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An *in vitro* Permeation of *Phyllanthus amarus* Extract through Human and Shed Snake Skins

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Abstract: An *in vitro* permeation of a hydroalcoholic extract of *Phyllanthus amarus* (PaE) was investigated using excised human epidermis and shed king cobra skin as the barrier membranes. Donor and receptor compartments of diffusion cells were pH–controlled to simulate the permeation environment of the human skin. The PaE was analyzed by using normal–phase densitometric TLC detected at λ 280 nm and toluene:ethyl acetate (17:3) as the mobile phase. There were four major components observed in the saturated solution of the donor at pH 5.5. Over 24 h, only one component, possibly phyllanthin, was found in the receptor solution after permeation across the human epidermis, while two components, possibly phyllanthin and an another unknown permeated, permeated through shed snake skin. When compared to the saturated donor concentration, phyllanthin gave permeation fluxes of 0.04±0.01 and 0.12±0.02 $\%\cdot cm^{-2}\cdot h^{-1}$ through the human and shed snake skins, respectively. It seems that only certain component(s) of the *P. amarus* extract could permeate through the skins, and by comparison, at a slower rate across the human skin than shed snake skin.

Key words: *Phyllanthus amarus*, human skin, shed snake skin, *in vitro* permeation

INTRODUCTION

Dermatological applications of herbal extracts for pharmaceutical and cosmetic purposes may have delivered some components which were not well documented. *In vitro* permeation studies by using various designs of diffusion cells have been one of the methods used to simulate percutaneous absorption of drugs or other compounds[1]. *In vitro* permeation studies of herbal extracts could be a supportive part for development of rationale use of phytotherapy. *Phyllanthus amarus* Schum & Thonn. (*P. amarus*), family *Euphorbiaceae,* was selected for study due to its traditional claims for treatment of skin diseases and recent reports on its activities as an anti-inflammatory[2,3], antibacterial[4], antifungus particularly dermatophytic fungi[5] and potent antioxidant properties [6]. Active components of *P. amarus* such as phyllanthin and hypophyllanthin have been isolated and identified[7,8]. Different species of *Phyllanthus* could be similar in morphology but different in pharmacological activities to *P.amarus*[2, 8], it is necessary to confirm the plants being used.

The skin plays a vital role in controlling the passage of substances. The permeation of any compounds through the skin involves physicochemical characteristics of the compounds, the vehicle and the barrier membrane [9, 10].

Passage of a dissolved compound across the human skin generally occurs from an outer skin pH of about 5 through various layers, and if possible, to a sink condition with a physiological pH of 7.4. Human skin is not as readily available as the waste material like shed snake skin, and both have been shown to give comparable *in vitro* permeation[9, 10, 11, 12]. This includes king cobras (*Ophiophagus hannah*) [12] which shed large pieces of skin readily usable for several experiments.

This study employed the human epidermis and shed snake skin as the barrier membranes and controlled the pH, which is likely to be one of the vital factors influencing the permeation of a hydroalcoholic extract of *P.amarus* (PaE). The permeation profile studied by this type of herbal extract could provide a vital information for further product development.

MATERIALS AND METHODS

Chemicals: Chemicals used include trichloromethane (Mallinckrodt Chemical Inc., U.S.A.), methanol (Fisher Scientific, U.K.), toluene (Merck, Sweden), glacial

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acetic acid (J.T. Baker, U.S.A.), ethyl acetate (Lab Scan Analytical Sciences, Ireland), agarose gel (ISC Bioexpress, U.S.A.) and ethidium bromide (Fluka Chemika, U.S.A.). All solvents were analytical grade. Thin layer chromatography (TLC) plates were silica gel 60 F_{254} (20×20×0.0025 cm, Merck, Germany).

Preparation of the human epidermis and shed snake skin: Fresh, surgically excised samples of human skin were obtained directly after breast plastic surgery with informed consent following ethical approval. Shed king cobras skin were kindly gifted and collected freshly from the Khok Sanga King Cobra Club, Khon Kaen, Thailand. These were kept at −21°C until use. The human epidermis was separated as previously described[15] while the dorsal scales of the shed snake skin were used as received.

Collection of plant: Whole plants of *P. amarus* were collected from Khon Kaen province, Thailand, during the rainy season (May−August) year 2005 and 2006. The identity of the plant was certified by one of us (P.C.) and the plant specimen was kept in the herbarium collection of Faculty of Pharmaceutical Science (collection number *PSH-KKU-05/AP01, PSH-KKU-06/AP01*).

DNA identification of P. amarus by sequence characterized amplified region (SCAR): Fresh leaves of *P. amarus* were used for genomic DNA extraction following a previously described method[16]. The PCR amplification and reactions were performed in a thermocycler (PCR Sprint, U.S.A.) and the products were electrophoretically separated in 1.2% (w/v) agarose gel and staining-visualized with ethidium bromide. The RAPD fingerprint pattern resulting from amplification was compared with the SCAR primers of *P. amarus* collection at the Department of Biology, Khon Kaen University.

Phyllanthus extract (PaE): Dried powder of whole plants of *P. amarus* was macerated in 50% ethanol (1:6, v/v) at 25±2° C for 7 days. The supernatant was concentrated by reduced pressure evaporator (Tokyo Rikakikai Co. Ltd., Japan), freeze−dried (FTS systems, U.S.A.) and pulverized. Average yields of PaE were 2.4% by fresh weight or 7.9% by dried weight.

Permeation studies: Side−by−side diffusion cells (area of 0.694 cm^2, Crown Glass Company, U.S.A.) with controlled circulation of solutions at 600 rpm and temperature at 32±1°C were employed. Donor compartments were filled with PaE saturated in citrate phosphate buffer at pH 5.6 and receptor compartments with phosphate buffer at pH 7.4. Samples were collected from the receptor compartments for analysis and replaced by the same volume of fresh phosphate buffer.

Analysis by normal phase thin layer chromatography (TLC) with densitometer: Extraction of samples was conducted by using several portions of chloroform until completion. The whole portions of the chloroform extract were collected, mixed and dried under nitrogen gas, then dissolved with 0.1 ml methanol. 0.02 ml of the methanol extract was taken and spotted on the TLC siliga gel plates. The mobile phase used for eluting the plates was a mixture of toluene: ethyl acetate (17:3). The plates were scanned at a wavelength of 280 nm using a densitometer TLC Scanner 4 (Camag, Switzerland) linked to WinCATS4 software.

RESULTS AND DISCUSSION

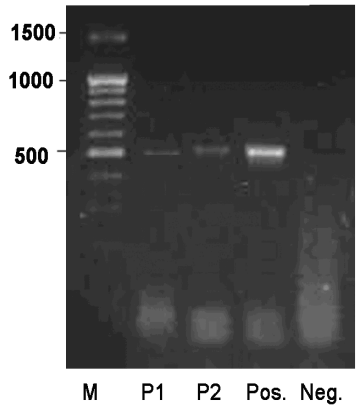

Fig. 1: Amplified DNA bands resulting from PCR amplification of genomic DNA of *P. amarus* collected at two occasions (P1 and P2). The PCR reactions were initiated with SCAR primers specific for *P. amarus*. M = DNA marker, Pos. = DNA of *P. amarus* reference plant, Neg.= negative control without DNA.

Collections of the plants from different occasions, P1 and P2, gave the same yield of PaE. Taxonomical

identification of *P. amarus* by an expert was confirmed by genomic DNA, as shown in Figure 1.

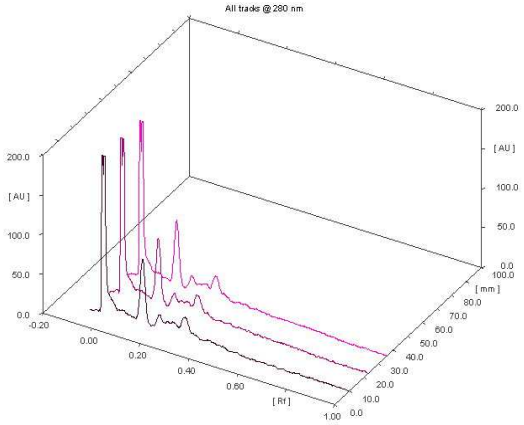

Fig. 2: TLC chromatogram of PaE saturated in the donor solution (1:10 dilution) at 6, 12 and 24 h of permeation study. Analysis conditions: normal phase densitometric TLC; $G60F_{254}$ silica gel plate (20×10 cm); mobile phase of toluene:ethyl acetate (17:3); and λ 280 nm.

PCR amplification of DNA of both collections (P1 and P2) using *P. amarus*−specific primers gave rise to a specific band of approximately 500 bp. The extraction process gave reproducible yields of a fine, hygroscopic and light-brown powder. PaE was hydrophilic, i.e. soluble in water, ethanol and insoluble in chloroform and n−hexane, and hygroscopic.

The TLC chromatograms of PaE saturated in the donor solution showed 2−3 resolution color bands on silica plate at R_f value about 0.1−0.5. The extract showed a good resolution of peak no. 1 and 2 at average R_f of 0.21 and 0.28, as shown in Figure 2, which resembles the peaks of phyllanthin [8] and an unknown. The receptor solutions using shed snake skin as the barrier at 6, 12 and 24 h could separate 3−4 peaks (as shown in Figure 3).

At sink conditions, the permeation of phyllanthin linearly increased with time from donor solution with the skin pH of 5.5 to receptor solution at pH 7.4. An attempt to estimate the permeation rates of peak no. 1 (at R_f 0.21) during 6−24 h, analyzed by linear regression, were 0.04±0.01 and 0.12±0.02 $\%\cdot cm^{-2}\cdot h^{-1}$ through the human epidermis and shed snake skin, respectively, with correlation coefficient of more than 0.999. Another unknown substance as shown by a presence of peak no.2 at R_f 0.28 permeated when using shed snake skin at a slower rate of 0.006 $\%\cdot cm^{-2}\cdot h^{-1}$.

It is concluded that phyllanthin, one of the major components of *P.amarus*, could permeate through the barrier structure of both human and shed snake skins. Further study is required to qualitatively identify each component and investigate the competitive permeation profile of the mixtures which are usually found when using any herbal extracts.

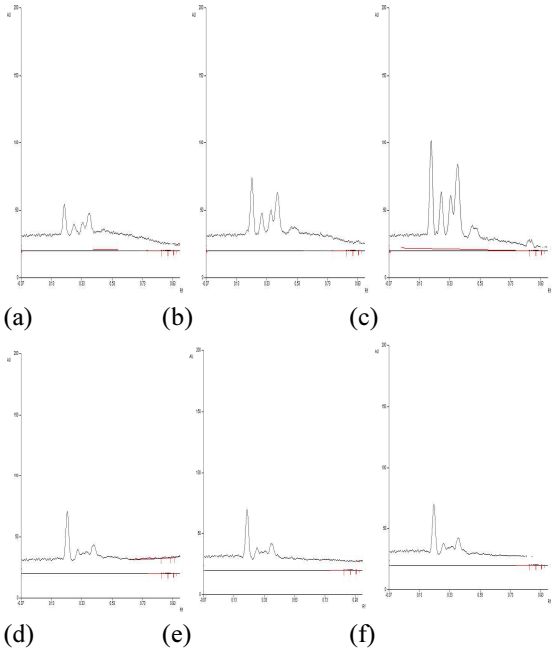

Fig. 3: TLC chromatograms of PaE permeated into the receptor solution (phosphate buffer pH 7.4) through shed snake skin (a−c) and the human epidermis (d−f) from the donor solution (buffer at pH 5.5) at (a) 6 h, (b) 12 h and (c) 24 h; at 32±1°C. Analysis conditions: normal-phase densitometric TLC: $G60F_{254}$ silica gel plate (20×10 cm); mobile phase of toluene:ethyl acetate (17:3); and λ 280 nm.

CONCLUSION

Some components of the extract of *P.amarus* were shown to permeate through the scales of the shed skin of king cobras and the human epidermis. The permeation could be a passive transport mechanism influenced by the physicochemical properties of the compounds involved. The human skin gave a slower

rate of permeation than the scales of shed snake skin which could be the result of differences in lipid compositions. This, however, suggests a potential development of a topical use of PaE, provided that the activities of the substance(s) were proved.

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