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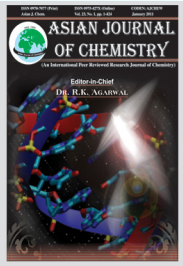

High Performance Thin-Layer Chromatography Method for Quantification of Betulinic Acid in Extracts of Leaves of *Orthosiphon stamineus* Benth.

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The standardization of herbal products is challenging due to unavailability or inadequacy of analytical methods and standards. Therefore, the present study aimed to develop a single step high performance thin-layer chromatography (HPTLC) method for quantification of betulinic acid in extracts of *Orthosiphon stamineus* (OS). Samples having minimum interfering species and higher content of betulinic acid were prepared by chromatography of the extract over silica gel with hexane and ethyl acetate (6:4 v/v). The quantification of betulinic acid. Lowest limit of detection (LOD) and lowest limit of quantification (LOQ) were found to be 0.02 and 0.20 µg, respectively. The method had shown linearity in a range of 0.2-500 µg with correlation coefficient 0.9979. Recovery and accuracy (intra and inter day) values were found to be 96.12-97.65, 97.34-98.84 and 97.16-98.37 %, respectively, with relative standard deviation (RSD) less than 5. Results of this study indicate that the method is sensitive, accurate and precise.

Key Words: HPTLC, *Orthosiphon stamineus*, Betulinic acid, Standardization.

INTRODUCTION

The standardization of herbal products, the biggest hindrance in their wider acceptance in modern healthcare, is always challenging due their complexity and inadequacy or unavailability of standards and analytical methods. The inadequacy of analytical methods is also preventing modernization of herbal manufacturing process because there is no way to establish bioequivalence between formulations prepared by modified or new method and the original method. Therefore, it is important to develop analytical methods that can be used for standardization of herbal products and their batch-batch reproducibility. This task can be achieved by developing analytical methods employing new analytical techniques using marker compounds of different categories[1,2]. Keeping it in view, the present study is undertaken to develop an analytical method to standardize extracts of an important medicinal plant, *Orthosiphon stamineus* Benth. (*Lamiaceae*).

The plant is being used extensively as a traditional medicine to treat various ailments in many countries[3,4]. The plant has also been investigated for a number of pharmacological activities and based on such activities use of leaves of the plant as nutraceuticals and tea is getting popular in many countries of the world. Main phyotochemicals of the plant are flavonoids, triterpenes and saponins. Among triterpenes, betulinic acid has a number of pharmacological activities such as antiretroviral, antimalarial, antiinflammatory, antitumor, antimicrobial, hepatoprotective, antiulcer, hypoglycemic, antihyperlipidemic and antimycobacterial[5-12].

In the literature, most of the methods for the quantification of betulinic acid and related triterpenes are high performance liquid chromatography (HPLC) based[13-17]. Cheng *et al.*[18] have reported a liquid chromatography-mass spectrometry method for the quantification of betulinic acid in plasma of mouse, rat and dog[18]. Akowuah *et al.*[19] and Akowuah and Zhari[20] have also reported HPLC methods for the quantification of betulinic acid in extracts of *Orthosiphon stamineus*. In these HPLC methods, detection of betulinic acid has been carried out at 210 nm. Most of the solvents absorb at this short wavelength, which may cause interference and instrument usually takes more time to stabilize the baseline. Despite the fact that HPLC is a sensitive technique with higher resolving power, thin-layer chromatography offers several advantages such as use of lesser amount of solvents, visual inspection and application of many samples and standards on a single plate for better comparison. Wojciak-Kosior[21] has reported a HPTLC method for separation of betulinic acid and other triterpenes using multiple gradient technique employing four mobile phases. Betulinic acid is present in minute quantities in *Orthosiphon stamineus* and its content is further reduced in aqueous extracts. The present study is undertaken to develop a single step method for the quantification of betulinic acid in extracts of *Orthosiphon stamineus*.

EXPERIMENTAL

Aqueous extract of *Orthosiphon stamineus* having code NHSIDE 06 was obtained from Herbal Secretariat, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. Analytical grade chemicals procured from Merck were formic acid, ethyl acetate, hexane, silica gel and silica gel 60 F_{254} plates of different sizes. Betulinic acid having more than 90 % purity was purchased from Sigma Aldrich. The analysis was performed on HPTLC system of CAMAG (Berlin, Germany) comprising of densitometer (CAMAG Model-3 TLC scanner) equipped with winCATS 4 software, semi-automatic sampler (Linomat-5) and image recorder (CAMAG PROSTER 3).

The standard stock solution of betulinic acid was prepared in methanol to a concentration of 1.00 mg/mL. A series of working standard solutions were prepared by diluting the stock solution with methanol to a concentration range of 0.20-500.00 µg/mL.

Validation of the method: The method was validated before its application to standardize extracts of *Orthosiphon stamineus*. The lowest limit of detection (LOD) was determined at signal to noise ratio (S/N) of 3:1 by application of a series of 2-fold dilutions of the standard solutions, whereas the lowest limit of quantification (LOQ) was taken at S/N of 10:1. Linearity of the method was evaluated over the whole range of investigated standard solutions.

Three concentrations of betulinic acid 30, 60 and 100 µg/mL were used to evaluate recovery, intraday and inter-day accuracy and precision. For recovery, 200 mg of the extract was spiked with the standard solutions and treated in the same way as to the preparation of samples of the extract. The spiked samples were compared with samples without spike to calculate percentage recovery of true value, spiked concentration. Three concentrations of the standard were applied on TLC plate and the plate was developed and scanned at 366 nm six times in a single day for intraday accuracy and precision and once daily for 5 consecutive days for inter-day accuracy and precision. Accuracy was evaluated by quantifying the applied concentration from calibration curve of concentration versus peak area or height and precision was evaluated by relative standard deviation (RSD) among the determined values.

Preparation of sample: 1 g of the extract was charged on silica gel column (2.5 cm × 50 cm) and eluted with 50 mL of mobile phase comprising of hexane:ethyl acetate (6:4 v/v). Elute was evaporated *in vacuo* at 40 ºC and the residue was used to prepare stock solution having concentration of 50.00 mg/mL. Working sample solution of concentration of 5.00 mg/mL was prepared by diluting the stock solution with methanol.

Application of samples and standards: The samples and the standards were applied band-wise on pre-coated TLC plate (60 F_{254}, 20 cm × 10 cm) as: 19 tracks, application volume 4 µL, 8 mm from lower edge, 15 mm on either side and 9.4 mm distance between the bands. The standard solution was applied in volume of 2, 4, 8 and 20 µL per application that was equivalent to 0.10, 0.20, 0.40 and 1.00 µg, respectively, whilst a known quantity of the standard solution (0.65 µg) was applied as a control. Except the control all the applications were in duplicate.

Chromatography and documentation: The plates was developed in saturated horizontal DS Teflon chamber with solvent system comprising of hexane, ethyl acetate and formic acid in a ratio of 3:2:0.02 v/v. The plate was allowed to develop to a distance of 5 cm from the lower edge. The plate was dried with gentle stream of warm air, sprayed with anisaldehyde reagent and kept in oven at 100 ºC for 10 min. Afterwards the plate was scanned at 366 nm and the quantification of betulinic acid was carried out by winCATS software using linear regression. Spots corresponding to peaks of the sample and the standards were assigned and scanned in a range of 400-200 nm for peak purity. Finally, images of the plate were taken at 254 and 366 nm for documentation.

Statistical analysis: All the samples and the standards were analyzed in duplicate and the results were presented as mean ± SD.

RESULTS AND DISCUSSION

LOD was found to be 0.02 µg at (S/N) 3:1, whereas LOQ was taken to be 0.20 µg at S/N 10:1. LOQ value was confirmed by analyzing the concentration 6 times. Linearity of the method, evaluated by analyzing a series of standard solutions and plotting calibration curves between concentration and peak area/peak height, was found to be in a range of 0.200-500.00 µg/mL with $R^2 = 0.9976$.

The results of recovery, accuracy and precision presented in Table-1 indicated these values in a range of 90.97-97.69 % with RSD less than 5 %. It is clear from these results that the method is accurate and the accuracy was not compromised in intraday and inter-day analysis.

The validated method was applied successfully to quantify betulinic acid in aqueous extract of *Orthosiphon stamineus*. For quantification of betulinic acid in the extracts, calibration curve was constructed between concentration and peak area. The quantification was carried out using linear regression equation, Y = 246.223 + 4.891X having correlation coefficient (R^2) 0.99801 and standard deviation (SD) ± 5.76. Recovery of the control was found to be 95.98 %. The content of betulinic acid in the extract was found to be 0.043 ± 0.0028 mg/g extract.

TABLE-1
RECOVERY, INTRA-DAY AND INTER DAY ACCURACY AND PRECISION VALUES OF BETULINIC ACID BY HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC)

Concentration (µg/mL)	Recovery (n = 3)		Intra-day (n = 6)		Inter-day (n = 6)	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
30	96.12	3.75	97.34	4.23	98.28	3.75
60	97.35	3.49	98.17	3.74	97.16	4.25
100	97.65	3.63	98.84	3.91	98.37	3.78

RSD: Relative standard deviation.

3D-Densitogram of the extract along with the standards, spectra of the standard and the sample and image of the plate presented in Fig. 1 indicated the resolution of betulinic acid and peak purity. The extraction method for the preparation of the sample having higher content of the analyte and lesser interfering species proved to be efficient. This extraction had resulted in quantification of betulinic acid in the sample that could be seen from image of the plate.

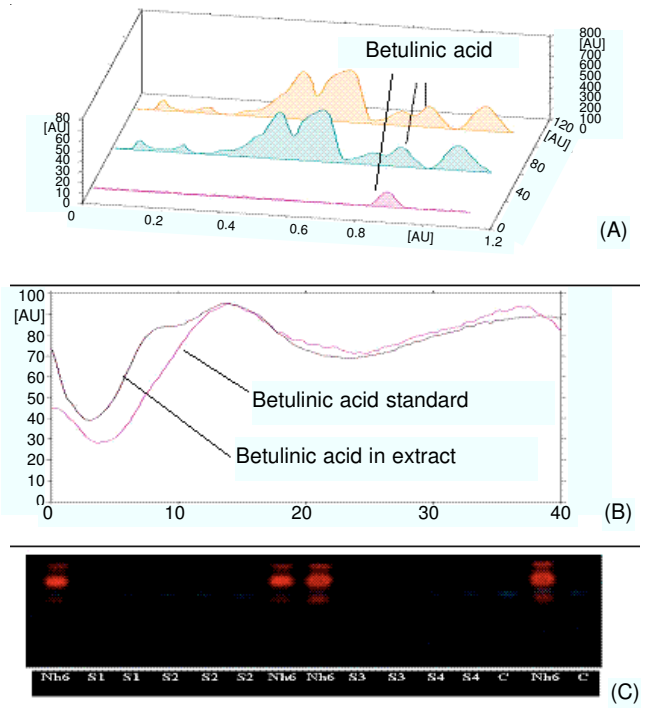

Fig. 1. Densitogram, spetra and image of HPTLC plate showing different concentrations of betulinic acid as a standard and extract of *Orthosiphon stamineus*, (A) 3D denditogram; (B) spectra; (C) image of the plate at 366 nm; in image S1-4 (concentrations of standard) and NH6 (extract)

Robustness of the method was also evaluated by slight variations in mobile phase, scanning wavelengths and development distance of the plate. It was found that these slight variations did not affect efficiency of the method.

As a traditional medicine aqueous extracts of leaves of the plant are being used, therefore, in the present study aqueous extracts have been selected to quantify their betulinic acid content. In any analytical process, preparation of sample is the most important step, particularly, if analyte is in a minute quantity and is further associated with interfering constituents. In the present experiment, betulinic acid, a pentacyclic triterpenoid, is found in association with other structurally related triterpenes such as ursolic acid, oleanolic acid, α-amyrin and β-amyrin *etc*., which make its separation difficult. Additionally, the presence of such compounds in minute quantities needs a highly sensitive method for their separation and detection on TLC plate. Moreover, betulinic acid is not UV active therefore, needs to be derivitized for detection in UV/vis region.

In the present study, a single step method of preparation of samples containing betulinic acid is used in higher concentration that is quantifiable by TLC. Additionally, the method makes it possible to minimize polar contaminants that are present in the sample. This sample on TLC has given optimum separation of betulinic acid from other triterpenes in a single step plate development instead of using multiple gradient mobile phases as reported by Wojciak-Kosior[21].

Various types of solvent systems were used to achieve optimum separation of the marker from the extract. The solvent system comprising of hexane:ethyl acetate:formic acid (5:2:0.02 v/v) had given optimum separation of betulinic acid with symmetrical and reproducible peaks.

The plate was developed in horizontal and vertical chambers and was found that the development in horizontal chamber gave better separation. Hence, horizontal chamber was used in this experiment. The plate was positioned in the chamber with the stationary phase facing inside and chamber was allowed to saturate for 10 min before starting the development. The solvent was allowed to migrate to a distance of 5 cm from the lower edge. Band-wise application was found to be better as compared to spot wise application. The plates were developed to a distance of 5 cm because the development of the plate for a distance of more than 5 cm was found to be affecting linearity of the method. Therefore, optimum distance for the development of the plate was kept at 5 cm.

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