© 2025 Journal of Pharmacy & Pharmacognosy Research, 13 (4), 1038-1044, 2025
ISSN 0719-4250
https://jppres.com

DOI: https://doi.org/10.56499/jppres24.2233_13.4.1038

Original Article

HPLC method validation for porcine ear permeability study of oxyresveratrol from *Artocarpus lakoocha* Roxb. heartwood extract

[Validación del método HPLC para el estudio de la permeabilidad de la oreja porcina al oxiresveratrol del extracto de duramen de *Artocarpus lakoocha* Roxb.]

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Abstract

Context: Oxyresveratrol or 2,3,4,5-tetrahydroxystilbene is the major constituent of *Artocarpus lakoocha* Roxb. (AL), which possesses several pharmacological activities. It also inhibits a key enzyme of melanogenesis, tyrosinase, resulting in skin lightening.

Aims: To develop and validate an HPLC method for the analysis of oxyresveratrol in full porcine ear skin permeation study of *A. lakoocha* heartwood extract.

Methods: A reversed-phase HPLC with a gradient system composed of acetonitrile and 0.05% trifluoroacetic acid at a flow rate of 1 mL/min was developed and validated according to the International Council for Harmonization guideline. All chromatograms were monitored at 320 nm. The analyses of oxyresveratrol permeation using side-by-side diffusion cells were conducted. The amounts of the active compounds were analyzed in the donor, receptor of the side-by-side diffusion cells, as well as accumulation in the skin.

Results: The yield of plant extract was 14.68%, with the amount of oxyresveratrol at 372 mg/g of the extract. Relative accuracy was between 98.79–105.74% with 1.87–2.83% intraday precision and 2.89-3.80% interday precision. The linearity showed a good correlation at 0.9994. The recovery of the active compound was approximately 88.53 ± 0.71%. Most of the compound was still on the donor side.

Conclusions: The result showed sufficient information for the analysis of oxyresveratrol in the skin permeation study. For further experiments, skin permeation enhancers could be incorporated to improve the efflux of oxyresveratrol into the skin.

Keywords: full porcine ear skin; resveratrol derivatives; skin permeation; stilbene; tyrosinase inhibitor.

Resumen

Contexto: El oxiresveratrol o 2,3,4,5-tetrahidroxiestilbeno es el componente principal de *Artocarpus lakoocha* Roxb. (AL), que posee varias actividades farmacológicas. También inhibe una enzima clave de la melanogénesis, la tirosinasa, lo que produce un aclaramiento de la piel.

Objetivos: Desarrollar y validar un método de HPLC para el análisis de oxiresveratrol en un estudio de permeación completa de la piel de la oreja de cerdo del extracto de duramen de *A. lakoocha*.

Métodos: Se desarrolló y validó un HPLC de fase inversa con un sistema de gradiente compuesto por acetonitrilo y ácido trifluoroacético al 0,05 % a una velocidad de flujo de 1 mL/min de acuerdo con las directrices del Consejo Internacional de Armonización. Todos los cromatogramas se monitorearon a 320 nm. Se realizaron los análisis de permeación de oxiresveratrol utilizando celdas de difusión lado a lado. Las cantidades de los compuestos activos se analizaron en el donante, receptor de las células de difusión lado a lado, así como la acumulación en la piel.

Resultados: El rendimiento del extracto de la planta fue del 14,68%, con la cantidad de oxiresveratrol en 372 mg/g del extracto. La precisión relativa estuvo entre el 98,79-105,74% con una precisión intradía del 1,87-2,83% y una precisión interdía del 2,89-3,80%. La linealidad mostró una buena correlación a 0,9994. La recuperación del compuesto activo fue de aproximadamente el 88,53 ± 0,71%. La mayor parte del compuesto todavía estaba en el lado del donante.

Conclusiones: El resultado mostró suficiente información para el análisis de oxiresveratrol en el estudio de permeación de la piel. Para experimentos posteriores, se podrían incorporar potenciadores de la permeación de la piel para mejorar el eflujo de oxiresveratrol en la piel.

Palabras Clave: derivados del resveratrol; estilbeno; inhibidor de la tirosinasa; permeación cutánea; piel de oreja de cerdo completa.

ARTICLE INFO
Received: October 1, 2024.
Accepted: January 27, 2025.
Available Online: February 20, 2025.

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INTRODUCTION

Artocarpus spp. are indigenous trees in Southeast Asia. They possess several biological properties such as anti-bacterial, anti-inflammatory, antioxidant, and anti-helminthic activities (Frimayanti et al., 2025). Monkey Jack (*Artocarpus lakoocha* Roxb.) is a plant in the family *Moraceae*. It is distributed in South- and Southeast Asia. In folk medicine, the heartwood of *A. lakoocha* extract is traditionally used for anthelminthic. In addition, the root and root bark of *A. lakoocha* were also used for antipyretics (Likhitwitayawuid, 2008). It contains phenolic compounds such as phenolic acid, flavonoids, tannins, and stilbenoids. The phytochemicals found in *A. lakoocha* are rutin, pyrogallol, gallic acid, resorcinol, quercetin, catechin, caffeic acid, resveratrol, and oxyresveratrol (Singhatong et al., 2010). For *A. lakoocha* heartwood extract, the major constituent is 2,3,4,5-tetrahydroxystibene or oxyresveratrol, which was found highly amount of 6–8% w/w (Rojsanga and Sithisarn, 2016).

Oxyresveratrol expressed several biological activities, for instance, anti-inflammation (Dvorakova and Landa, 2017; Gautam and Patel, 2014), antibacterial (Bostanghadiri et al., 2017), anti-herpes simplex virus (Chuanasa et al., 2008) and neuroprotection (Andrabi et al., 2004). It also inhibits tyrosinase enzyme, which plays an important role in hyperpigmentation. It exhibited stronger inhibitory activity against mushroom tyrosinase, approximately 32-fold, compared with a positive control kojic acid (Kim et al., 2002). The mechanisms of action on tyrosinase inhibitory activity were related to antioxidant and anti-glycation, which resulted in anti-skin aging and skin whitening (Sungthong and Phadungkit, 2015; Suwannalert et al., 2012). Skin is the body's largest organ, with the main function as the primary barrier protecting the body from the external environment (Arda et al., 2024). Therefore, studies on the permeable enhancement of drugs or phytochemicals through the skin are crucial to obtaining such compounds in systemic circulation or deeper tissues (Alkilani et al., 2015). Recently, oxyresveratrol from *A. lakoocha* incorporated into cosmetics for skin whitening purposes has been increasing in Thailand. There were various studies in cosmetic product development for skin permeation of oxyresveratrol. Teeranachaideekul et al. (2013) developed an encapsulation system using liposomes, which increased the permeation of *A. lakoocha* from 2.14 to 3.58 μg/cm^2. There was also a study of *A. lakoocha* extract nano-emulsion for skin permeation that improved the skin-lightening process.

The quality of plant extracts is primarily assessed by the analysis of bioactive compounds. The amount of a particular bioactive compound not only reflects the quality but also ensures the efficacy of such extracts (Govindaraghavan and Sucher, 2015). Hence, the determination of oxyresveratrol in an *A. lakoocha* extract was one of the crucial steps in quality assessment. An HPLC method was the most frequently used for the analysis of oxyresveratrol. Sangsen et al. (2015) used C_{18} as a stationary phase with acidified acetonitrile mobile phase for plasma oxyresveratrol analysis. Regarding the analysis of oxyresveratrol in skin permeation studies, analytical methods of oxyresveratrol are limited. Therefore, the objectives of this study were to develop and validate the analytical method of oxyresveratrol for full porcine skin permeation study by using the HPLC technique.

MATERIAL AND METHODS

Plant extract preparation

A. lakoocha heartwood was purchased from Napakha-orn Pharmacy, Bua Yai district, Nakhon Ratchasima province (15° 35′ 41.05″N, 102° 25′ 47.04″E). It was identified by Assistant Professor Dr. Wanida Caichompoo, Faculty of Pharmacy, Mahasarakham University (voucher specimen code MSU.PH-MOR.AL01) and deposited at the Faculty of Pharmacy, Mahasarakham University. Prior to extraction, the heartwood was washed and cut into small pieces. Then, it was further dried at 60°C for 1 hour. After the drying process, it was blended to obtain fine powder and further sieved with 60 mesh. The fine powder was macerated with 95% ethanol with a solid-to-liquid ratio of 1:10 w/v for three days and then filtered. The marc was then repeated with two additional macerations. After filtration, the filtrate was further evaporated under a reduced pressure condition. Finally, it was lyophilized to obtain the dried powder extract. The sample was kept at -20°C and protected from light before use.

Standards, chemicals and reagents

Oxyresveratrol was purchased from Chengdu Biopurify Phytochemical, China. Methanol and Acetonitrile (HPLC grade) were obtained from ACI Labscan (Bangkok, Thailand). Trifluoroacetic acid (TFA) was from Sigma-Aldrich (St. Louise, USA). NaCl, Na_2HPO_4, KH_2PO_4, and KCl were from Carlo Erba Reagenti (Val de Reuil, France). A commercial grade 95% ethanol was purchased from Alcoh-Al (Bangkok, Thailand). All other chemicals and solvents were analytical grade. Ultra-purified water was generated by Milli-Q with a 0.22 μm Millipak® filter (Millipore, Darmstadt, Germany).

Porcine skin preparation and permeation study

The study was approved by the Ethic Committee on Animals for Scientific Purposes of Mahasarakham University with exemption (Protocol number: 0008/2560). Full porcine ears were purchased from a slaughterhouse within 2 hours after killing. The ear skin was cleaned and prepared to obtain 1.00 ± 0.20 mm thickness and kept at -20°C (Tuntiyasawasdikul et al., 2014). Before the permeation experimental study using side-by-side diffusion cells, the skin was soaked in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 1 hour. *In vitro* permeation studies were conducted using porcine ear epidermis with saturated solutions of *A. lakoocha* or oxyresveratrol standard in 0.01 M PBS, pH 7.4 at 32.0 ± 0.5°C. The experiment was performed according to the method previously described by Tuntiyaswasdikul et al. (2017) with slight modifications. Briefly, the sample (1 mL) containing oxyresveratrol was collected from donors and receptors in different durations within 12 hours. After sampling, the medium (PBS, pH 7.4) was immediately added to donors and receptors. In the end, full porcine ear skins were finally extracted, and the residual oxyresveratrol was analyzed. The accumulation of oxyresveratrol in the skin was also conducted by sampling the skin at 0.25, 0.5, 0.75, 3, 6, and 12 hours. The details of the extraction of oxyresveratrol from the skin are described in the percent recovery section. All samples were diluted to obtain the desired concentration within the linearity range of the study.

Instrumentation

Experiments were operated using a high-performance liquid chromatographic system (Shimadzu Corp., Kyoto, Japan) consisting of a binary pump liquid chromatograph (SCL-10AVP) with a photodiode array detector (SPD-20A). Samples were introduced into the analytical system with Rheodyne® loop, 20 µL. The analytical column was packed with a 5 µm C_{18} column (150 × 4.6 mm, 5 µm) (Dr Maisch HPLC GmbH, Germany). Mobile phases composed of acetonitrile (A) and 0.05% TFA (B) with gradient conditions, as follows; 0-6 min, 82-75% B; 6-15 min, 75-60% B; 15-18 min, 60-82% B; 18-21 min, 82% B. The flow rate of the mobile phase system was set at 1.0 mL/min under ambient temperature conditions. The chromatograms were collected at 320 nm. All samples were filtered through 0.45 µm PTFE before introducing to HPLC. A porcine permeation study was performed using side-by-side diffusion cells by Logan SDC-6 (Somerset, NJ, USA).

Method development and method validation

Standard stock solutions of oxyresveratrol were prepared and subsequently diluted to desired concentrations with 50% acetonitrile (0.16, 0.08, 0.04, 0.02, and 0.01 mg/mL). The mobile phase condition was slightly modified from Huang et al. (2008) as described above. The optimized method was further validated according to the International Council for Harmonization Guidelines (2023). The validation parameters were composed of precision, %recovery, stability of the standard solution, linearity, and range, the limit of detection (LOD), and the limit of quantitation (LOQ).

Precision

The precision of analytical methods was assessed regarding repeatability and interday precision. Two concentrations at 85.50 and 21.38 µg/mL of standard oxyresveratrol within a linearity range were evaluated. Each concentration was analyzed in six replications on the same day for repeatability and continued with each of the six replications for two consecutive days for interday precision. Standard deviation, pooled standard deviation, and relative standard deviation (RSD) of intraday and interday precisions were calculated.

Percent recovery

Calculation of the percent recovery of a standard oxyresveratrol (equation [1]) was performed in side-by-side diffusion cells. Oxyresveratrol in donors, porcine ear skin, and receptors at 12 hours were analyzed using an HPLC technique. Residual oxyresveratrol in porcine ear skins was rinsed with methanol and water, respectively. Then, the skins were cut into small pieces and vortexed with methanol for 5 min. Subsequently, the samples were extracted using ultrasonication for 1 hour. Finally, the samples were centrifuged at 6,000 rpm for 5 min and filtered through a 0.45 µm syringe filter before oxyresveratrol analysis.

$$\text{Recovery (\%)} = \frac{D + S + R}{y} \times 100 \qquad [1]$$

Where D: oxyresveratrol in donor at 12 hours (mg/mL); S: oxyresveratrol in the skin at 12 hours (mg/mL); R: oxyresveratrol in receptor at 12 hours (mg/mL); y: oxyresveratrol in donor at 0 hour (mg/mL)

Linearity and range

Linearity and range were evaluated over 0.16, 0.08, 0.04, 0.02, and 0.01 mg/mL of oxyresveratrol standard with triplicates of each concentration. As recommended by the International Council for Harmonisation Guidelines (2023), a minimum of five concentrations must be performed. The equation of concentrations versus the peak area of all standard solutions was plotted. The coefficient of determination of the line was also calculated.

Limit of detection and limit of quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) were assessed according to the ICH guideline Q2(R1). There are several methods for determining LOD and LOQ, such as signal-to-noise and standard deviation of response and slope. In this study, LOD and LOQ were calculated according to the standard deviation of response and slope approach (LOD = 3.3σ/S and LOQ = 10σ/S), where σ and S are the standard deviation and slope of the calibration line, respectively.

Statistical analysis

The data were statistically expressed in terms of mean ± standard deviation (SD). The independent t-test was performed to compare the difference between groups with three replications using IBM SPSS Statistics, Version 29 (SPSS, Chicago, IL, USA). The level of significance was set at 0.05.

RESULTS AND DISCUSSION

The yield of plant extract was 14.68% with the amount of oxyresveratrol at 372 mg/g of the extract. The chromatograms of oxyresveratrol in standard solution and extract are shown in Fig. 1. The method validation parameters of HPLC are shown in Table 1. Our proposed method showed a similar relative accuracy, while relative errors were relatively lower than the previous method (Huang et al., 2008). The relative errors were lower than 10%, and the relative accuracy was between 90–110%, which is suitable for further application of the method. For linearity and range, five concentrations of oxyresveratrol (0.1710, 0.0855, 0.0428, 0.0214, and 0.0107 mg/mL) were plotted against peak area. The calibration line showed a good correlation of 0.9994 with the equation [2].

$$y = 28{,}428{,}308.62x - 54{,}043.67 \quad [2]$$

where y: peak area; x: concentration of oxyresveratrol.

LOD and LOQ were calculated according to the standard deviation of response and slope approach, which were found to be 3.38 and 10.24 µg/mL, respectively. This was comparable to a previous study by Huang et al. (2008), the analytical method development using acetonitrile and 0.5% aqueous acetic acid for pharmacokinetics of oxyresveratrol after oral administration of *Smilax china*. The method showed that relative accuracy at concentrations of 1.265, 6.325 and 12.65 µg/mL was about 104.7, 106.4 and 95.3% and relative error 6.70, 4.23 and 9.87%, respectively.

Percent recovery of oxyresveratrol at 12 hours of the permeation study was 88.53 ± 0.71% (n = 3), which was a relatively high %recovery. With regard to the amount of oxyresveratrol in side-by-side diffusion cells, it was mainly in the donor side with approximately 95.26 ± 0.85%, accumulation within the skin at about 4.71 ± 0.01%, and less than 0.01% on the receptor side. In stability testing of oxyresveratrol, the amount remained at approximately 93.11% after being kept at 25°C for 12 hours. The loss of oxyresveratrol during extraction could be from the degradation of the compound under the experimental condition as well as strong binding with skin components. For linearity, our proposed method showed a sufficient correlation of determination at 0.9994, while the analysis of oxyresveratrol using HPTLC and HPLC for *Morus* spp. showed the linearity at 0.999 (Ayinampudi et al., 2011).

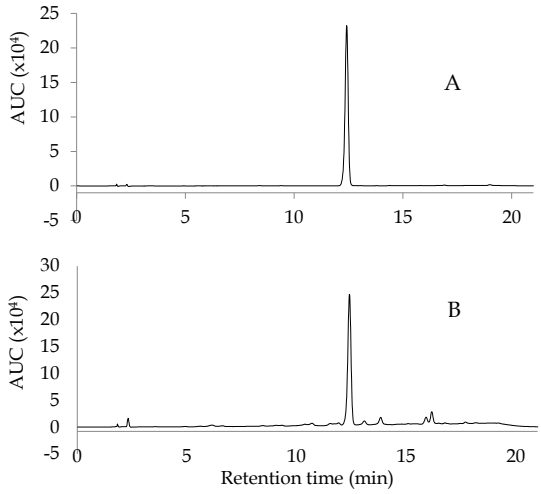


Figure 1. Chromatograms of a standard oxyresveratrol **(A)** and *A. lakoocha* extract **(B)**.

For the skin accumulation study of oxyresveratrol, the standard was added to the donor side at a concentration of 37.55 mg/mL. The porcine ear skins were collected at 0.25, 0.5, 0.75, 3, 6 and 12 hours. Subsequently, the skins were extracted and analyzed with HPLC. The data are shown in Table 2. The result revealed that oxyresveratrol was the most likely accumulated within the skin rather than permeated into the receptor side. Deposition of oxyresveratrol within the skin is a desired site of action, where melanocytes are found in basal cells of the epidermis. The active component oxyresveratrol could inhibit tyrosinase enzymes produced by melanocytes, which resulted in hypopigmentation (Sungthong and Phadungkit, 2015). However, the amount of oxyresveratrol deposited in skins was relatively low, 4.71 ± 0.01%. Increasing permeation of the active component using skin permeation enhancers such as essential oils, terpenoids, surfactants, alcohols, and hygroscopic liquid could be further evaluated (Arda et al., 2024).

Table 1. Method validation parameters of HPLC analysis of oxyresveratrol.

Parameters	Concentration (µg/mL)	Results	
Accuracy	85.50	%relative error	1.53%
		%relative accuracy	98.79%
	21.38	%relative error	5.75%
		%relative accuracy	105.74%
Repeatability, n = 6 (%RSD)	85.50	1.87%	
	21.38	2.83%	
Interday precision, n = 3 (%RSD)	85.50	3.80%	
	21.38	2.89%	
LOD	3.38 µg/mL		
LOQ	10.24 µg/mL		
Linearity (R^2)	0.9994		

Table 2 The amount of oxyresveratrol within porcine ear skins in different sampling times.

Time (hour)	The extract	Standard oxyresveratrol
	Amount of oxyresveratrol (µg/mL)	Amount of oxyresveratrol (µg/mL)
0.25	25.02 ± 3.88	22.41 ± 2.27
0.5	37.61 ± 2.83	30.94 ± 3.09
0.75	47.55 ± 0.99*	27.57 ± 3.16*
3	105.32 ± 20.87	84.19 ± 6.91
6	169.07 ± 26.07	138.85 ± 37.31
12	202.06 ± 4.68	255.38 ± 80.64

Data are expressed as mean ± SD (n = 3). *p<0.05 statistically significant difference.

Kongkwamcharoen et al. (2023) investigated hydrogel patches containing *Crinum asiaticum* L. extract on skin permeation studies. The results revealed that the patch containing glycerin as a penetration enhancer can improve the permeation of the bioactive compound of lycorine. In another study, the permeation rate of lidocaine HCl and ketorolac were significantly increased in the presence of organic alcohol, hexanol, due to the alteration of skin integrity (Assaf et al., 2021).

CONCLUSION

The proposed method can be applied to analyze oxyresveratrol in a porcine ear skin permeation study. For further experimentation, skin permeation enhancers could be incorporated to improve the efflux of oxyresveratrol into the skin or increase the skin's accumulation of oxyresveratrol.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This research project was financially supported by Mahasarakham University with grant number 6517017.

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AUTHOR CONTRIBUTION:

Contribution	Mekjaruskul C	Caichompoo W	Tuntiyasawasdikul S	Long S	Sungthong B
Concepts or ideas	x		x		x
Design	x		x		x
Definition of intellectual content	x	x			x
Literature search	x				x
Experimental studies	x	x	x		x
Data acquisition	x	x	x	x	x
Data analysis	x		x	x	x
Statistical analysis	x				x
Manuscript preparation	x				
Manuscript editing	x	x	x	x	x
Manuscript review	x	x	x	x	x

Citation Format: Mekjaruskul C, Caichompoo W, Tuntiyasawasdikul S, Long S, Sungthong B (2025) HPLC method validation for porcine ear permeability study of oxyresveratrol from *Artocarpus lakoocha* Roxb. heartwood extract. J Pharm Pharmacogn Res 13(4): 1038–1044. https://doi.org/10.56499/jppres24.2233_13.4.1038

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