Research Article

การวิเคราะห์หาปริมาณของพลัมบาจินในสารสกัดรากเจตมูลเพลิงแดงด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ

นัดตา สุขเกษม[1], วรัญญา จตุพรประเสริฐ[2], กนกวรรณ จารุกำจร[3]*

[1] นักศึกษาหลักสูตรเภสัชศาสตรมหาบัณฑิต สาขาวิชาเภสัชภัณฑ์ กลุ่มวิจัยฤทธิ์ทางยาของผลิตภัณฑ์ธรรมชาติโดยเทคโนโลยีชีวภาพทางเภสัชศาสตร์ (PANPB) คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น 40002

[2] ปร.ด., อาจารย์, คณะแพทยศาสตร์ มหาวิทยาลัยมหาสารคาม จังหวัดมหาสารคาม 44000

[3] ปร.ด., รองศาสตราจารย์, กลุ่มวิจัยฤทธิ์ทางยาของผลิตภัณฑ์ธรรมชาติโดยเทคโนโลยีชีวภาพทางเภสัชศาสตร์ (PANPB) คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น 40002

*** ติดต่อผู้นิพนธ์:** กนกวรรณ จารุกำจร คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002

โทรศัพท์: 043-202305, โทรสาร: 043-202379, อีเมล์: kanok_ja@kku.ac.th

บทคัดย่อ

การวิเคราะห์หาปริมาณของพลัมบาจินในสารสกัดรากเจตมูลเพลิงแดงด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ

นัดตา สุขเกษม[1], วรัญญา จตุพรประเสริฐ[2], กนกวรรณ จารุกำจร[3]*

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พลัมบาจิน (5-hydroxy-2-methyl-1,4-naphthoquinone) เป็นสารสีเหลืองในกลุ่มควิโนนอยด์ที่มีฤทธิ์ทางชีววิทยาหลากหลาย พบได้มากในรากของเจตมูลเพลิงแดง หรือ *Plumbago indica* L. ซึ่งเป็นพืชสมุนไพรที่ถูกใช้เป็นยาแผนโบราณเพื่อรักษาโรคมากมาย **วัตถุประสงค์:** การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาและประเมินวิธีวิเคราะห์ปริมาณของพลัมบาจินโดยใช้เทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ (RP-HPLC) วิธีวิเคราะห์ที่ผ่านการประเมินถูกนำไปใช้หาปริมาณพลัมบาจินในสารสกัดหยาบของรากเจตมูลเพลิงแดง **วิธีการทดลอง**: ระบบ RP-HPLC ประกอบด้วยคอลัมน์ชนิด C18 เป็นวัฏภาคคงที่และวัฏภาคเคลื่อนที่เป็นอะซีโทไนไตรล์และน้ำ (50:50 โดยปริมาตร) ที่กำหนดอัตราการไหลเท่ากับ 1 มิลลิลิตรต่อนาที และตรวจวัดพลัมบาจินที่ความยาวคลื่น 254 นาโนเมตร **ผลการทดลอง:** โครมาโทแกรมไม่พบพีคของสารรบกวนที่เวลารีเทนชั่น (retention time) ของพลัมบาจิน (t_R = 6.2 นาที) และมีความสัมพันธ์เชิงเส้นตรงที่ดี (r^2 = 0.99823) ขีดจำกัดของการตรวจวัด (LOD) และขีดจำกัดของการวิเคราะห์ปริมาณ (LOQ) เท่ากับ 21.85 และ 72.82 นาโนกรัมต่อมิลลิลิตร ตามลำดับ ความเที่ยงตรงภายในวันและระหว่างวันแสดงด้วยร้อยละของค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (% relative standard deviation) เท่ากับร้อยละ 0.37-0.65 และ 0.17-1.25 ตามลำดับ และค่าความถูกต้องแสดงด้วยร้อยละการคืนกลับเท่ากับร้อยละ 98.71±4.83 **บทสรุป:** วิธีวิเคราะห์นี้ยืนยันได้ว่ามีความจำเพาะ ความไว ความเที่ยงตรง และความถูกต้อง สำหรับการวิเคราะห์หาปริมาณของพลัมบาจิน วิธีวิเคราะห์ที่ผ่านการประเมินนี้ถูกใช้วิเคราะห์หาปริมาณพลัมบาจินในสารสกัดหยาบเมธานอลและเอธานอลของเจตมูลเพลิงแดงและพบปริมาณพลัมบาจินร้อยละ 0.15±0.00 และ 0.21±0.01 ของน้ำหนักแห้ง ตามลำดับ

คำสำคัญ: เจตมูลเพลิงแดง, พลัมบาจิน, โครมาโทกราฟีของเหลวสมรรถนะสูงวัฏภาคย้อนกลับ

Quantitative determination of plumbagin in *Plumbago indica* L. root extract using reverse phase-high performance liquid chromatography

Nadta Sukkasem[1], Waranya Chatuphonprasert[2], Kanokwan Jarukamjorn[3]*

[1] Graduate student (M.PS. program in Pharmaceuticals), Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand

[2] Ph.D., (Pharmaceutical Sciences), Lecturer, Faculty of Medicine, Mahasarakham University, Mahasarakham 44000 Thailand

[3]Ph.D., (Pharmaceutical Sciences), Associate Professor, Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand

***Corresponding author**: Kanokwan Jarukamjorn, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand.
Tel: 043-202305, Fax: 043-202379, Email: kanok_ja@kku.ac.th

Abstract

Quantitative determination of plumbagin in *Plumbago indica* L. root extract using reverse phase-high performance liquid chromatography

Nadta Sukkasem[1], Waranya Chatuphonprasert[2], Kanokwan Jarukamjorn[3]*

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Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a yellowish quinonoid compound with extensive biological activity that is abundant in the root of *Plumbago indica* L. or scarlet leadwort, an herbal plant used as a traditional remedy to treat a variety of illnesses. **Objectives:** To develop and validate an analytical method for quantification of plumbagin using reverse phase-high performance liquid chromatography (RP-HPLC). The validated method was utilized for determination of plumbagin in *P. indica* root crude extracts. **Methods:** The RP-HPLC system consisted of a C18 column as stationary phase with a mobile phase of acetonitrile and water (50:50, v/v) at a flow rate of 1 mL/min. Plumbagin was detected at 254 nm. **Results:** No interference peak was observed in chromatograms at the retention time of plumbagin (t_R = 6.2 min) with good linearity (r^2 = 0.99823). Limit of detection (LOD) and limit of quantification (LOQ) were 21.85 ng/mL and 72.82 ng/mL, respectively. Within-day and between-day precision, expressed as % relative standard deviation, were of 0.37-0.65% and 0.17-1.25%, respectively, with the accuracy as %recovery of 98.71±4.83%. **Conclusion:** The RP-HPLC method proved to be specific, sensitive, precise, and accurate for quantitative determination of plumbagin. Utilization of the validated method demonstrated the content of plumbagin in *P. indica* methanolic and ethanolic crude extracts to be 0.15±0.00% and 0.21±0.01% dry weight, respectively.

Keywords: *Plumbago indica*, plumbagin, reverse phase HPLC

Introduction

Plumbago indica L. or scarlet leadwort is an herbal plant that belongs to the family Plumbaginaceae. The root of *P. indica* has been used in ancient Indian remedies to treat a variety of illnesses (Dutt, 1877; Lorsuwannarat *et al*., 2013).

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Fig. 1) is a yellowish quinonoid compound found in roots of Plumbago species (Lorsuwannarat *et al*., 2013). Extensive studies have revealed various biological activities of plumbagin, including antibacterial (Kaewbumrung and Panichayupakarananta, 2014), anthelmintic (Zhang and Coultas, 2013; Lorsuwannarat *et al*., 2014), antimalarial (Sumsakul *et al*., 2014), anti-inflammatory (Wang *et al*., 2014; Zhang *et al*., 2015), immunosuppressive (McKallip *et al*., 2010), abortifacient (Sheeja *et al*., 2009), anticancer (Wang *et al*., 2008; Xu and Lu, 2010; Lai *et al*., 2011; Hafeez *et al*., 2013; Wang *et al*., 2014; Checker *et al*., 2015), and antidiabetic (Sunil *et al*., 2012).

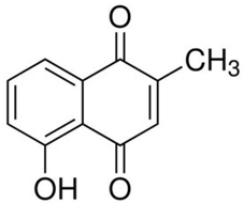

Fig 1. Chemical structure of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)

There are several methods to quantify plumbagin, including spectrophotometry (Israni *et al*., 2010), TLC-densitometry (Yogananth and Basu, 2009), and high-performance liquid chromatography (HPLC) (Unnikrishnan *et al*., 2008). However, HPLC analysis of plumbagin has only been applied to *P. zeylanica* extracts, to date (Wang and Huang, 2005; Jain *et al*., 2014).

Reverse phase-HPLC (RP-HPLC) is an analytical technique that allows separation, identification, and quantification of chemicals in mixtures, and is one of the most precise techniques for quantitative analysis of plant constituents in plant extracts (Hajimehdipoor *et al*., 2010; Weon *et al*., 2013; Wang, 2014). It is a chromatographic technique based on pumping samples through a column filled with solid adsorbent material. The column separates each component in the sample according to their physicochemical properties (Bird, 1989). Several studies have developed and validated the use of RP-HPLC to determine the chemical constituents of plant extracts (Maji *et al*., 2012; Al-Rimawi, 2014)

Here we describe a validated RP-HPLC method to determine the plumbagin content in crude methanolic and ethanolic extracts of *P. indica* root.

Material and Methods

Chemicals and reagents

Standard plumbagin (purity 97%) was purchased from LKT Laboratories (St. Paul, Minnesota, USA). Methanol (AR grade) and ethanol (AR grade) were obtained from ACI labscan (Thailand). Acetonitrile (HPLC grade) was a product of Merck (Darmstadt, Germany). All other laboratory chemicals were of high purity from commercial suppliers

Plant material

The root of *P. indica* was obtained from Mor-Tong-In Thai Traditional Medicine (Mahasarakam, Thailand) in June, 2014 and identified by Dr. Waraporn Putalun, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. The reference specimen (PANPB-PI 2014–002) was deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University.

Instrument

RP-HPLC system consisted of Hypersil ODS (Agilent Technologies, CA, USA) C18 column (5 µm, 250 × 4.0 mm) using an Agilent 1260 Infinity system (Agilent Technologies) and a UV-VIS detector (Agilent 1260 Infinity, Agilent Technologies). The chromatogram was analyzed using ChemStation software (Agilent Technologies).

Preparation of standards

Standard plumbagin was accurately weighed as 1 mg and dissolved in 1 mL of 50% (v/v) methanol or ethanol to obtain a stock solution of 1 mg/mL. The stock solution was serially diluted to obtain standard solutions of 1, 10, 25, 50, and 100 µg/mL for further analysis.

Extraction and preparation of the crude extract

The roots of *P. indica* were dried at 50ºC in an oven before being shredded and extracted with methanol or ethanol for 3 hours using a soxhlet apparatus. The extracts were then evaporated and freeze-dried into powder. The dry crude powder (200 mg) was extracted with 1 mL of 50% (v/v) methanol or ethanol. The mixture was vortexed for 5 min and then centrifuged at 10,000 *g* for 20 min. The supernatant was collected and filtered through a 0.45 µm-membrane filter before injection into the RP-HPLC system.

RP-HPLC conditions

An isocratic linear solvent system of acetonitrile and water (50:50, v/v) with a flow rate of 1 mL/min was employed. Chromatograms were monitored at 254 nm and analyzed with ChemStation software (Agilent Technologies).

Results

Method validation of the quantitative determination of plumbagin in *P. indica* extract

The method validation was based on the standard method (Szepesi, 2000). Identification and quantification of plumbagin in the *P. indica* extracts was performed on the basis of the retention time (t_R) and peak area of plumbagin authentic standards.

Specificity

The method showed high specificity for plumbagin as there was no peak interference around the plumbagin retention time in either the chromatograms of the blanks (methanol Fig. 2A and ethanol Fig. 2C) or the plumbagin standards (methanol Fig. 2B and ethanol Fig. 2D). The retention time of plumbagin in the extracts correlated to those of the plumbagin standards, in which the retention times of the standard plumbagin in methanol and ethanol were 6.294 and 6.282 min, respectively.

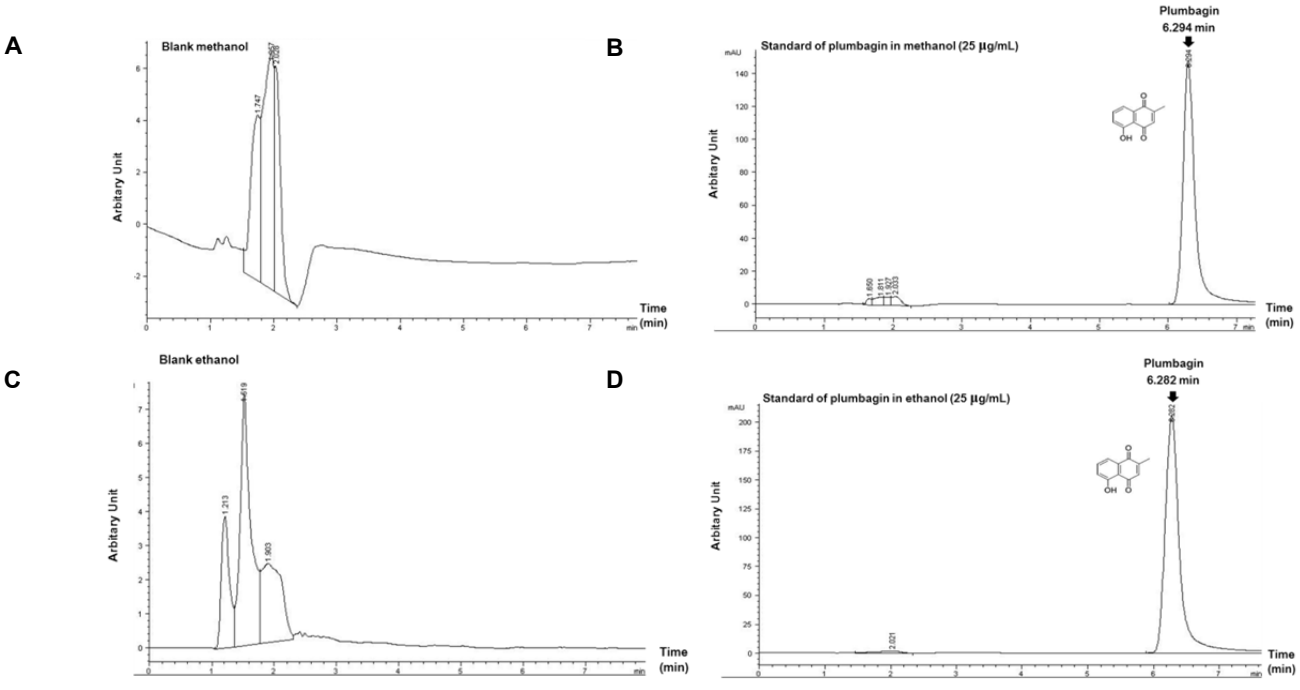

Fig 2. Chromatograms of blank methanol (A), standard plumbagin in methanol (B), blank ethanol (C), and standard plumbagin in ethanol (D).

Linearity

The method showed a good linear relationship between the concentration of plumbagin in the range of 1 to 100 µg/mL and the HPLC peak area at 254 nm with the linear regression equation of Y = 97.496X + 130.34 (r^2 = 0.99823, Table 1).

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ of the method were carried out by determining the standard deviation (SD) of the response (n = 3) and the slope (S) of the linear equation according to the formulas: LOD = 3.3 SD/S and LOQ = 10 SD/S. The LOD and LOQ of the method were 22.05 ng/mL and 66.83 ng/mL, respectively (Table 1), demonstrating good sensitivity of the method.

Table 1. Validation parameters of the analytical method for quantification of plumbagin in *P. indica* crude extract.

Parameters	
Sensitivity	
LOD (ng/mL)	22.05
LOQ (ng/mL)	66.83
Specificity	
at λ 254 nm	No peak interference
Linearity (concentration 1-100 µg/mL)	
Linear regression equation	Y = 97.496X + 130.34
Coefficient of determination (r^2)	0.99823
Precision (%RSD) (concentration 1-100 µg/mL)	
Peak area	
Within-day	0.37 - 0.65
Between-day	0.17 - 1.25
Retention time	
Within-day	0.03 - 0.32
Between-day	0.37 - 1.44
Accuracy (%recovery)	98.71±4.83

Precision

Within-day and between-day reproducibility of the method (precision) was calculated as the percentage relative standard deviation (%RSD) of the peak area and retention time of plumbagin at each concentration of the standard solutions (1-100 µg/mL, n = 5 for each concentration). The within-day and between-day precision determinations were 0.37- 0.65 %RSD and 0.17-1.25 %RSD for peak area, and 0.03 - 0.32 %RSD and 0.37 – 1.44%RSD for retention time, respectively, indicating high precision of the method (Table 1).

Accuracy

Accuracy of the method was evaluated as %recovery of the plumbagin added. The accuracy was 98.71±4.83% (n = 9), indicating very good accuracy of the method (Table 1).

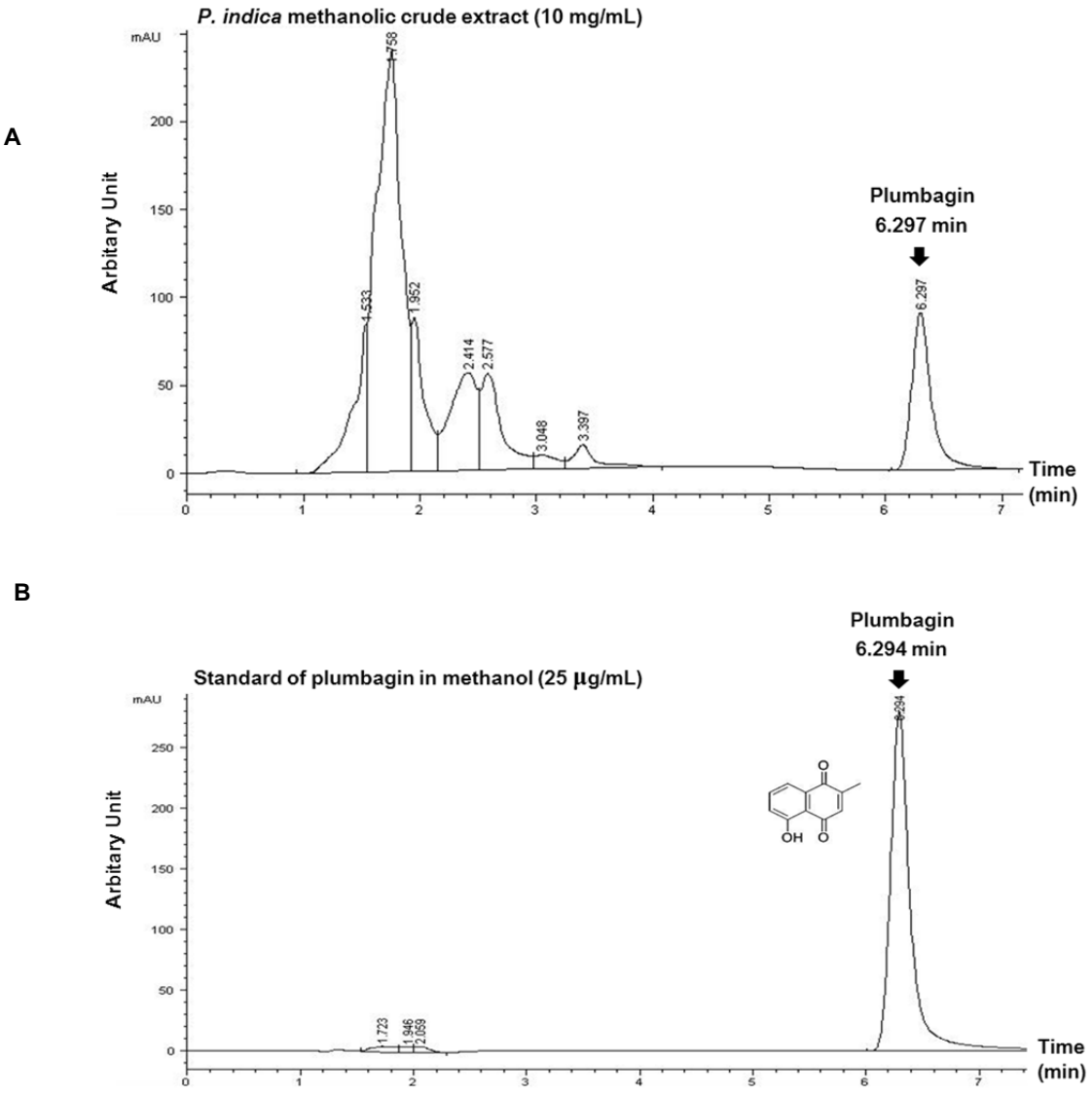

Fig. 3 Chromatograms of methanolic *P. indica* crude extract (A) and plumbagin standard in methanol (B)

Quantification of plumbagin in *P. indica* root crude extracts

The chromatogram of the *P. indica* methanolic crude extract (Fig. 3A) shows that the main constituent of the *P. indica* crude extract was plumbagin (t_R = 6.297 min) by comparison to the retention time of the standard plumbagin in methanol (t_R = 6.294 min, Fig. 3B). The content of plumbagin in the *P. indica* methanolic crude extract was 0.15±0.00% dry weight of the extract (n = 5).

The chromatogram of the *P. indica* ethanolic crude extract (Fig. 4A) shows that plumbagin (t_R = 6.287 min) was the main constituent of the *P. indica* extract by comparison to the retention time of the standard plumbagin in ethanol (t_R = 6.282 min, Fig. 4B). The content of plumbagin in the *P. indica* ethanolic crude extract was 0.21±0.01% dry weight of the extract (n = 5).

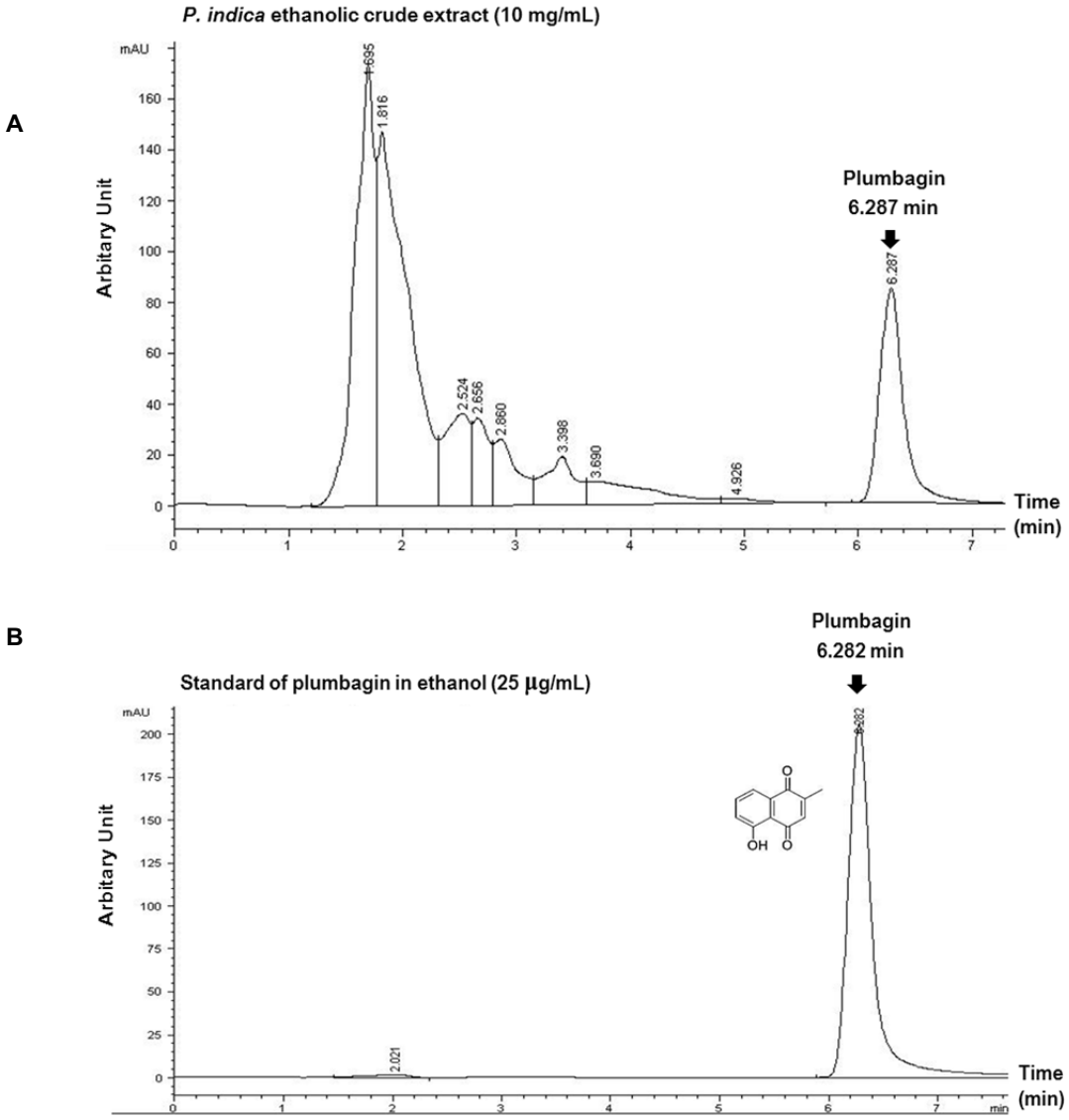

Fig. 4 Chromatograms of the ethanolic *P. indica* crude extract (A) and plumbagin standard in ethanol (B)

Discussion

Plumbagin is the main active constituent of Plumbago species used in traditional medicinal remedies, including *P indica* (Ariyanathan *et al*., 2010). In this study, crude extracts of *P. indica* root were analyzed for plumbagin content using a novel RP-HPLC method. The amount of plumbagin in methanolic and ethanolic extracts of *P. indica* root were 0.15±0.00% and 0.21±0.01% dry weight, respectively. These levels correlated well with the 0.17% reported in a previous study that assayed plumbagin levels in chloroform extracts of *P. indica* root using TLC (Ariyanathan *et al*., 2010). Unnikrishnan *et al*. (2008) compared HPTLC and HPLC methods for determination of plumbagin content in *Plumbago* spp. and found the plumbagin content of *P. indica* root ethanolic extracts was 0.19% dry weight by HPTLC and 0.20% dry weight by HPLC, and that HPLC was more sensitive and precise than HPTLC. HPLC is considered to be superior to either HPTLC or TLC due to its better separation capacity, greater precision and accuracy, and less time per sample (Rashmin *et al*., 2012). These observations suggest the validity of our RP-HPLC method consisting of a simple mobile phase system with greater separation.

Conclusion

The quantification of plumbagin using RP-HPLC was validated for specificity, sensitivity (LOD and LOQ), linearity, precision, and accuracy (Table 1). When the validated method was used to determine the amount of plumbagin in *P. indica* root crude extracts, it proved to be precise, accurate, and simple to perform.

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