

Rapid separation and determination of betulinic acid from a complex matrix using combination of TLC and RP-HPLC

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Abstract: Hitherto, only a few studies are reported about using the combination of TLC and RP-HPLC for the separation and determination of analyte(s) from a complex matrix. The present study is aimed to develop a simple and rapid method for the separation and determination of betulinic acid from a complex matrix, extracts of *Orthosiphon stamineus*, using a combination of the two techniques. The samples having higher contents of the analyte and fewer interfering species were prepared using TLC. The samples were then eluted through C₁₈ column using isocratic solvent system comprising acetonitrile, methanol and acetic acid acidified water of pH 2.8 in a ratio of 70 : 20 : 10 (v/v/v), respectively, and detection was carried out at 210 nm. The method was validated and applied successfully to quantify betulinic acid in various types of extracts of the plant. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.0005 and 0.0050 µg/ml, respectively. The method exhibited linearity in a concentration range of 0.005-100.00 µg/ml ($R^2 = 0.9999$). The recovery was found to be 97.10 - 97.60 % (RSD < 5%), whereas, intra-day and inter-days accuracy values were 97.13 - 98.67% (RSD < 5%) and 96.45 - 98.00% (RSD < 5%), respectively. The results of the present study indicate that the developed method is simple, rapid, sensitive and accurate, and may be of a value to natural product industry and researchers for the standardization of extracts containing betulinic acid in a lesser time and consuming fewer solvents.

Keywords: Betulinic acid; HPLC, *Orthosiphon stamineus*, UV detection.

INTRODUCTION

Herbal products or phytomedicines can be standardized using marker compounds of various categories as analytical standards by means of modern analytical tools. Among these categories, pharmacological markers - compounds having certain biological properties – can be used to maintain batch-batch constancy in efficacy of herbal products. Betulinic acid, a triterpenoid possessing a number of pharmacological activities, is found in many plants, hence can be used as an analytical marker for their standardization (Cantrell *et al.*, 2001; Chowdhury *et al.*, 2002; Baglin *et al.*, 2003; Okunade *et al.*, 2004; Liu, 2005; Billo *et al.*, 2005; Tanachatchairatana *et al.*, 2008). *Orthosiphon stamineus* Benth. (Family: *Lamiaceae*), a traditional medicinal plant of tropical and sub-tropical region, having therapeutic as well as commercial importance also contains betulinic acid (Bwin, 1967; Goh *et al.*, 1994; Nirdnoy and Muangman, 1991; Zasshi *et al.*, 1999; Sriplang *et al.*, 2007; Yam *et al.*, 2007). Based on pharmacological properties, aerial parts of the plant are being used to prepare various dosage forms and tea (*Miasi kushing Tea* and *Java Tea*). All such products can be standardized by determining and maintaining their betulinic acid contents.

Despite proven benefits and substantial growth in the past few years, herbal products are unable to get wider acceptance both in the main stream of pharmaceuticals and modern healthcare system due to lack of standardization. And it is always challenging due to complex and un-

known nature of plant materials, and inadequacy or unavailability of standards and methods of analysis. Bearing in mind the importance of analytical methods for the standardization of herbal products, the present study is undertaken to develop a method for the separation and determination of betulinic acid from a complex matrix in a short time accurately.

A variety of methods are reported for the quantification of betulinic acid as well as structurally related triterpenes. Such methods include gas chromatography (Perez-Camino and Cert, 1999; Takeoka *et al.*, 2000; Janicsak *et al.*, 2003), capillary zone electrophoresis (Guo *et al.*, 2005; Yang *et al.*, 2007), miscellar electrokinetic chromatography (Liu *et al.*, 2003; Du *et al.*, 2009), liquid chromatography-mass spectrometry (Cheng *et al.*, 2003), high performance thin-layer chromatography (Wojciak-Kosior, 2007; Hussain *et al.*, 2011) and HPLC (Cen and Xia, 2003; Claude *et al.*, 2004; Liao *et al.*, 2005; Zhao *et al.*, 2006; Monte *et al.*, 1997). Akowuah *et al.* (2003) and Akowuah and Zhari (2008) reported two HPLC methods for the quantification of betulinic acid in extracts of *Orthosiphon stamineus*, whereby elution was carried out using mobile phase comprising acetonitrile and phosphate buffer of pH 2.8 (80 : 20 v/v) and detection was carried out at 210 nm. It was observed that during detection at this low wavelength many constituents of the extracts showed absorption, hence, resulted in complex chromatograms. In our experiments - particularly analysis of aqueous extracts - less retained compounds i.e. polar ones necessitated the retention of betulinic acid fairly distant

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from the solvent front, which often resulted in peak broadening. The presence of betulinic in minute quantity and its association with other structurally related compounds further demanded the preparation of samples having its higher contents and fewer interfering species. To achieve such goals, various methods can be applied, but we have selected TLC and solvent extraction. Hence, the present study aimed to develop a rapid and accurate method for the separation and determination of betulinic acid in various types of extracts of *Orthosiphon stamineus* using a combination of two chromatographic techniques, TLC - to prepare samples- and HPLC- to quantify the analyte.

MATERIALS AND METHODS

Plant material

Various types of extracts of the plant such as ethanol extract (*UiTM-1*), 50% acetone extract (*UiTM-2*) and three aqueous extracts having code numbers *NHSIDE-5*, *NHSIDE-6* and *HVM-7* were obtained from the Pilot Plant, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. The codes of the extracts referred to the name of organization whereby such extracts were subjected to spray drying e.g. *UiTM* (Universiti Teknologi Mara, Malaysia), *NHSIDE* (Nusantara Technologies Sdn. Bhd. Malaysia) and *HVM* (Hovid Sdn. Bhd. Malaysia). The dried aerial parts of the plant were macerated with all the stated solvents for 24 h at room temperature with occasional agitation. Then the extracts were filtered and got spray dried.

Materials

Analytical/HPLC grade chemicals/materials procured from Merck were formic acid, ethyl acetate, hexane, methanol, acetonitrile, acetic acid, phosphoric acid and TLC plates of 20 × 10 cm and 10 × 5 cm (silica gel 60F₂₅₄, 0.50 mm). Different types of columns procured from Agilent Technologies included LiChrosorb (250 × 4 mm), LiChrospher (250 × 4 mm), Nucleosil (250 × 4 mm) and LiChrosorb (120 × 4 mm). Betulinic acid of more than 90% purity was purchased from Sigma Aldrich. In-house prepared ultra pure water was used.

Instrumentation (HPTLC)

The samples were prepared using HPTLC system of CAMAG (Berlin, Germany) equipped with semi-automatic sampler (Linomat-5), densitometer (Model-3 TLC scanner) and image recorder (CAMAG PROSTER 3). The system was operated using software, winCATS-4. The plates were developed in horizontal DS Teflon chamber (CAMAG).

HPTLC conditions

The samples were applied on TLC plate using semi-automatic sampler with the help of nitrogen gas. The plate was developed in horizontal chamber to a distance of 5 cm, dried with a stream of hot air and sprayed with anisaldehyde reagent. Then it was kept in an oven at 100°C

to facilitate derivative formation to be viewed and subsequent documentation at 366 nm.

Instrumentation (HPLC system)

The samples were analyzed using HPLC system, 1100 series (Agilent Technologies, Waldronn, Germany) equipped with degasser (G1379 A), quaternary pump (G1311 A), auto-sampler (G1313 A), column oven (G1316 A) and UV detector (G 1314 A).

HPLC conditions

The samples were eluted through reversed phase C₁₈ column (Hibar Rt 250 X 4 mm i.d., LiChrosorb RP-18, 10 µm) using an isocratic mobile phase comprising acetonitrile, methanol, acetic acid acidified water of pH 2.8 (70 : 20 : 10 v/v/v). The flow rate was maintained at 1.00 ml/min and temperature of the column was kept at 25°C. The samples were injected in a volume of 15 µl and detection was carried out at 210 nm. The data acquisition was performed by chemStation version A.08.03.

Preparation of sample solutions

The solvent system comprising methanol and water in a ratio of 1 : 1 v/v was used to prepare stock solutions of the extracts because such solvent system had given optimum solubility. The stock solution of each of the extracts was prepared by dissolving 500 mg of extract in 5 ml of the solvent system.

The samples and the standards were applied band-wise on pre-coated TLC plate (10 × 5 cm) as: 4 tracks (3 standards and 1 sample), application volume 4 µl, band length 6 mm, 8 mm both from lower edge, 10 mm from each side, and distance between the bands 3 mm. The plate was developed in saturated horizontal DS Teflon chamber as described by Hussain *et al.* (2011) using mobile phase comprising hexane, ethyl acetate and formic acid in a ratio of 3 : 2 : 0.02 (v/v/v). The plate was developed to a distance of 6 cm from the lower edge, which was then dried with gentle stream of warm air, sprayed with anisaldehyde reagent and kept in oven at 100°C for 10 min. Afterwards, the plate was viewed in UV chamber under 366 nm for the calculation of R_f value.

Afterward, the stock solution of each of the extract was separately applied band-wise on a silica gel TLC plate (20 × 10 cm) as: application volume (100 µl), distance from the lower edge 8 mm, leaving 15 mm on either side of the plate and band length 17 cm. Then the plates were developed to a distance of 6 cm in a saturated horizontal chamber with a solvent system comprising hexane, ethyl acetate and formic acid (3.00 : 2.00: 0.02 v/v/v) (Hussain *et al.*, 2011). The plate was then dried under stream of air and a region of the plate from 4-5 cm - corresponding to R_f value of betulinic acid (0.75) - from the lower edge was scratched and the material suspended in 10 ml of acetonitrile by sonication. The suspension was filtered and the residue was again suspended in 10 of acetonitrile

and filtered. Both the filtrates were dried in vacuo at 40°C, and the residue was reconstituted with 100 µl of mobile phase, used for HPLC analysis.

Preparation of standard solutions

The stock solution of betulinic acid was prepared in acetonitrile because of its sparing solubility in methanol to a concentration of 1 mg/ml. Then a series of working standard solutions of concentration 0.005-100.00 µg/ml were prepared by diluting the stock solution with mobile phase, used for elution.

Determination of LOD, LOQ and linearity

The LOD was determined at a signal/noise (S/N) ratio 3 : 1 by injecting the standard solution after successive two fold dilutions with the mobile phase, whilst LOQ was taken at S/N ratio 10 : 1, which was then established by analyzing the analyte 6 times at LOQ. The standard solutions of different concentration range 0.005-100.00 µg/ml were used to evaluate linearity of the method which was accomplished by plotting concentration versus peak area/height.

Validation of HPLC method

Three concentrations of the standard solution, 0.05, 0.25 and 2.50 µg/ml, were used to determine recovery, intra-day and inter-days accuracy and precision of the method. Each of the standard solutions was analyzed in triplicate for recovery (6 times in a single day for intraday accuracy and precision) and once every day for 6 consecutive days for inter-days accuracy and precision. The quantification of these standards was carried out from 5 data point calibration curves that were constructed on each day of the analysis.

To assess extraction recovery, the extracts were spiked separately with three concentrations (0.05, 0.25 and 2.50 µg/ml) of the standard solution. The spiked samples were analyzed and results were compared to that of un-spiked extract that served as a control. The extraction recovery values of such standards were calculated as a percentage of the true value (spiked amount) to that of the values obtained after analysis.

Analysis of samples

Each of the samples was analyzed at chromatographic conditions mentioned above, and the quantification of betulinic acid was carried out from the calibration curve using linear regression.

STATISTICAL ANALYSIS

For recovery studies each of the samples was analyzed in triplicate and results were presented as a mean \pm standard deviation (SD). For intraday and inter days accuracy and precision samples were analyzed 6 times on the same day and once daily for 6 consecutive days, respectively, and

the results were mentioned as mean \pm SD. Each of the extracts was analyzed in triplicate and the results were presented as a mean \pm SD.

RESULTS

The various types of extracts of the plant were analyzed at chromatographic conditions described by Akowuah *et al.* (2003) using mobile phase comprising acetonitrile and phosphoric acid acidified water (80 : 20 v/v). The resulting chromatograms of the standard, ethanolic extract and aqueous extract (*NHSIDE-6*) given in figs. 1a, 1b and 1c exhibited the retention time of betulinic acid (11.984 min). The absence of peak at 11.984 min in ethanolic extract indicated either the absence of betulinic acid or the contents were too low, whilst *NHSIDE-6* extract showed the peak which was quite above the baseline. Hence, the results of figs 1a and 1b showed the suitability of the method for the standard and ethanolic extract, whereas, chromatogram of the *NHSIDE-6* (fig. 1c) indicated the unsuitability of the method for the quantification of betulinic acid in aqueous extracts. This problem was assigned to two main factors such as the peaks of polar compounds near the solvent front and minute contents of betulinic acid. Additionally, pressure of the pump was observed to be quite high due to resistance to flow of mobile phase in the column. Some modifications were made including changes in column temperature (20°C, 25°C, 30°C and 35°C) and pH of mobile phase (1.8, 2.0, 2.5, 3.0, 4 and 4.5), but all such attempts proved fruitless. Moreover, pH of mobile phase below 2 was found to be effecting the stabilization of the base line at 210 nm. Last but not least, the use three types of columns such as LiCrosorb (250 \times 4 mm), LiChrospher (250 \times 4 mm), Nucleosil (250 \times 4 mm) and LiChrosorb (120 \times 4 mm) was also remained fruitless in achieving the desired outcome. Finally, two activities were planned- increase the contents of betulinic acid and reduce interfering species by partitioning, and change in mobile phase composition- to improve specificity, efficiency and peak symmetry and reduce retention time (t_R).

To proceed with, *NHSIDE-6* (10 g) was suspended in distilled water (100 ml) and partitioned with hexane (100 ml) and chloroform (100 ml) separately, the procedure was repeated thrice. Both, hexane fraction and chloroform fraction were dried in vacuo at 40°C and the residues were reconstituted with 0.50 ml of mobile phase. These samples were eluted with mobile phase comprising acetonitrile, methanol and phosphoric acid acidified water of pH 2.8 (75 : 5 : 20 v/v/v). The use of this mobile phase resulted in reduction both of pressure of the pump and t_R of betulinic acid (9.901 min). The chromatograms of the standard, *NHSIDE-6* and its chloroform fraction and hexane fraction are given in figs. 2a, 2b, 2c and 2d respectively. The results shown in figures 2c and 2d indicated that peaks corresponding to the standard were quite higher than that of extract (*NHSIDE-6*), fig. 2b. Hence, such modification in mobile phase and partitioning had im-

proved the situation encountered at the beginning. Further modifications in the mobile phase by successive increments in the proportion of methanol resulted in reduction of t_R of the standard. Finally, using mobile phase comprising acetonitrile, methanol and phosphoric acid acidified water of pH 2.8 (70 : 20 : 10 v/v/v), t_R of betulinic acid was found to be 6.558 min, which was significantly lesser than 11.30 min as stated by Akowuah *et al.* (2003). In addition to decrease in t_R , the mobile phase had resulted in the reduction of pressure of the pump that would be certainly needed to enhance the life of HPLC system.

Since, phosphoric acidified water required the washing of the column for a long-time, acetic acid was used to pre-

pare acidified water of pH 2.8 for the mobile phase. It was worth noting that acidified water (pH 2.8) prepared by both the acids had shown comparable results. Hence, in the present study, we have preferred acetic acid because it did not precipitate in pump, column and tubing, and needed lesser solvent and time for flushing the instrument. Furthermore, the use of acetic acid might be advantageous in liquid chromatography-mass spectrometry (LC-MS) where phosphates were troublesome. Therefore, for further analysis, a mobile phase comprising acetonitrile, methanol and acetic acid acidified water of pH 2.8 (70 : 20 : 10) was used.

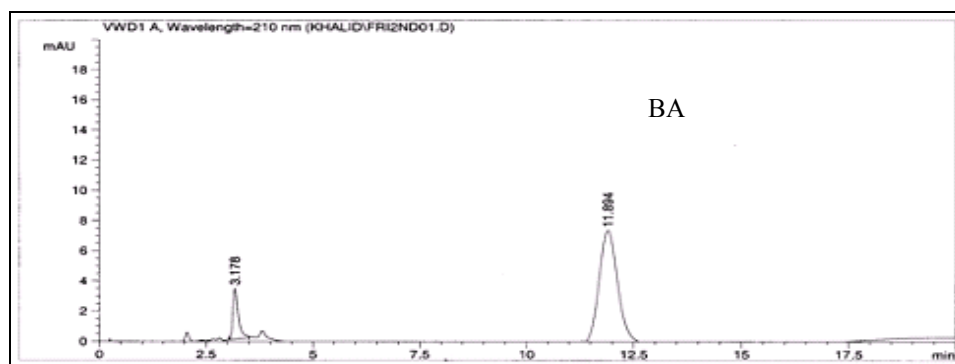


Fig. 1a: Chromatogram of betulinic acid (BA) at 210 nm.

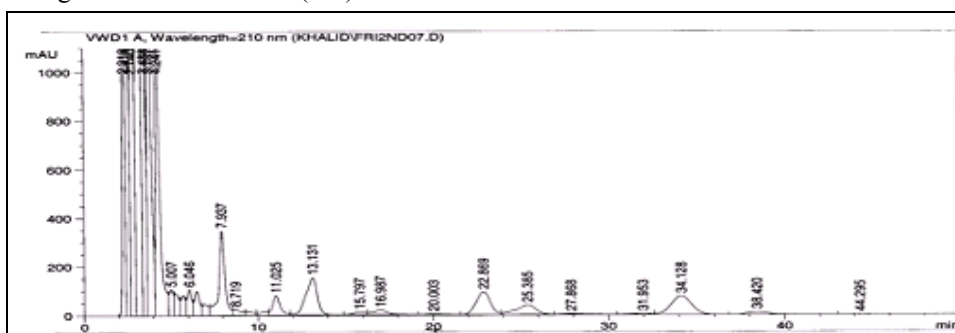


Fig. 1b: Chromatograms of ethanol extract of *Orthosiphon stamineus* at 210 nm, BA (Betulinic acid).

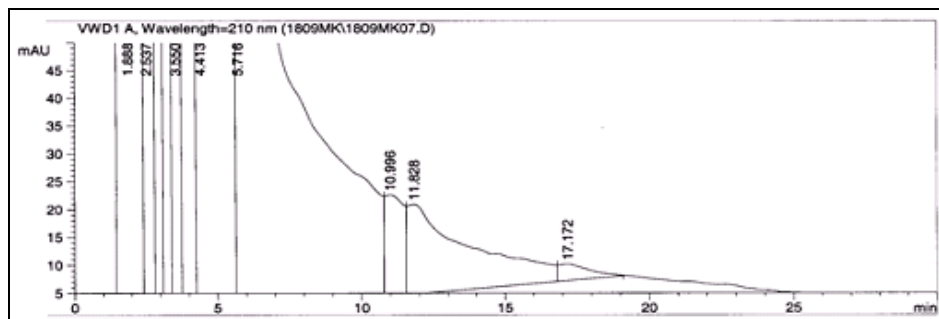


Fig. 1c: Chromatograms of aqueous extract of *Orthosiphon stamineus* at 210 nm, HVM-7 (Code number assigned to the aqueous extract): BA (Betulinic acid)

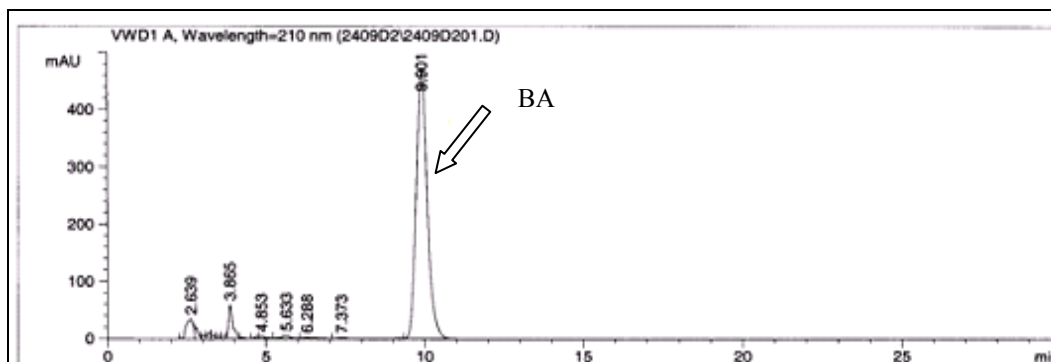
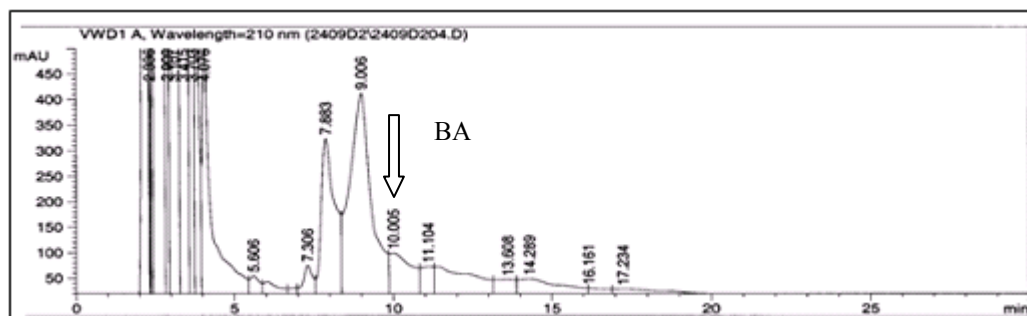


Fig. 2a: Chromatograms of Betulinic acid (BA at 210 nm



having both betulinic acid and *NHSIDE 6* extract is shown in fig. 3. This plate was used to calculate R_f value of betulinic acid which was found to be 0.75. This R_f was then used to prepare samples of the extracts.

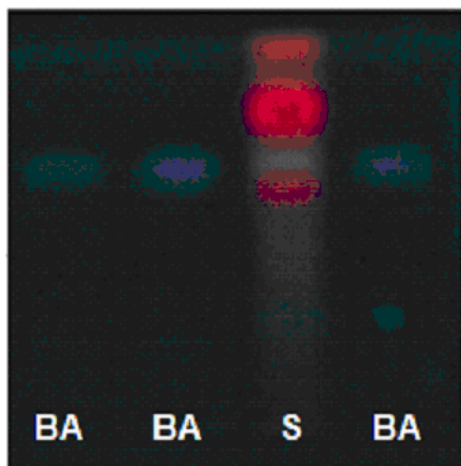


Fig. 3: Image of thin layer chromatography plate at 366 nm. S (sample *NHSIDE-6*); BA (betulinic acid)

The method was then evaluated for linearity, LOD, LOQ, accuracy and precision to find the repeatability and reproducibility. The results of validation of the method presented in table 1 indicated that the method was accurate, precise, repeatable and reproducible. The method was found to be linear over the whole investigated range. Furthermore, the method was found to be robust because slight changes in proportions of components of the mobile phase, pH of the acidified water, temperature of the column had not shown much variation in the retention time (t_R) and peak symmetry.

The validated method was then applied for the quantification of betulinic acid in samples of various types of extracts of *Orthosiphon stamineus* prepared by TLC. The chromatograms of samples prepared from various extracts using TLC and the standard are shown in figs. 4a and 4b. The results of these figs. indicated that the peaks were quite good with t_R of 6.558 min. The contents of betulinic

acid in different types of extracts of the plant are presented in table 2. The results of this table showed that the extracts contained betulinic acid in varying amounts.

DISCUSSION

The preparation of samples is the most important step in any analysis. For better detection, analyte must be away from interfering species and in a quantity within sensitivity range of a detector, being used. Such goals can be achieved by analyzing higher amount of sample or decreasing the interfering species by selective solvent extraction, solid phase extraction and TLC. The instrument used for solid phase extraction is expensive and not available in less equipped laboratories. The use of bigger samples in HPLC analysis is also not recommended. However, selective solvent extraction by partitioning and TLC can be performed in almost all laboratory settings therefore, we have used these techniques in the present study.

It is lucid from results of the present study that both the techniques-partitioning and TLC- are good to increase the contents of the analyte and reduce interfering species. However, among both the techniques, TLC is not only less tedious but requires lesser sample, solvent and time for removing interfering substance and increasing the contents of analyte.

The mobile phase comprising acetonitrile, methanol and acetic acid acidified water of pH 2.8 (70 : 20 : 10 v/v/v) resulted in the reduction of pressure of the pump, which was looked-for enhancing the life of the HPLC system. It was further noted that betulinic acid can be quantified using any of the columns used in the present study. The results proved that the preparation of samples for HPLC analysis using TLC had increased the specificity of the method. It is apparent from the chromatograms that by using the combination of TLC and HPLC, analysis of complex extract can be accomplished in lesser time and using less solvent. The reduction of impurities in the sample can enhance the life of the column. In addition to the tubing, pump and column can be flushed in lesser time as

Table 1: Recovery, intra-day accuracy and precision, inter-day accuracy and precision and linearity of the HPLC method using betulinic acid as a standard (detection at 210 nm)

Concentration $\mu\text{g/ml}$	Recovery (n = 3)		Intraday (n = 6)		Inter day (n = 6)	
	Mean	RSD	Accuracy	Precision	Accuracy	Precision
	%	%	%	RSD %	%	RSD %
0.05	97.10	1.73	97.27	1.14	97.47	0.32
0.25	97.12	1.50	97.13	1.43	96.45	1.15
2.50	97.67	0.91	98.67	0.68	98.00	0.68
Standard	Linear regression equation		R^2	Linear range ($\mu\text{g/ml}$)	LOD ($\mu\text{g ml}$)	LOQ ($\mu\text{g /ml}$)
Betulinic acid	$Y=896.79X+11.181$		0.9999	0.005-100	0.0005	0.005

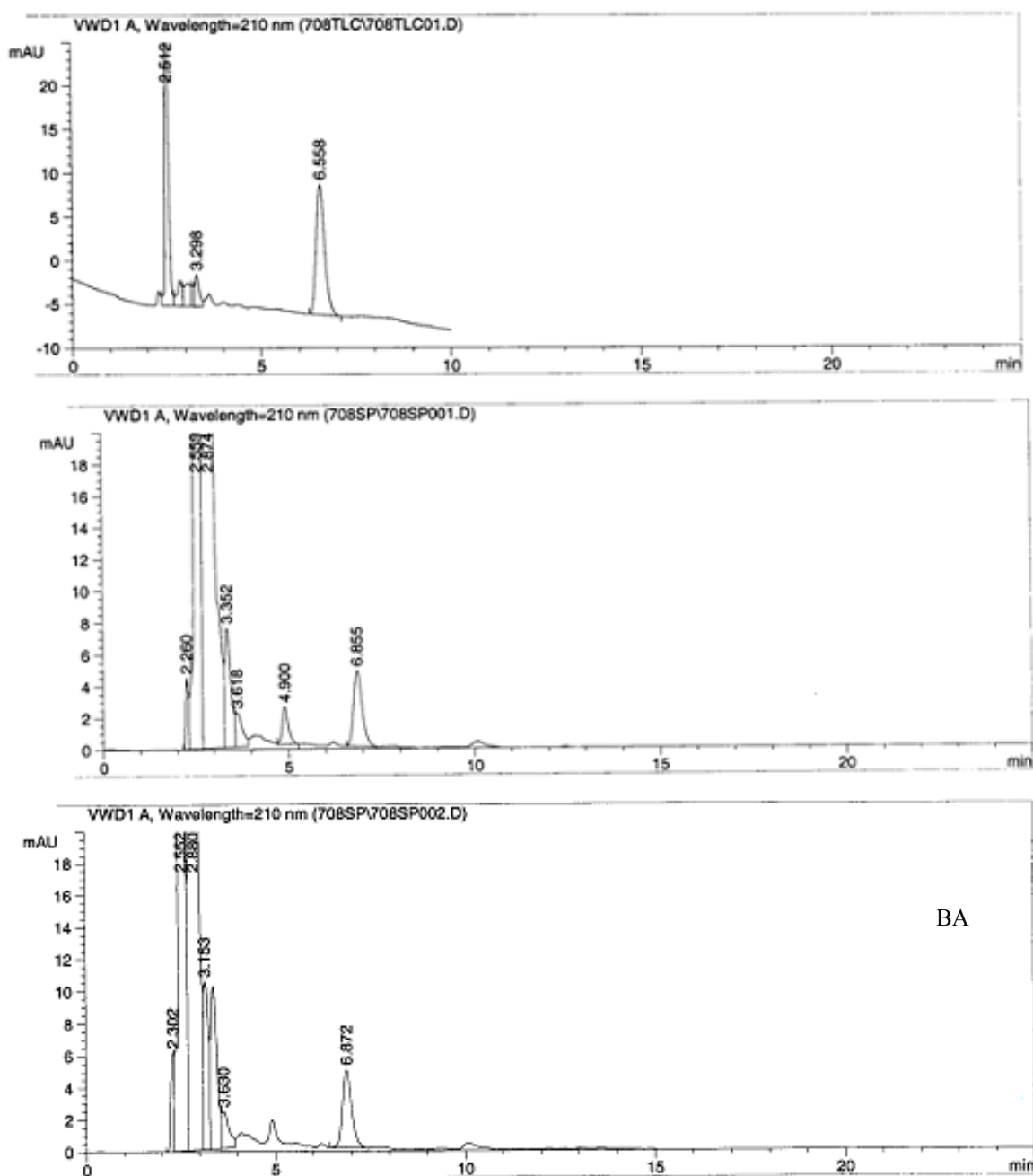


Fig. 4a: Chromatograms of betulinic acid (BA) and samples of extracts of *Orthosiphon stamineus* prepared by thin layer chromatography

compared to that following the analysis of samples prepared directly from extracts. The time and solvents required for flushing the HPLC system is important particularly in case of plant extracts because of their complexity.

CONCLUSION

It is concluded from the results of the present study that the combination of TLC and HPLC may efficiently be used to quantify a specific analyte from a complex matrix

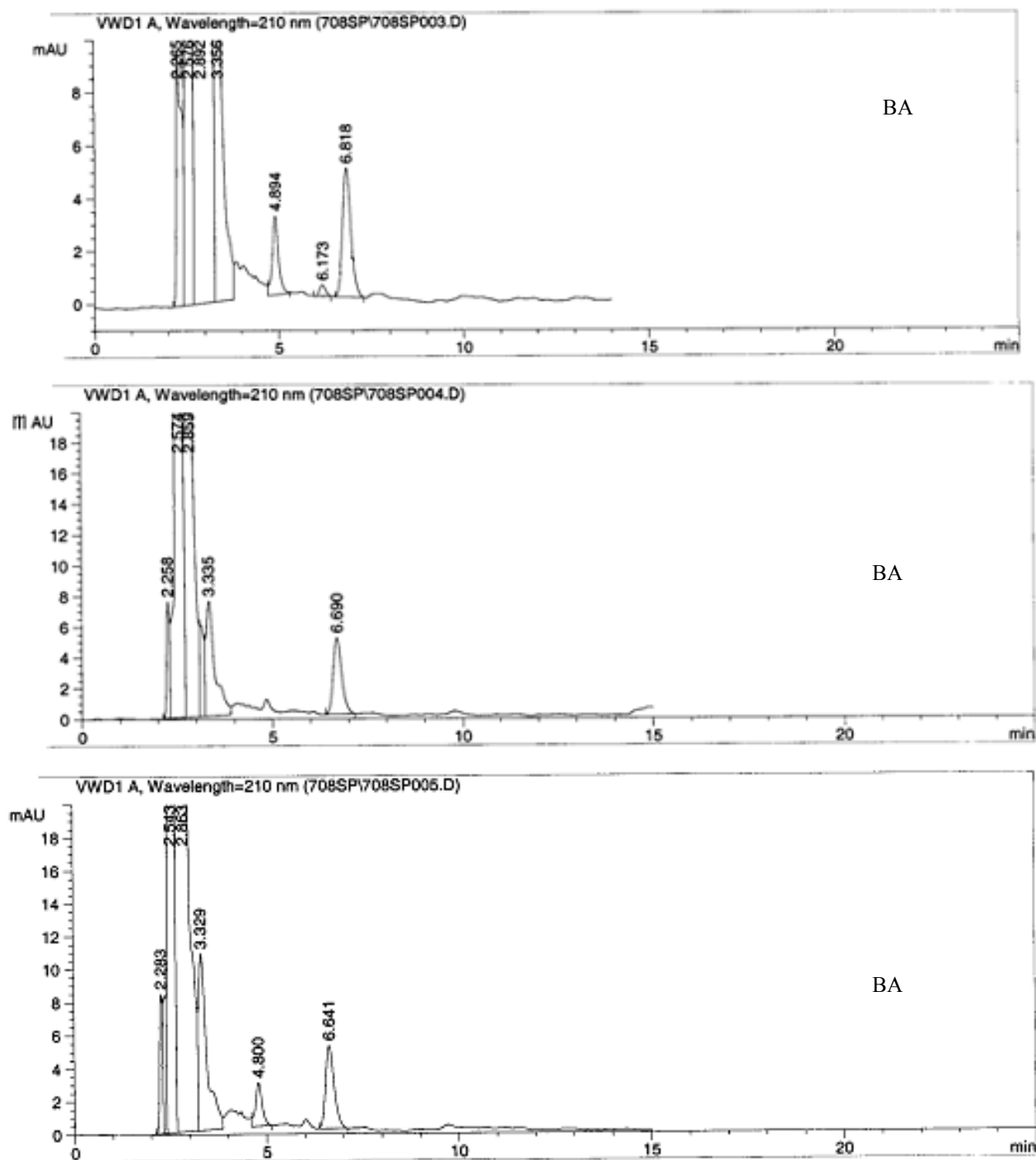


Fig. 4b: Chromatograms of samples of various extracts of *Orthosiphon stamineus* prepared by thin layer chromatography. Aqueous extracts (NHSIDE-5 and NHSIDE-6); aqueous extract (HVM-7)

such as plant extracts and formulations of extracts. Moreover, the method is found to be simple, rapid, sensitive, accurate and precise, therefore, may be applied to standardize extracts/products made from *Orthosiphon stamineus*.

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Table 2: Contents of betulinic acid in different types of extracts of *Orthosiphon stamineus* by HPLC (detection at 210 nm)

Name of extract (n = 3)	mg/g (extract)	± SD
Ethanol (<i>UiTM-1</i>)	3.70	1.27
50% acetone (<i>UiTM-2</i>)	2.61	0.28
Aqueous (<i>NHSIDE-5</i>)	2.43	0.25
Aqueous (<i>NHSIDE-6</i>)	2.63	0.15
Aqueous (<i>HVM-7</i>)	2.25	0.04

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