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High-performance liquid chromatography for quantification of plumbagin, an anti-*Helicobacter pylori* compound of *Plumbago zeylanica* L.

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Abstract

The plant *Plumbago zeylanica* L. is a semi-climbing shrub that grows throughout Asia and Africa. In our previous study, *P. zeylanica* L. exhibited high anti-*Helicobacter pylori* and good bactericidal activities over a wide pH range (pH 2–7). Plumbagin – the major ingredient derived from the roots of *P. zeylanica* L. – is a naphthoquinone compound. In this study, we investigated plumbagin's anti-*H. pylori* activity and developed a reversed-phase high-performance liquid chromatography (HPLC) method for quantification of plumbagin from *P. zeylanica* L. We also observed that plumbagin has strong anti-*H. pylori* activity, with 0.02–0.16 mg/ml as minimum inhibitory concentrations and 0.16–1.28 mg/ml as minimum bactericidal concentrations. Reversed-phase HPLC was performed with a gradient mobile phase composed of water and methanol, and peaks were detected at 254 nm. Standard curves were linearized in the range of from 10 to 200 μg/ml (regression coefficient $r^2 = 0.99995$). After spikes of 50, 100, and 150 μg/ml of plumbagin standard solution, recovery rates were between 97.45 and 99.24%. Both intra- and inter-day precisions had coefficient variation of less than 1% at concentrations of 50, 100, and 150 μg/ml. The limits of detection and quantitation were 0.02 and 0.06 μg/ml, respectively. Based on validation results, this analytical method is a precise, accurate and stable method to quantify plumbagin derived from *P. zeylanica* L.

Keywords: Plumbagin; Anti-*Helicobacter pylori* activity; HPLC; *Plumbago zeylanica* L.

1. Introduction

Plumbago zeylanica L. is a semi-climbing shrub that grows throughout Asia and Africa. This plant is distributed in thickets and grasslands at low elevations in Taiwan [1]. The whole plant and its roots have been used as a folk medicine for the treatment of rheumatic pain, dysmenorrhea, carbuncles, contusion of the extremities, ulcers and elimination of intestinal parasites [2]. In traditional Indian medicine, *P. zeylanica* L. has been assigned medicinal properties and is used in formulations for a number of ayurvedic compounds [3]. In Africa, *P. zeylanica* L. is used in southwestern Nigerian folk medicine for parasitic diseases, scabies and ulcers [4]. Pharmacological studies carried out by several workers have indicated that *P. zeylanica* L. extract has antiplasmodial [5], antimicrobial [2,6,7], antihyperglycemic [8], insecticidal [9], antiallergic [10], central nervous system stimulatory [11] and cytotoxic properties (last against tumor cells) [12].

In our previous study, we found *P. zeylanica* L. has strong antibacterial and bactericidal activities against *Helicobacter pylori*, with low minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs), with bactericidal activity over a wide pH range (pH 2–7) and with good stability over the range of pH 1–7 [13]. *H. pylori* is associated with a wide spectrum of gastroduodenal diseases. Infection with *H. pylori* is now recognized as the primary cause of type B gastritis, and *H. pylori* gastritis is strongly associated with gastric and duodenal ulcers [14,15]. A great deal of epidemiological data show that high prevalence of *H. pylori* infection might be related to high rates of gastric cancer and gastric adenocarcinoma [16–18].

Compounds isolated from *P. zeylanica* L. are composed of naphthoquinones such as plumbagin, 3,3-biplumbagin, 3-chloroplumbagin, chitranone, elliptinone, and isoshi-

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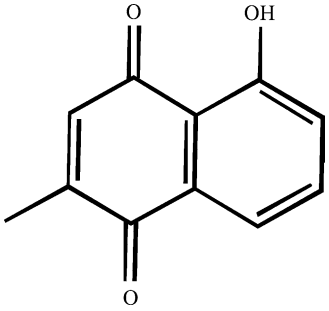

Fig. 1. Structure of plumbagin.

nanolone; and coumarins such as seselin, 5-methoxyseselin, suberosin, and xanthyletin [6,19–22]. Other compounds such as 2,2-dimethyl-5-hydroxy-6-acetylchromene, plumbagin acid, β-sitosterol, β-sitosteryl glucoside, bakuchiol, 12-hydroxyisobakuchiol, saponaretin, isoorientin, isoaffinetin and psoralen have also been isolated and identified [19,22]. Of these compounds, plumbagin is a major ingredient of *P. zeylanica* L. [6,21–23] derived from the roots of the plant. Both in vitro and in vivo studies of plumbagin have indicated that the compound exhibits high levels of bioactivities such as anti-tumor [23], anti-hepatoma [24], anti-intestinal carcinogenesis [25], antimicrobial [6,21,26,27] and increased macrophage bactericidal [28] properties. The structure of plumbagin is shown in Fig. 1.

Therefore, we hypothesized that plumbagin might be the major contributor to the observed anti-*H. pylori* activity of *P. zeylanica* L. A normal-phase HPLC method for quantification of plumbagin from *P. zeylanica* L. had been reported by Gupta et al. [22], which a μSpherogel column was chosed, a mobile phase composed of *n*-hexane-choloform-2-propanol (30:70:2, v/v/v) was eluted. The results shown that plumbagin was not completely separeted with another compound derived from *P. zeylanica* L., 2,2-dimethyl-5-hydroxy-6-acetylchromene, and base line shifted. The quantity detected was low, 0.032%.

The aims of the current study were to document anti-*H. pylori* activity of plumbagin and to develop a reversed-phase HPLC method for quantification of plumbagin from *P. zeylanica* L. To validate an HPLC quantitative method, the specificity, linearity, accurancy, precision, limits of detection and quantitation of plumbagin were investigated. Plumbagin content for thirteen *P. zeylanica* L. samples were also examined.

2. Materials and methods

2.1. Chemicals and reagents

Standard plumbagin was purchased from Acros (NJ, USA), with amoxicillin purchased from Sigma (St. Louis, MO, USA), methanol (HPLC grade) from Tedia (Fairfield, OH, USA), absolute ethanol (HPLC grade) from Sigma–Aldrich (Seelze, Germany), tryptic soy broth (TSB) from Difco (Sparks, MD, USA), Columbia agar from bioMérieux (I'Etoile, France) and a gas generating kit from Oxoid (Basingstoke, UK). Chemicals were reagent grade.

2.2. Bacterial strains

Five *H. pylori* strains, BCRC 17021, 17023, 17026, 17027 and 15415, were obtained from the Bioresources Collection and Research Center (BCRC), Hsinchu, Taiwan, ROC, which is a member of the World Federation for Culture Collections (WFCC) since 1984.

2.3. Bacterial cultivation

The five strains of *H. pylori* were cultured in 5 ml tryptic soy broth (each liter containing: a pancreatic digest of casein (17 g), an enzymatic digest of soybean meal (3 g), dextrose (2.5 g), sodium chloride (5 g) and dipotassium phosphate (2.5 g); pH 7.3), with Columbia agar (each liter containing: bio-polyone (10 g), bio-lysat (10 g), bio-myotone (3 g), corn starch (1 g), sodium chloride (5 g) and agar (13.5 g); pH 7.3) slant containing 5% (v/v) defibrinated sheep blood formed at the bottom of the test tube. The broth was then incubated in a microaerophilic jar system (BBL, Sparks, BD, USA), featuring a gas composition of 5% O_2 and 10% CO_2 in air (an Oxoid BR 056A gas-generating kit was used for this purpose), at 37 °C for 72 h. The cell suspension was then diluted with 0.1% peptone to provide a cell concentration of 0.5–1.0 × 10^6 CFU/ml for antimicrobial testing.

2.4. Minimum inhibitory concentration (MIC) testing

MICs for plumbagin against the five strains of *H. pylori* were examined. A broth-dilution method [29] was used for MIC testing. Plumbagin was dissolved with dimethylsulfoxide (DMSO) and diluted with two-fold dilutions of Columbia agar containing 5% (v/v) defibrinated sheep blood. DMSO was used as negative control. The media were poured into petri dishes to form plates. An 0.1-ml volume of cell suspensions (0.5–1.0 × 10^6 CFU/ml) were spread onto the plates. After incubation in the microaerophilic jar system in 5% O_2 and 10% CO_2 in air at 37 °C for 72 h, the resulting colonies were enumerated. The MICs for the plumbagin against the five *H. pylori* strains were determined. The MIC was defined as the lowest concentration of plumbagin at which no colony of the test bacteria on the plate was formed.

2.5. Minimum bactericidal concentration (MBC) testing

MBCs for plumbagin against the five strains of *H. pylori* were also determined. For MBC testing, as well as the MIC testing, a broth-dilution method [29] was used. Plumbagin was dissolved with DMSO and diluted with two-fold dilutions of Columbia agar containing 5% (v/v) defibrinated sheep blood or TSB. It was then individually added to test

tubes with Columbia agar slants at the bottom of the tubes and the TSB layer covered on the slants; both the slant and broth contained the same concentrations of plumbagin. DMSO was used as control. *H. pylori* suspensions were added to the TSB layer to produce 0.5–1.0 × 10^6 CFU/ml of the initial bacterial count. Following incubation in the microaerophilic jar system in 5% O_2 and 10% CO_2 in air at 37 °C for 72 h. A 0.1-ml volume of each broth was spread onto Columbia agar plates containing 5% (v/v) defibrinated sheep blood without plumbagin. After incubation in the microaerophilic jar system in 5% O_2 and 10% CO_2 in air at 37 °C for 72 h, the resulting colonies were enumerated. MBC was defined as the lowest concentration of plumbagin at which no colonies of test bacteria formed on the cultivation medium [30].

2.6. Apparatus and chromatographic conditions

The HPLC system consisted of a Hewlett-Packard Model 1100 system (Darmstadt, Germany), equipped with a multisolvent delivery system and an ultraviolet (UV) detector. The column was a LiChrospher®100RP18e, 5 μm, 4.0 mm internal diameter (i.d.) × 250 mm (Merck, Darmstadt, Germany).

The mobile phase was composed of water-methanol with gradient elution as follows: 0 min, 2:98; 10 min, 50:50; 30 min, 100:0; 40 min, 100:0. The mobile phase was filtered under vacuum through a 0.45-μm membrane filter before use. The flow rate was 1 ml/min with UV absorbance detection at 254 nm. The operating temperature was maintained at room temperature.

2.7. Plant materials

Thirteen samples of *P. zeylanica* L. were obtained from different herbal markets and identified by Technician Nien-Yung Chiu from the Institute of Chinese Pharmaceutical Science, China Medical College. Sample Number 6 was a voucher specimen (no. 250481) deposited at the Institute of Ecology and Evolutionary Biology, College of Life Science, National Taiwan University. This sample was investigated for anti-*H. pylori* activity and used for validation of analytical method, limit of detection (LOD) and limit of quantitation (LOQ). The other twelve *P. zeylanica* L. samples were used for quantitation of plumbagin of *P. zeylanica* L. Except for samples Numbers 8 and 13, which were derived from the stem of *P. zeylanica* L., the other samples were derived from a mixture of roots and stem of *P. zeylanica* L. Samples of *P. zeylanica* L. were dried at below 65 °C and chopped into small pieces before use.

2.8. Preparation of P. zeylanica L. samples

A total of 50 ml ethyl acetate was added to 2.5 g *P. zeylanica* L. powder (passed through a 60-mesh screen), and the mixture was stirred at room temperature for 3 h. After passage through a filter paper, the filtrate was concentrated to dryness in a rotary vacuum evaporator at less than 40 °C. The concentrate was dissolved with absolute ethanol and filtered through a 0.45-μm membrane filter before use.

2.9. Standard solutions prepared

A stock solution of plumbagin (concentration, 1.0 mg/ml) was prepared with absolute ethanol and stored at −18 °C until use.

2.10. Validation of analytic method

Validation of the analytical method for plumbagin derived from *P. zeylanica* L. was examined for specificity, linearity, accuracy, precision, LOD and LOQ.

For specificity validation, a standard plumbagin solution (0.1 mg/ml) and *P. zeylanica* L. sample solution were prepared with absolute ethanol. The absolute ethanol was used as a control. A volume of 10 μl was injected into the HPLC column individually.

For linearity validation, standard plumbagin solutions of 10, 50, 100, 150, and 200 μg/ml were prepared and 10 μl was injected into the HPLC column. Triplicate analyses were performed in three different days. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

For accuracy validation, plumbagin concentrations of 50, 100, and 150 μg/ml were prepared and mixed with *P. zeylanica* L. sample solution at a ratio of 1:1 (v/v). The three injections for each concentrations were done per day over three different days (3 injections × 3 concentrations × 3 days). Recoveries of plumbagin were calculated as the following equation:

$$\text{Recovery (\%)} = \frac{2 \times \text{measured plumbagin concentration} - \text{plumbagin concentration of } \textit{Plumbago zeylancia} \text{ L.}}{\text{plumbagin theoretical concentration}} \times 100$$

coefficient of variation (CV) was calculated as standard deviation (SD) to the mean value from the results of triplicate testing.

For precision validation, plumbagin concentrations of 50, 100, and 150 μg/ml was prepared and 10 μl was injected into the HPLC column. Concentrations of plumbagin from the experiments were calculated with a linear equation of the standard curve. Triplicate analyses were conducted. The intra- and inter-day precisions were obtained by triplicate analyses in a day and per day over 3 days, respectively.

2.11. LOD and LOQ

LOD and LOQ for the analytical method were studied. Serial dilutions of plumbagin were made with abso-

lute ethanol, and were then analyzed with HPLC method as described above. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

2.12. Plumbagin quantitation from P. zeylanica L.

Plumbagin content of the thirteen samples of *P. zeylanica* L. were determined with the HPLC method as described above. Moisture content of the thirteen samples was examined by drying in an oven at 105 °C. The plumbagin contents of *P. zeylanica* L. samples were established on a dried material.

3. Results and discussion

3.1. MICs and MBCs for plumbagin

As cited in previous literatures, plumbagin is a major ingredient of *P. zeylanica* L. [6,21–23] derived from the roots of the plant. In our previous study, we found *P. zeylanica* L. exhibited strong antibacterial and bactericidal activities against *H. pylori* [13]. Therefore, we strongly supposed that plumbagin is highly linked with the anti-*H. pylori* activity of *P. zeylanica* L. From the results of MICs and MBCs of plumbagin (Table 1), indeed, we found plumbagin both having high levels of anti-*H. pylori* and bactericidal activities. MICs and MBCs for plumbagin against the five strains of *H. pylori* ranged from 0.02 to 0.16 mg/ml and 0.16 to 1.28 mg/ml, respectively. In our previous studies of *P. zeylanica* L., of the water, ethanol, acetone and ethyl acetate extracts, the ethyl acetate extract exhibited the lowest MICs and MBCs, ranging from 0.32 to 1.28 mg/ml and 5.12 to 20.48 mg/ml, respectively [13]. These values were higher than the MICs for plumbagin much more (Table 1). The plumbagin contents of these four water, ethanol, acetone and ethyl acetate extracts were 0.116 ± 0.003, 1.816 ± 0.004, 1.790 ± 0.032, and 2.618 ± 0.002 mg/g, respectively (data not shown), the ethyl acetate extract exhibited the greatest plumbagin content. These results indicated that the higher plumbagin was contained in the sample, the higher anti-*H. pylori* activity was demonstrated. Therefore, we strongly proposed that plumbagin is a major compound contributing to the observed anti-*H. pylori* and bactericidal activities of *P. zeylanica* L.

Table 1
Minimum inhibitory concentrations and minimum bactericidal concentrations for plumbagin against *H. pylori*

Strain	MIC (mg/ml)	MBC (mg/ml)
Helicobacter pylori BCRC 17021	0.02	0.16
H. pylori BCRC 17023	0.08	0.64
H. pylori BCRC 17026	0.16	1.28
H. pylori BCRC 17027	0.08	1.28
H. pylori BCRC 15415	0.04	0.64

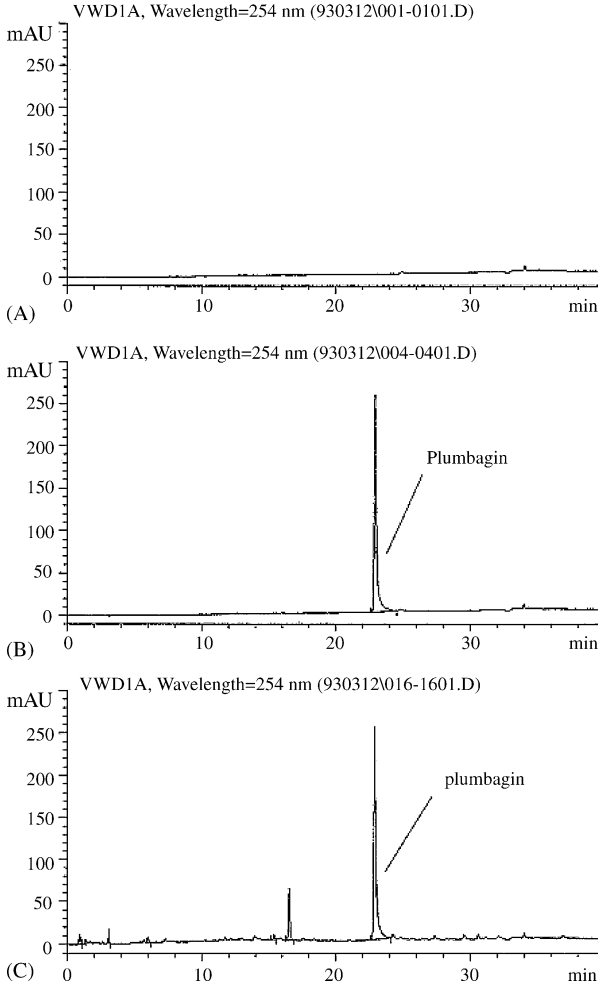

Fig. 2. The specificity validation for the HPLC analytical method for plumbagin, an anti-*H. pylori* compound of *P. zeylanica* L.: (A) blank solution; (B) plumbagin standard solution; and (C) *P. zeylanica* L. sample solution.

3.2. Validation of analytic method

3.2.1. Specificity validation

Based on the structure of plumbagin (Fig. 1), this compound would be characterized by moderate-low polarity. Therefore, a C18 reversed-phase HPLC was chosed for quantification of plumbagin derived from *P. zeylanica* L. A moderately-to-highly polar mobile phase composed of methanol and water was eluted with 1 ml/min flow rate. The results of HPLC chromatograms are shown in Fig. 2. In Fig. 2C, it is apparent that plumbagin is a major compound of *P. zeylanica* L., with 22.92 min of retention time, no other interferring peaks observed around the peak of plumbagin; similar results are shown in Fig. 2A and B; again, there are no interfering peaks to be found around the peak of 22.92 min. From the results of photodiode array detection, we found the same UV spectra of plumbagin's peaks from HPLC chromatograms between the standard and the sample solutions. Purities of plumbagin's peaks of standard and sample solutions were 99.93 and 99.99%, respectively (data not shown).

Table 2
Parameters of quantitation for plumbagin

Parameter	Result
Linear range (μg/ml)	10–200
Equation	$Y = 32.022X - 66.562$[a]
Linearity (r^2)	0.99995
LOD (μg/ml)[b]	0.02
LOQ (μg/ml)[c]	0.06

[a] $Y = AX + B$, where Y is peak area, X is the concentration of the analyzed material.
[b] Limit of detection (LOD): signal to noise ratio = 3.
[c] Limit of quantitation (LOQ): signal to noise ratio = 10.

From the results reported by Gupta et al. [22], plumbagin was not completely separated with another compound derived from the plant, 2,2-dimethyl-5-hydroxy-6-acetylchromene, and base line shifted. The results we obtained shown good separation effect for plumbain from *P. zeylanica* L. As shown in Fig. 2, the reversed-phase HPLC method has validated specificity for the analysis of plumbagin from *P. zeylanica* L.

3.2.2. Quantitation parameters

The linearity of the plumbagin standard curve was examined. The results are shown in Table 2. Three separate calibration curves obtained on different days by plotting the peak area vs. concentration were found to be linear when evaluated by linear regression analysis. The linear equation of $Y = 32.022X - 66.562$ and correlation coefficient (r^2) of 0.99995 were obtained. The coefficient variation for the triplicate analyses ranged from 0.06 to 0.61% (data not shown). As shown by the results of Table 2, the standard curve of plumbagin is linear at the concentrations range of 10–200 μg/ml.

The limit of detection represents the lowest concentration of plumbagin that can be detected by the instrument and the analytical method, whereas the limit of quantitation represents the lowest concentration of plumbagin that can be determined with acceptable precision and accuracy by the instrument and method. The results of LOD and LOQ analysis for plumbagin derived from *P. zeylanica* L. were found to be 0.02 and 0.06 μg/ml (Table 2), respectively, indicating that the analytical method for the quanititation of plumbagin of *P. zeylanica* L. exhibited good sensitivity.

3.2.3. Accuracy validation

The accuracy of the analytical method was studied. Plumbagin solution at concentrations of 50–150 μg/ml were spiked into *P. zeylanica* L. sample solutions to evaluate recoveries of plumbagin for this method, which recoveries nears to 100% indicating a good accuracy of this method obtained. From the results of Table 3, good plumbagin recoveries exhibited, for which ranging from 97.75 to 98.99%, with 0.17–0.86% of coefficient variations. It demonstrates that the analytical method has good accurracy.

3.2.4. Precision validation

Both the intra- and inter-day precisions of the analytical method were studied, which obtained by triplicate analyses in a day and per day over three days, respectively. The results shown in Table 4, both intra- and inter-day precisions were higher than 99%, for which 0.25–0.97% and 0.17–0.47% of coefficient variations, respectively. The results indicated that the method for quantitation of plumbagin from *P. zeylanica* L. has good precision.

3.3. Plumbagin content of P. zeylanica L.

Plumbagin content was examined with the above HPLC method for the thirteen different sources of samples of *P. zeylanica* L. The results are shown in Table 5. There was wide variation in plumbagin content for the 13 different samples, with the plumbagin contents ranging from 0.629 to

Table 3
Validation of the accuracy of the analytical method for plumbagin

Spiked level (μg/ml)	Recovery (%)[a]			Mean (%)	CV (%)[b]
	1	2	3		
50	97.56 ± 0.87	97.82 ± 0.95	97.88 ± 0.74	97.75 ± 0.17	0.17
100	98.88 ± 0.23	99.24 ± 0.45	98.84 ± 0.33	98.99 ± 0.22	0.23
150	99.11 ± 0.29	98.00 ± 0.44	97.45 ± 0.56	98.18 ± 0.85	0.86

[a] All values are mean ± SD as obtained by triplicate analyses.
[b] Coefficient of variation = SD/mean × 100%.

Table 4
Validation of precision of the analytical method for plumbagin

Theoretical concentration (μg/ml)	Intra-day[a] ($n = 3$)		Inter-day[b] ($n = 9$)	
	Measured concentration(μg/ml)	CV(%)[c]	Measured concentration (μg/ml)	CV(%)
50	50.33 ± 0.13	0.25	48.57 ± 0.18	0.38
100	100.99 ± 0.98	0.97	103.65 ± 0.48	0.47
150	152.52 ± 0.46	0.30	150.28 ± 0.25	0.17

[a] All values are mean ± SD as obtained by triplicate analyses in a day.
[b] All values are mean ± SD, obtained by triplicate analyses per day over 3 days.
[c] Coefficient of variation = SD/mean × 100%.

Table 5
Plumbagin content of different sources of *P. zeylanica* L.

Sample	Plumbagin content (mg/g dried material)[a]
No. 1[c]	0.861 ± 0.01[b]
No. 2[c]	3.349 ± 0.34
No. 3[c]	4.401 ± 0.25
No. 4[c]	4.975 ± 0.13
No. 5[c]	1.072 ± 0.08
No. 6[c]	2.620 ± 0.17
No. 7[c]	2.729 ± 0.10
No. 8[c]	0.640 ± 0.01
No. 9[c]	3.811 ± 0.13
No. 10[c]	1.699 ± 0.09
No. 11[c]	3.347 ± 0.24
No. 12[d]	2.824 ± 0.18
No. 13[d]	0.629 ± 0.07

[a] All data are calculated as the standard linear equation: $Y = 31.64X - 119.79$, $r^2 = 0.9995$, where Y is peak area, X is the concentration of the analyzed material.
[b] All data are mean ± SD as obtained by triplicate analyses.
[c] Roots and stem of *P. zeylanica* L.
[d] Stem of *P. zeylanica* L.

4.975 mg/g dried plant material. Two *P. zeylanica* L. samples contained more than 4 mg/g of plumbagin, and three samples ranged from 3 to 4 mg/g. Five samples had a lower concentration of plumbagin, ranging from 1 to 3 mg/g, and three samples had even less, with less than 1 mg/g. Of the thirteen samples, the plumbagin contents of Numbers 8 and 13 were the lowest, with 0.640 and 0.629 mg/g plumbagin contents, respectively. These two samples came from part of the stem of *P. zeylanica* L., whereas the other eleven samples, all of which had much higher plumbagin contents, were derived from a mixture of roots and stem. Based on these results, we proposed that plumbagin is mainly presented in the roots of *P. zeylanica* L., which the plumbagin content of the roots of *P. zeylanica* L. is much greater than that of the stem. Empirically, the roots of *P. zeylanica* L. have been the effective part of the plant for eradication of *H. pylori* infection.

4. Conclusion

Plumbagin is mainly presented in the roots of *P. zeylanica* L., which showed strong anti-*H. pylori* and bactericidal activities. A reversed-phase HPLC method with gradient elution for the quantitation of plumbagin from *P. zeylanica* L. was successfully developed. With good linear range, specification, accuracy, inter- and intra-day precisions, and lowly LOD and LOQ were obtained. From this method, we can easily quantify anti-*H. pylori* compound~plumbagin from *P. zeylanica* L.

References

[1] H.L. Li, in: Editorial Committee of the Flora of Taiwan (Eds.), Flora of Taiwan, second ed., vol. IV, Editorial Committee of the Flora of Taiwan, 1998, p. 79.
[2] N.Y. Chiu, K.H. Chang, The Illustrated Medicinal Plants of Taiwan, vol. 2, fifth ed., SMC Publishing Inc., Taipei, 2003, p. 152.
[3] K.R. Kirtikar, B.D. Basu, Indian Medicinal Plants, vol. 2, Shiva Publishers, Dehradun, India, 1993, p. 1466.
[4] S.R. de Paiva, S. da S. Marques, M.R. Figueiredo, M.A.C. Kaplan, Floresta e Ambiente 10 (2003) 98.
[5] H.T. Simonsen, J.B. Nordskjold, U.W. Smitt, U. Nyman, P. Palpu, P. Joshi, Varughese, J. Ethnopharmacol. 74 (2001) 195.
[6] R. Durga, P. Sridhar, H. Polasa, Indian J. Med. Res. A 91 (1990) 18.
[7] N. Atkinson, Aust. J. Exp. Biol. 34 (1956) 17.
[8] J.A. Olagunju, A.A. Jobi, O.O. Oyedapo, Phytother. Res. 13 (1999) 346.
[9] I. Kubo, M. Uchida, J.A. Klocke, Agric. Biol. Chem. 47 (1983) 911.
[10] Y. Dai, L.F. Hou, Y.P. Chan, L. Cheng, P.P.H. Bur, Biol. Pharm. Bull. 27 (2004) 429.
[11] C.P. Bopaiah, N. Pradhan, Phytother. Res. 15 (2001) 153.
[12] L.C. Lin, L.L. Yang, C.J. Chou, Phytochemistry 62 (2003) 619.
[13] Y.C. Wang, T.L. Huang, FEMS Immunol. Med. Microbiol. 43 (2005) 407.
[14] B.J. Johnstone, P.I. Reed, M.H. Ali, J. Gantroenterol. 23 (Suppl. 142) (1988) 69.
[15] M. Clyne, B. Drumm, Infect. Immunol. 61 (1993) 4051.
[16] J.M. Scheiman, A.F. Culter, Am. J. Med. 106 (1999) 222.
[17] B.M. Lee, J.J. Jane, J.S. Kim, Y.C. You, S.A. Chun, H.S. Kim, H.M. Han, M.Y. Ahn, S.H. Byun, Jpn. J. Cancer Res. 89 (1998) 597.
[18] D. Forman, D.G. Newekk, F. Fullerton, J.W.G. Yarnell, A.R. Stacey, N. Wald, F. Sitas, Br. Med. J. 302 (1991) 1302.
[19] L.C. Lin, C.J. Chou, Chin. Pharm. J. 55 (2003) 77.
[20] G.M.K.B. Gunaherath, A.A.L. Gunatilaka, M.U. Sultanbawa, S. Balasubramaniam, Phytochemistry 22 (1983) 1245.
[21] M.A. Iyengar, G.S. Pendse, Planta Med. 14 (1966) 337.
[22] M.M. Gupta, R.K. Verma, G.C. Uniyal, S.P. Jain, J. Chromatogr. 637 (1993) 209.
[23] L.C. Lin, L.L. Yang, C.J. Chou, Phytochemistry 62 (2003) 619.
[24] R. Parimala, P. Sachdanandam, Mol. Cell. Biochem. 125 (1993) 59.
[25] S. Sugie, K. Okamoto, K.M.W. Rhaman, T. Tanaka, K. Kawai, J. Yamahara, H. Mori, Cancer Lett. 127 (1998) 177.
[26] K. Mohana, K.K.P. Purushothaman, Indian J. Exp. Biol. 18 (1980) 876.
[27] N. Didry, L. Dubreuil, M. Pinkas, Pharmazie 49 (1994) 681.
[28] K.M. Abdual, R.P. Ramchender, Immunopharmacology 30 (1995) 231.
[29] P.M. Davidson, M.E. Parish, Food Technol. 43 (1989) 148.
[30] P.E. Coudron, C.W. Stratton, Diagn. Microb. Infect. Dis. 31 (1998) 39.