Determination of Cytotoxic Compounds of Thai Traditional Medicine Called Benjakul Using HPLC

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Benjakul is a Thai Traditional medicine preparation, used for balanced health. From selective interviews of folk doctors in southern Thailand, it was used as the adaptogen drug for cancer patients. In our previous study, the ethanolic extract of Benjakul preparation exhibited high cytotoxic activity against lung cancer cell lines (COR-L23). Piperine has been identified as the main compound in the extract. In addition, plumbagin was found as the most cytotoxic compound. In this study, a reversed-phase high performance liquid chromatography (HPLC) method for quality control such as chemical fingerprint, quantification and stability of the ethanolic extract of Benjakul preparation was developed. The reversed-phase HPLC was performed with a gradient mobile phase composed of water and acetronitrile, and peaks were detected at 256 nm. Based on validation results, this analytical method is precise, accurate and stable for quantitative determination of piperine and plumbagin which are cytotoxic compounds isolated from the ethanolic extract of Benjakul preparation. This method could be suitable for analysis of Benjakul extract.

Keywords: *Benjakul preparation, HPLC, Quantification, piperine, plumbagin*

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Due to the policy of the Ministry of Public health of Thailand, standardization of active compounds in Thai Traditional Medicine is required for standardization before registration as herbal medicine product (HMP). For this aspect, the Thai Herbal Pharmacopoeia was established for the quality control of some HMP. Typically, the chemical finger print and determination of active ingredients of HMP were needed[1].

Benjakul (BEN), a Thai Traditional medicine preparation, is composed of five plants; *Piper chaba* fruit (PC), *Piper sarmentosum* root (PS), *Piper interruptum* stem (PI), *Plumbago indica* root (PL) *and Zingiber officinale* rhizome (ZO). It is commonly used for a balanced health preparation in Thai traditional medicine. From selective interviews of folk doctors in Southern Thailand, Benjakul was used as the adaptogen drug for cancer patients[2]. It was also used as mainly drug for treatment the other diseases by application with patient before using the other drug[3].

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In previous study, the investigators found that the ethanolic extract of Benjahul preparation exhibited high cytotoxic activity against lung cancer cell lines (COR-L23) with IC_{50} value of 19.80 μg/ml[4]. Two compounds, piperine (as major compound) and plumbagin, were isolated from ethanolic extract of Benjakul preparation. Plumbagin exhibited the highest cytotoxic activity against COR-L23 with IC_{50} of 2.55 μM, whereas piperine showed slightly activity with IC_{50} of 43.44 μM. Both piperine and plumbagin (structures showed in Fig. 1) should be used as markers due to the high amount piperine and the highest cytotoxic activity against COR-L23 of plumbagin[4].

The aim of the present study was to develop a reversed-phase HPLC method for studying on chemical fingerprint and quantification of piperine and plumbagin. In addition, the validation of this assay was performed in terms of specificity, linearity,

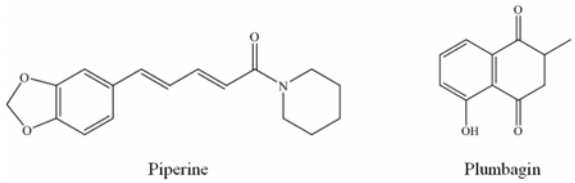

Fig. 1 Structure of piperine and plumbagin

accuracy, precision and limits of detection. The piperine and plumbagin contents from crude extract were determined by HPLC.

Materials and Method

Chemicals and reagents

Standard piperine was purchased from Merck (Darmstadt, Germany), with plumbagin purchased from Sigma-Aldrich (Seelze, Germany), acetronitrile and purified water (HPLC grade) from Labscan (Bangkok, Thailand).

Apparatus and chromatographic conditions

The studied was carried out using a High performance liquid chromatography (HPLC) system (Constametric® 4100 Bio), with ultraviolet visible detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500). A reversed-phase column was Phenomenex Luna 5µ C18(2) 100A analytical column (250 x 4.60 mm 5 micron; Phenomenex, Inc., USA), protected by a Security Guard Cartridge (C18, 4 x 3.0 mm; Phenomenex, Inc., USA).

The mobile phase was composed of water-acetronitrile with gradient elution as follows: 0 minute, 60: 40; 30 minutes, 50: 50; 50 minutes, 5: 95; 60 minutes, 0: 100. The mobile phase was filtered under vacuum through a 0.45 µm membrane filter before use. The flow rate was 1 mL/minute with UV absorbance detection at 256 nm. The operating temperature was maintained at room temperature.

Plant Materials

The parts of plants, which were reported to be used against anticancer by folk doctors in Thailand, were collected from all parts of Thailand in January to March 2006. Place of collection were all of part of Thailand and the voucher numbers , *Piper chaba*. Fruit (Amphor Thongphaphoom, Kanjanaburee Province, SKP 146160301), *Piper sarmentosum* root (Amphor Hadyai, Sonkhla Province, SKP 146161901), *Piper interruptum* stem (Amphor Maerim, Chaingmai Province, SKP 146160901), *Plumbago indica* root (Amphor Bankoknoi, Bangkok, SKP148160901), *Zingiber officinale* rhizome (Amphor Khaokho, Petchaboon Province, SKP206261501). Authentications of plant materials were carried out at the herbarium of the Department of Forestry Bangkok, Thailand where the herbarium vouchers have been kept to specify plant and species identified. Another one of these plants have been keep to specimen in the herbarium of Southern Center of Thai Medicinal plant at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla.

Preparation of Benjakul extracts

Five plant materials were dried at 50°C 100 g of each plants was combined as Benjakul preparation, powdered and macerated with 95% ethanol. It was then filtered and concentrated to dryness under reduced pressure to obtain the ethanolic extracts. The percentage of yields were 7.73. The ethanolic extract of Benjakul preparation was dissolved with acetronitrile, sonicated for 15 minutes and filtered through a 0.45 µm membrane filter before use.

Standard solutions prepared

A stock solution of piperine and plumbagin (concentration 1.0 mg/ml) was prepared with acetronitrile and stored at -18°C until use.

Validation of HPLC method

The validation of the analytical method for plumbagin and piperine from Benjakul extract was examined in terms of specificity, linearity, accuracy, precision as well as the limit of detection (LOD) and limit of quantitation (LOQ).

Specificity

Standard piperine solution (200 µg/ml), plumbagin solution (50 µg/ml) and the sample solutions of the ethanolic extract of Benjakul (10 mg/ml) were prepared in acetronitrile. The acetronitrile was also used as a control. A volume of 10 µl was injected into the HPLC column individually.

Linearity

The linearity was validated by preparing the standard piperine and plumbagin solutions at least 5 concentrations. A volume of 10 µl of each concentration 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.

Precision

The precision was validated by preparing the standard piperine and plumbagin solutions at least 3 concentrations. A volume of 10 µl of each concentration was injected into the HPLC column. Concentrations of standard compound 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. Coefficient of variation (CV) was calculated as standard deviation (SD) to the mean value from the results of triplicate testing and not more than 2%.

Accuracy

The standard of piperine and plumbagin with the known amount were spiked to the ethanolic extract of Benjakul sample solution, where the contents of piperine and plumbagin had been previously determined before adding the standard compounds. The three injections for each concentration were performed per day over three different days (3 injections x 3 concentrations x 3 days) and calculated % recovery of standard piperine and plumbagin.

LOD and LOQ

For limit of detection (LOD) and limit of quantitation (LOQ), serial dilutions of piperine and plumbagin were made with acetronitrile and then analyzed using the HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

Results

Validation of Analytic Method

Specificity Validation

The results of HPLC chromatograms for specificity validation are shown in Fig. 2. It was apparent that piperine was a major compound of the ethanolic extract of Benjakul preparation, with retention time of 28.04 min. Plumbagin is a minor compound in the ethanolic extract of Benjakul preparation, with retention time of 24.59 min. However, there are interfering peaks observed around the peak of piperine and plumbagin.

Quantitation parameters

The linearity of the piperine and plumbagin standard curves were examined. Serial dilutions of standard piperine and plumbagin were prepared and analyzed. The results are shown in Table 1. Three separate calibration curves of each standard obtained on different days by plotting the peak area versus concentration were found to be linear when evaluated by linear regression analysis. The linear equation of $Y = 23035X\text{-}102552$ and correlation coefficient ($r2$) of 1 were obtained from piperine standard curve. For plumbagin, the linear equation of $Y = 35887X\text{-}96639$ and correlation coefficient ($r2$) of 0.9998 were obtained from standard curve. As shown by the results of Table 1, the standard curve of piperine and plumbagin are linear at the concentrations range of 50-400 µg/mL and 10-200 µg/mL, respectively.

The limit of detection represents the lowest concentration of piperine and plumbagin that can be detected by the instrument and the analytical method, whereas the limit of quantitative represents the lowest

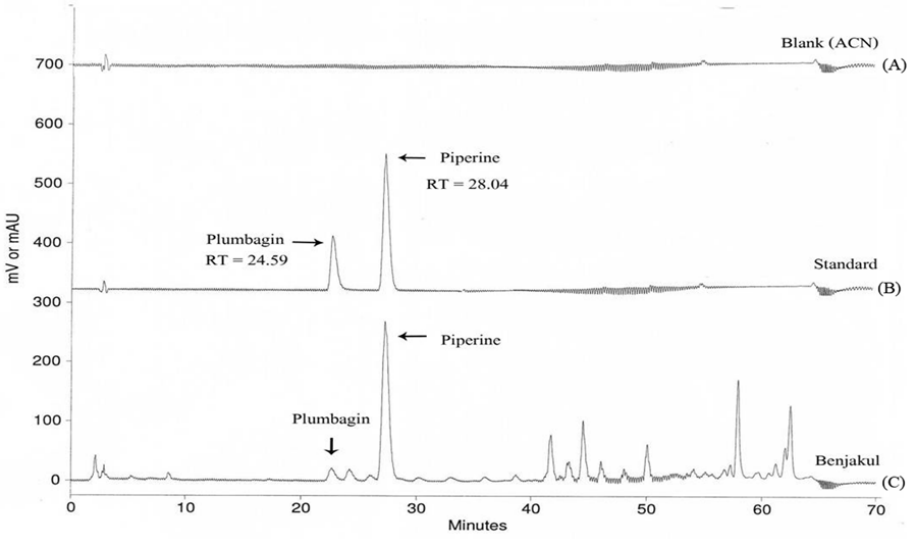

Fig. 2 The specificity validation for the HPLC analytical method for piperine and plumbagin: (A) blank solution; (B) piperine and plumbagin standards solution and (C) ethanolic extract of Benjakul preparation sample solution.

Table 1. Parameters of quantitation for piperine and plumbagin

Parameter	Piperine	Plumbagin
Linear range (µg/ml)	50-400	10-200
Equation	Y = 23035X – 102552[a]	Y = 35887X-96639[a]
Linearity (r^2)	1	0.9998
LOD (µg/ml)[b]	0.80	0.22
LOQ (µg/ml)[c]	2.66	0.75

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

Table 2. Validation of precision of the analytical method for piperine

Theoretical concentration (µg/ml)	Intra-day[a] (n = 3) Measured concentration (µg/ml)	CV (%)[c]	Inter-day[b] (n = 9) Measured concentration (µg/ml)	CV (%)[c]
50	51.03 ± 0.59	1.15	50.69 ± 1.00	1.97
100	100.53 ± 0.31	0.31	100.14 ± 0.60	0.60
200	200.57 ± 1.31	0.65	199.23 ± 2.09	1.05

[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 x 100%.

concentration of piperine and plumbagin that can be determined with acceptable precision and accuracy by the instrument and method. The results of LOD and LOQ analysis for piperine were found to be 0.80 and 2.66 µg/mL, respectively. For plumbagin, LOD and LOQ analysis were found to be 0.22 and 0.75 µg/mL, respectively. The results indicated that the analytical method for the quantitative of piperine and plumbagin exhibited good sensitivity for UV-visible detector that used in this work.

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 showed in Table 2, 3, both intra- and inter-day precisions of piperine and plumbagin were higher than 93%, for which 0.31-1.42% and 0.60-1.97% of coefficient variations, respectively. The results indicated that the method for quantitative determination of piperine and plumbagin from ethanolic extract of Benjakul preparation have good precision.

Accuracy validation

The accuracy of the method was determined by investigating the recovery of samples of spiking standard piperine and plumbagin into ehtanolic extract of Benjakul preparation and comparing the measured value to the true value, which recoveries nears to 100% indicating a good accuracy of this method obtained. The results showed in Table 4,5, piperine and plumbagin had good recoveries, for which ranging from 93.68 to 100.92%, with 0.20-1.36% of coefficient variations. It demonstrates that the analytical method has good accuracy.

Determination of the ehtanolic extract of Benjakul preparation content

The ethanolic extracts of Benjakul preparation was determined contents of piperine and plumbagin by using HPLC methods. The results was found the amount of piperine and plumbagin were 47.61 mg/g

Table 3. Validation of precision of the analytical method for plumbagin

Theoretical concentration (μg/ml)	Intra-day[a] (n = 3) Measured concentration (μg/ml)	CV(%)[c]	Inter-day[b] (n = 9) Measured concentration (μg/ml)	CV(%)[c]
25	23.46 ± 0.16	0.70	23.38 ± 0.18	0.76
50	49.95 ± 0.60	1.19	49.69 ± 0.58	1.16
100	100.22 ± 1.42	1.42	100.21 ± 1.23	1.23

[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 x 100%.

Table 4. Validation of the accuracy of the analytical method for piperine

Spiked level (μg/ml)	Recovery (%)[a]			Mean (%)	CV (%)[b]
	1	2	3		
50	93.39 ± 0.62	93.29 ± 0.43	94.36 ± 0.67	93.68	0.63
100	95.52 ± 0.84	95.97 ± 0.11	96.59 ± 0.17	96.02	0.56
200	97.15 ± 0.05	97.22 ± 0.03	97.52 ± 0.07	97.30	0.20

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

Table 5. Validation of the accuracy of the analytical method for plumbagin

Spiked level (μg/ml)	Recovery (%)[a]			Mean (%)	CV (%)[b]
	1	2	3		
25	97.93 ± 0.95	96.36 ± 0.82	97.75 ± 1.12	97.35	0.89
50	100.52 ± 1.28	99.80 ± 1.97	102.44 ± 0.92	100.92	1.36
100	96.78 ± 1.16	98.92 ± 0.89	99.18 ± 1.20	98.30	1.34

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

and 2.46 mg/g as show in Table 6.

Discussion

A reverse-phase high performance liquid chromatographic (RP-HPLC) procedure was used for study chemical fingerprint of the ehtanolic extract of Benjakul preparation. The method was validated and showed good linearity, precision, accuracy and recovery. The calibration curves are linear over the ranges of 50-400 μg/ml for piperine and 25-200 μg/ml for plumbagin, respectively with $r^2 > 0.999$. The limit of detection (LOD) and limit of quantitation are 0.80 and 2.66 μg/ml for piperine and 0.22 and 0.75 μg/ml for plumbagin, respectively. The precision of the HPLC

Table 6. Plumbagin and piperine contents of Benjakul extraxt

Plumbagin content (mg/g)[a]	Piperine content (mg/g)[b]
2.46 ± 0.02	47.61 ± 0.42

[a] All data are calculated as the standard linear equation: Y = 35994X-92792, r^2 = 0.9997, where Y is peak area, X is the concentration of the analyzed sample.
[b] All data are calculated as the standard linear equation: Y = 22878X-95581, r^2 = 0.9999, where Y is peak area, X is the concentration of the analyzed sample.
All data are mean ± SEM as obtained by triplicate analyses

method for determining piperine and plumbagin, confirmed by analyzing both intra- and inter-day, were higher than 93%. All the coefficient variation for piperine and plumbagin were less than 2%. The accuracy of the method for piperine and plumbagin were studied by spiking standard piperine and plumbagin into the ehtanolic extract of Benjakul preparation. The percentage recoveries for piperine and plumbagin were found to be ranging from 93.68 to 100.92%, with 0.20-1.36% of coefficient variations. It demonstrates that the proposed method has good precision and accuracy.

Conclusion

A reverse-phase HPLC procedure was used for study chemical fingerprint of the ehtanolic extract of Benjakul preparation. The method was validated and showed good linearity, precision, accuracy, recovery and lowly LOD and LOQ. This analysis method could be considered for quantitative determination of piperine and plumbagin in the Benjakul extract.

Acknowledgements

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การหาปริมาณสารที่ออกฤทธิ์เป็นพิษต่อเซลล์ของตำรับยาแผนไทยชื่อเบญจกูล โดยใช้เทคนิค HPLC

อรุณพร อิฐรัตน์, อินทัช ศักดิ์ภักดีเจริญ

เบญจกูลเป็นตำรับยาแผนไทยใช้เป็นตำรับยาปรับสมดุลของร่างกาย จากการสัมภาษณ์หมอพื้นบ้านภาคใต้ที่ถูกคัดเลือกพบว่ามีการใช้เป็นยาปรับธาตุสำหรับผู้ป่วยมะเร็งมีการศึกษาพบว่า สารสกัดเบญจกูลด้วยเอทธานอล แสดงฤทธิ์ต้านมะเร็งปอด (COR-L23) สารสำคัญที่ออกฤทธิ์คือ piperine และ plumbagin ออกฤทธิ์ต้านมะเร็งได้ดีที่สุดในการศึกษาครั้งนี้เป็นการพัฒนาเทคนิค reversed-phase high-performance liquid chromatography (HPLC) ในการควบคุมคุณภาพได้แก่การดูลายพิมพ์นิ้วมือการหาปริมาณสารสำคัญของสารสกัดชั้นเอทธานอลของตำรับเบญจกูล โดยใช้ตัวทำละลายเคลื่อนที่เป็นน้ำและ acetronitrile ภายใต้ความยาวคลื่น 256 นาโนเมตร การรับรองผลโดยการวิเคราะห์ ความเที่ยงตรงความแม่นยำ และปริมาณของ piperine และ plumbagin ซึ่งเป็นสารสำคัญที่ออกฤทธิ์ต้านมะเร็ง วิธีการที่ได้นี้สามารถใช้ในการวิเคราะห์ปริมาณสารสำคัญของสารสกัดเบญจกูลได้