnight but allowed free access to tap water prior to experimental study. The PNF5 powder (50 mg kg^{-1}) and PNF5-G44/14 solid dispersion (contained 50 mg kg^{-1} of PNF5) were filled into mini-rat capsules[20]. Firstly, four rats (as group 1) were orally administrated with PNF5 mini-rat capsules, while another four rats (Group 2) were administrated with PNF5 solid dispersion mini-rat capsules. After 2 weeks of washout period, the rats from group 1 were administered with PNF5 solid dispersion mini-rat capsules and rats from group 2 were administered with PNF5 mini-rat capsules.

Blood samples of 0.2 mL, collected from the rat's tail vein at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 h after oral administration were kept in heparinized (approximately 10 mg) micro-centrifuge tubes and were then centrifuged at 10000 rpm for 15 min. The upper layer of the plasma was collected and stored at -80°C prior to HPLC analysis. The pharmacokinetic parameters of the bioactive lignans after orally rats administrated PNF5 and PNF5 solid dispersion were calculated and compared. The relative bioavailability of 1 and 2 in the test dosage forms were measured by comparing the AUC after oral administration of PNF5 and PNF5 solid dispersion using Kinetica 5.0 software.

RESULTS

HPLC method and validation: The phyltetralin (1) and phyllanthin (2) (Fig. 2), were chosen to evaluate the standardised PNF5, since their highly enriched in PNF5, with higher potency as potential antihyperuricemic agents[5,21]. The calibration curves of 1 and 2 by using HPLC-fluorescence detection method were linear (r^2 = 0.9998 and 0.9998, respectively) over the range of 0.010-11.66 and 0.010-20 μg mL^{-1}, respectively. The limit of detection (LOD) of 1 and 2 were 0.010 and 0.005 μg mL^{-1}, respectively, while the limits of quantification (LOQ) were 0.010 and 0.010 μg mL^{-1}. The recovery, between-day and within-day accuracy were in the range of 98.02-106.80, 98.02-106.80 and 98.02-106.80%, respectively.

Solubility of PNF5 and log p-value of lignans: The results showed that water solubility of lignans in the PNF5 was 0.12±0.02 mg mL^{-1}. The log p of lignans at pH 1, 4 and 7 were 3.82, 3.74 and 3.80 for phyltetralin (1) ($p>0.05$), respectively and 3.79, 3.82 and 3.85 for phyllanthin (2) ($p>0.05$), respectively, indicating its high lipophilicity.

***In vitro* P-glycoprotein efflux study:** The absorption of 1 and 2 (μg cm^{-2} gut sac) was evaluated using everted gut sac method. The absorption levels of 1 and 2, when the PNF5 in combination with P-glycoprotein inhibitors: Verapamil and quinidine, were significantly higher than that of the PNF5 samples alone ($p<0.001$ for verapamil and $p<0.05$ for quinidine; one-way ANOVA) (Fig. 3).

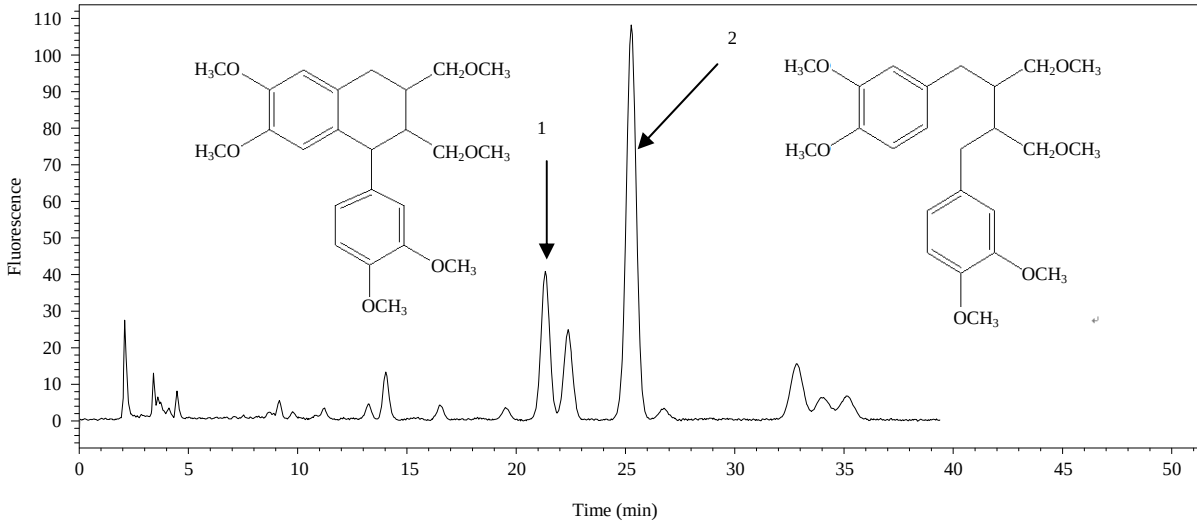

Fig. 2: HPLC chromatogram of PNF5 (78 μg mL^{-1}) using fluorescence detector, 1: Phyltetralin and 2: Phyllanthin

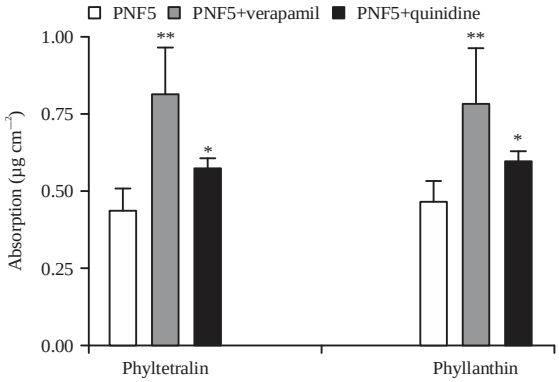

Fig. 3: Evaluation the absorption of phyltetralin (1) and phyllanthin (2) from serosa side using everted gut sac method. The PNF5 (0.1 mg mL^{-1}), verapamil (100 μM) and quinidine (1.3 mM). (*$p<0.05$, **$p<0.001$, one-way ANOVA and *post hoc* Dunnett's multiple comparison test). Values are presented as Mean±SD (n = 6)

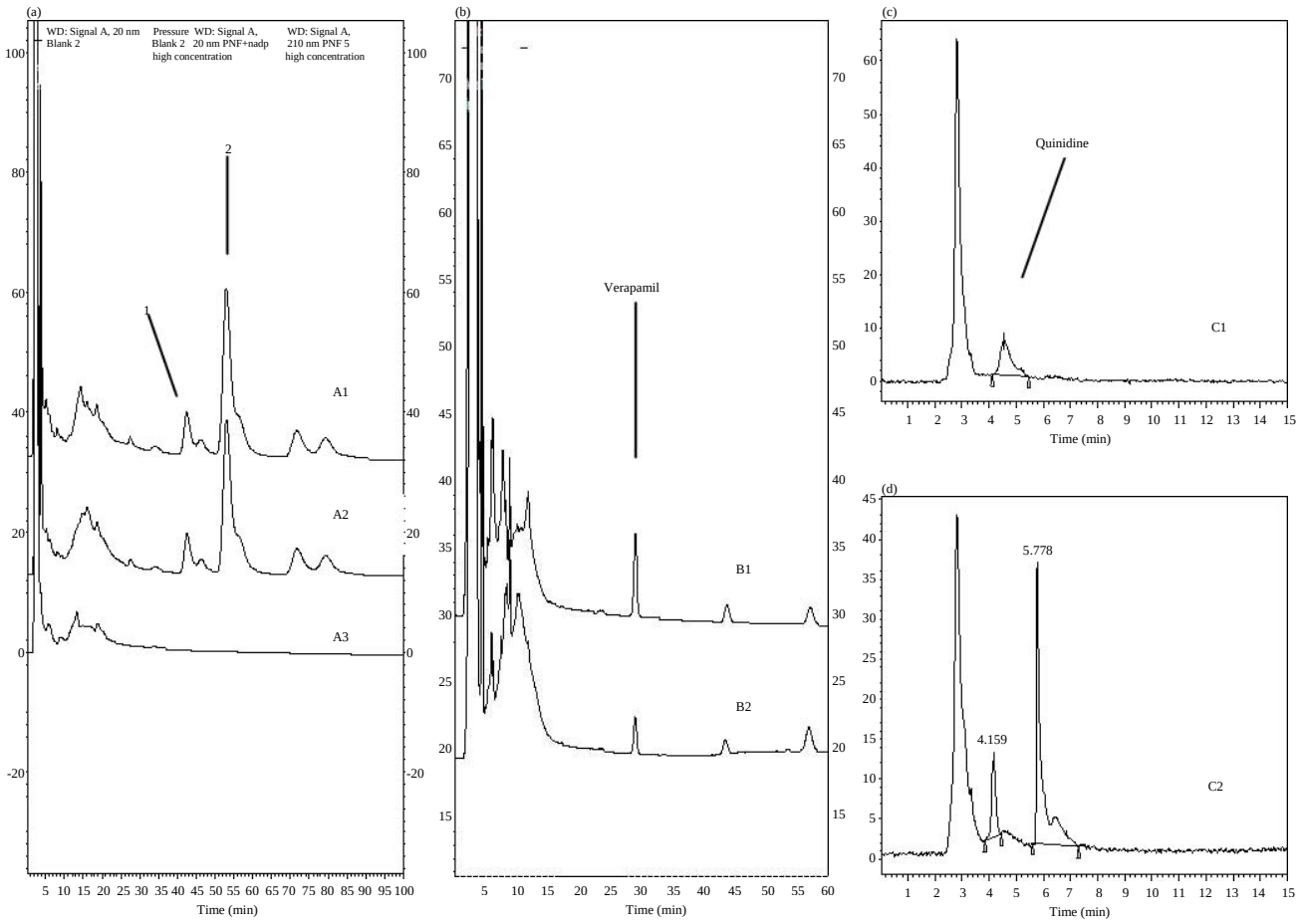

Fig. 4(a-d): *In vitro* metabolic studies of phyltetralin (1) and phyllanthin (2) in PNF5 using rat liver homogenate incubated at 37°C for 50 min (n = 3). Chromatogram A1 and A2 show the 1 and 2 in PNF5 (200 μg mL^{-1}) in absence and presence of NADPH. The 1 and 2 are shown at 43.75 and 54.48 min. Chromatogram A3 is blank liver homogenate. Chromatogram B1 and B2 show the verapamil peak at 70 μg mL^{-1} in the absence and presence of NADPH, respectively at 29.06 min. Chromatogram C1 and C2 show the quinidine peak at 147 μg mL^{-1} in the absence and presence of NADPH, respectively. The quinidine is shown at 4.56 min; the metabolites are shown at 4.15 and 5.78 min

***In vitro* first pass metabolism:** In chromatogram B (Fig. 4), verapamil concentration was significantly reduced ($p<0.0001$, t-test) when the NADPH was presence in the incubated sample. For quinidine (Fig. 4, chromatogram C), the peak disappeared after incubation in the homogenized liver with NADPH and two new peaks were detected at 4.15 and 5.78 min. In contrast, chromatogram A (Fig. 4) showed no significant differences in peak height for 1 ($p = 0.4363$, t-test) and 2 ($p = 0.5396$, t-test) and no lignans metabolites were observed when incubated with homogenized liver in absence (A1) and presence (A2) of the NADPH.

Dissolution and optimization of PNF5 Gelucire® solid dispersion: The results shown that the PNF5-G44/14 solid dispersion at ratio of 10:90 could achieve the fastest dissolution rate (Fig. 5). However, the carrier efficiency was low. Thus the drug:carrier ratio of 20:80 (F2) was adopted as the optimized formulation for the future study, since it has the similar dissolution profiles as F1.

The results of the dissolution test on the capsules containing PNF5-SDC and PNF5-PFC were summarised in Fig. 5. Results showed that 1 and 2 in different proportions of PNF5-SDC could be fast released in the dissolution medium within 45 min. The results showed that the difference (f1) and similarity (f2) factors of phyltetralin comparing PNF5-SDC (F2) and PNF5-PFC were 94 and 1, respectively, whilst those of phyllanthin were 67 and 13, respectively.

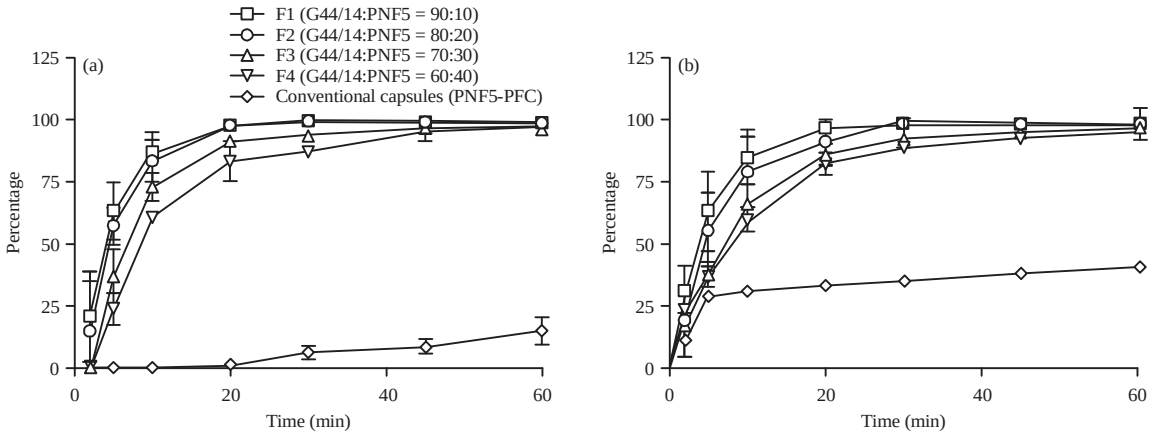

Fig. 5(a-b): Dissolution profiles of phyltetralin (1) and phyllanthin (2) in PNF5 Gelucire® Solid Dispersion Capsules (PNF5-SDC) and PNF5 conventional powder-filled capsules (PNF5-PFC). The F1, F2, F3 and F4 are the formulations of PNF5-SDC. Values are presented as Mean±SD (n = 6)

Differential Scanning Calorimeter (DSC) study of PNF5-SDC: The thermograms of PNF5-SDC with a ratio of PNF5 in G44/14 of 20:80 was achieved by DSC were shown in Fig. 6. Thermogram A of G44/14 was monitored where one sharp valley peak was identified at 41°C, similarly to that previously described[22]. Thermograms B was the heating profile of PNF5. A short valley peak was found in thermogram C at 39°C. No endothermic peak was found around 39°C except for the fusion endothermic peak of G44/14. Thus, the endothermic peak at 39°C may still be the fusion endothermic peak of G44/14. In thermogram D, the G44/14 fusion endothermic valley peak was absent. However, an endothermic peak with shoulders was identified at 110°C.

Relative oral bioavailability: The plasma concentration-time profiles of 1 and 2 after oral administration were shown in Fig. 7. The AUC of 1 and 2 in the PNF5-SDC were significantly increased when compared with those in the PNF5-PFC. The relative oral bioavailability (F_{rel}) of 1 and 2 were increased by 3.85 and 2.42 fold, respectively, showing remarkable improvement after oral administration with the PNF5-SDC.

DISCUSSION

The saturated solubility of the drug in the diffusion layer is the key factors for the drug dissolution rate, according to Noyes-Whitney equation, whereas the dissolution is the most important rate-limiting step for drug absorption. Thus, the solubility study was preformed. The result indicated that lignans in the PNF5 powder was sparingly soluble in water, according to the criteria of British Pharmacopeia, implicated that the solubility of lignans was the rate limited step for the poor oral bioavailability.

The poor oral bioavailability of lignans may also related to the gastrointestinal (GIT) membrane permeability, which can be estimated by the n-octanol-water partition coefficients (log p) value, the more lipophilic of the compound, the faster its absorption. Meanwhile, the pH value of GIT fluids may markedly change the dissociating of drug molecular and subsequently affect the drug absorption, since only the unionized form that is appreciably absorbed. Thus, the log p-values of 1 and 2 were evaluated at different pH. The results suggested that 1 and 2 could fast penetrate the lipid membrane of the small intestine and the dissociation of drug molecular at different pH value of GIT lumen were not significant affected the lignans absorption ($p>0.05$) and the pH stability was not the rate-limiting step for the poor oral bioavailability of lignans. Lignans of 1 and 2 in PNF5 could thus be categorised as the biopharmaceutical classification system II substances[23], which possessing poor aqueous solubility and high lipophilicity.

For the *in vitro* P-glycoprotein efflux study, the improved absorption of 1 and 2 may be due to the inhibition of P-gp efflux by verapamil and quinidine, suggested that lignans were the substrate of P-gp. The result was in accordance with another *in vitro* study using Caco-2 cell monolayers as reported[24]. However, when PNF5 combined with quinidine, the absorption of 1 and 2 showed relatively less improvement compared with that of the combination with verapamil, due to the quinidine was the relatively weak

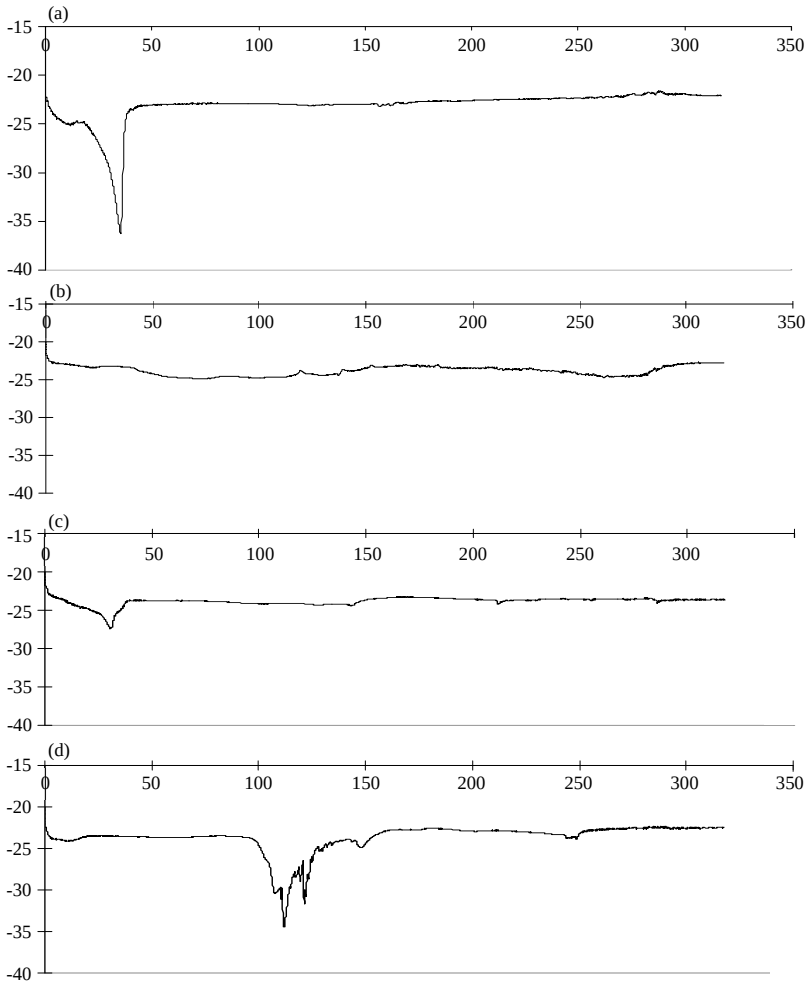

Fig. 6(a-d): DSC thermograms of (a) G44/14, (b) PNF5, (c) PNF5+G44/14 physical mixture and (d) PNF5+G44/14 solid dispersion (n = 3)

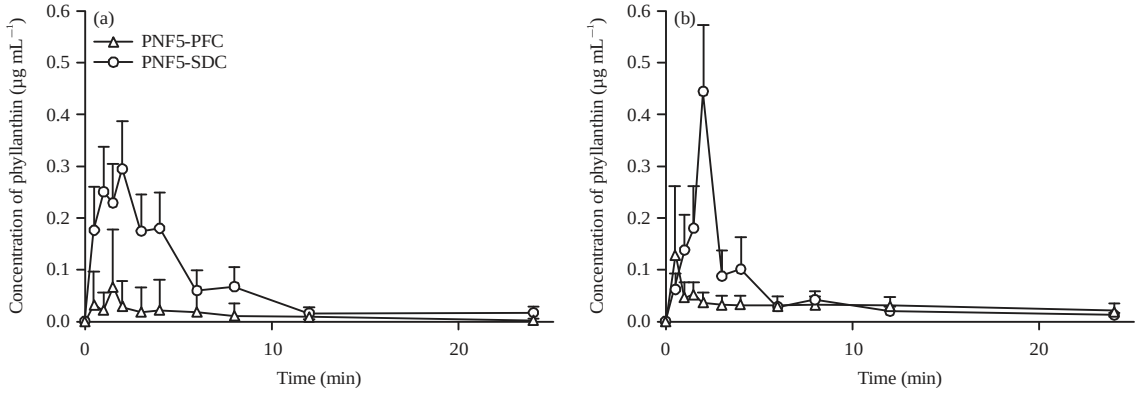

Fig. 7(a-b): Plasma concentration-time curve (AUC) of phyltetralin (1) and phyllanthin (2) after oral administration of 50 mg kg^{-1} PNF5 using Gelucire® Solid Dispersion Capsules (PNF5-SDC) and PNF5 conventional powder-filled capsules (PNF5-PFC), respectively. Values are presented as Mean±SD (n = 8)

inhibitor of P-gp efflux[25]. The lipophilicity and molecular weights may also assist the compounds binding efficiency with P-gp, as many have reported that the lipophilicity and molecular weight of drug candidate were important

parameters that may contribute to their binding efficiency on P-gp studies[26,27]. Those with high binding for P-gp normally possessed high log p-values[28] exceeding 3 and their molecular weights generally exceeded 400. The lignans of 1 and 2 were therefore predicted as potential candidates for P-gp binding efflux following their high log p-values and molecular weights (418.534 and 416.518 for 1 and 2, respectively). Collectively, as the substrate of P-gp, lignans affect its own oral absorption.

The results of *in vitro* first pass metabolism study shown that the P450 3A4 substrate of quinidine and verapamil, as two positive controls were significantly metabolized by the homogenized liver matrix, indicating the method was valid for indentifiy the substrate of P450 3A4 enzyme. These results suggested that 1 and 2 of lignans were not metabolized by the liver matrix. Thus, the first pass metabolism may not be the major contributing factor towards the poor bioavailability of 1 and 2.

The above results collectively revealed that the poor oral bioavailability of lignans in PNF5 was mainly due to the poor aqueous solubility and P-gp efflex. It could thus be overcome by the formulation design. It is well known that some methods such as cyclodextrins, nanoparticles, liposomes were frequently used for the non-polar drugs formulation. However, the compound loading efficiency of these methods was low. In addition, the PNF5 is the lignan-rich bioactive fraction containing total lignans of 43.55±2.01% (w/w). Thus, loading efficiency of PNF5 lignans entrapped by these formulation methods would be very less. Solid dispersion was an appropriate method for the formulation of plant extract, as the small particles or active molecules of extract could entirely dispersed in the matrix and forms physical or molecular level of mixture or complex. The dissolution and absorption of the solid dispersion would not be affected by the entrapment efficiency. However, it would be affected by the proportion of gust molecular and solid dispersion matrix.

Gelucire®44/14 (G44/14), a commercially used solid dispersion[29] was used in the present study, as it could form semi-solid state at room temperature by a relatively low melting point of 44°C and improve the aqueous solubility of the lipophilic bioactive compounds such as 1 and 2 in PNF5 and subsequently to achieve higher bioavailability.

The dissolution profiles indicated all the PNF5-SDC exhibited fast release, compared with conventional formulation and the dissolution rate was proportional to the amount of G44/14. In contrast, the conventional PNF5-PFC barely dissolved in the dissolution medium (Fig. 6) due mainly to its poor water solubility.

The dissolution profiles of 1 and 2 in PNF5-SDC and PNF5-PFC were evaluated using difference (f1) and similarity (f2) factors. The difference factor (f_1) is the percent (%) difference between two curves at each time point and is a measurement of the relative error between two curves. The similarity factor (f_2) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in percent (%) dissolution between two curves[30]. The dissolution profiles are normally considered similar when the f1 value is close to zero, whilst f2 value is close to 100. The results indicated a significant change in PNF5-SDC when compared to PNF5-PFC. Thus, the differences in the bioavailability of these formulations were expected.

The current thermal analysis study was only directed towards the melting behaviors of the solid dispersion and physical mixture. This endothermic profile of thermogram D confirmed that the test sample (PNF5-SDC) was not the physical mixture of PNF5 and G44/14. The G44/14 fusion endothermic peak at 39°C was absent and significant shifted to 110°C. This phenomenon may be due to other dominant physical or chemical forms present in the sample[31], such as the solid dispersion complex.

The results of *in vivo* bioavailability study indicated that the PNF5-SDC formulation could potentially increase the efficacy of PNF5 in clinical applications. This result in accordance with other researches finding, in which the bioavailabilities of water insoluble drugs were also increased by incorporating with G44/14[32]. The improved *in vivo* bioavailability may be due to the increased dissolution rate of 1 and 2 by the solid dispersions, reduction in particle size of active ingredient and/or pre-dissolved state of compounds present in the semi-solid dispersion of G44/14[29,33]. Moreover, G44/14 may partially changed the highly ordered crystalline structures of 1 and 2 to amorphous and subsequently improved their dissolution rate[34]. The improved bioavailabilities of lignans were also due to the inhibition of P-gp efflux by G44/14, although the mechanism which is currently unknown[35]. In addition, the unique self-emulsifying properties of G44/14 may lead to the forming of microemulsion on contact with liquids in the GIT which may also result in absorption improvement[36].

CONCLUSION

The aqueous solubility and P-gp efflux were the major contributing factors for the low oral bioavailability of lignans in PNF5. The G44/14 solid dispersion significantly increased the solubility of lignans, inhibits the P-gp efflux activity and subsequently improved the oral bioavailability of lignans in PNF5.

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