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Metabolite Fingerprinting of *Piper nigrum* L. from Different Regions of Thailand by UHPLC-QTOF-MS Approach and *In Vitro* Bioactivities

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Abstract

This study aimed to profile the metabolites, piperine content, and biological activities (antioxidant, anti-inflammatory, anticancer, antibacterial, and apoptotic cell) of 15 samples of *Piper nigrum* L. from various geographic regions of Thailand. UHPLC-QTOF-MS was applied to establish alkaloid profiles of the *P. nigrum* L. samples. PCA and HCA analysis showed that the samples from the PN-1, PN-2, and PN-11 sources differed significantly from those of other sources in terms of compound type and alkaloid concentration profile. Comparative analysis based on a validated HPLC-DAD method was used for the standardization of 15 varieties of *P. nigrum* L. with piperine as a standard phytomarker which revealed that PN-4 source possessed a high content of piperine. PN-2 showed the highest antioxidant capacity (DPPH activity) while PN-10, PN-12, and PN-15 showed the highest FRAP activity. PN-11 source showed high inhibition of anti-inflammatory and anticancer properties. Furthermore, PN-3, and PN-4 also showed the highest both anti-inflammatory and antibacterial activity. Almost samples have been shown to induce late apoptosis and necrosis in SW480 cell line. This study will also be useful for the development of quality control parameters through the standardization of varieties of *P. nigrum* L. as raw materials in traditional medicine formulations.

Keywords: *Piper nigrum* L., HPLC, UHPLC-Quadrupole time of flight (QTOF)-MS, Piperine, Metabolite fingerprint

Introduction

Piper nigrum L. (from the Piperaceae family), namely, "black pepper", is a climbing shrub that is found mostly in India, Indonesia, Malaysia, Vietnam, and Thailand [1]. It is used in traditional medicine (TMs) worldwide, such as in Ayurveda & Unani from India, traditional Chinese medicine, and Kampo. In Unani, it is known as FilfilSiyah and is used to treat various conditions, such as flatulence (Nafkh-e-Shikam), indigestion (Zof-e-Hazam), and skin problems (Bars-o-bahak) [2]. It is known as Maricain in Ayurveda and is used to treat cough, cold, and digestive problems in well-known formulations such as Trikatu [3]. In Thai traditional medicine, fruits and vines are used as carminative, antiflatulent, appetizer, and nourishment pills [2]. Phytochemical studies of *P. nigrum* L. identified alkaloid groups, which included Piperine and a large amount Piperazine [4], and essential oils, which included monoterpenes such as limonene, pinene, and sabinene [5]. *P. nigrum* L. has been evaluated in terms of anti-inflammatory [6], antitumour [5], anticancer [6], antibacterial, antioxidant [7], anti-apoptotic [8], anti-depressant [3,9], anti-fungal [10], analgesic [11], anti-pyretic [11], anti-diarrhoeal, anti-spasmodic [12], anti-mutagenic [13], anti-spermatogenic [14], anti-thyroid [15], and larvicidal activities [16].

Scientific validation of *P. nigrum* L. to ensure its rational use in therapeutic efficacy, safety in healthcare along with its quality evaluation are crucial [17]. The importance of TMs is gaining relevance in health sectors due to several therapeutic benefits. Validation of TM involves chemical profiling to systematically investigate and standardize in terms of quality, safety, and efficacy of traditional herbal formulations by quantification of bioactive compound, spurious drug determination, comparative fingerprint analysis, stability and the quality consistency of TMs [17]. Maintaining the quality of botanical extracts are challenging as the raw materials are collected from wild sources [18]. Several environmental and geographical parameters include soil conditions, availability of light and water, temperature variations, nutrients, and geographical location, which affect the chemical composition of plants [19]. Whereas, change in physical appearances and chemical constituents of plants can happen by cultivation harvesting techniques and storage methods used. Thus, the quality parameter should be monitored from the raw material to plant extracts and final products as they show significant variance in terms of chemical constitution, quality, and therapeutic effects [20,21]. Therefore, standardization of herbal drugs consider authentication of raw material, and assessment quality assurance of the intermediates like extracts and final products involving simple techniques of pharmacognosy and physicochemical studies to analytical chemical fingerprinting techniques like high-performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS/MS) [22].

P. nigrum L. is a common herbal ingredient in the remedies that are listed in the national list of essential medicines of the Thailand Food and Drug Administration, such as the Prab-Chom-Phu-Ta-Weep and Ar-Phai-Sa-Lee remedies, which is used to relieve first-stage cold symptoms and allergic rhinitis. However, to meet the increasing demand in the market for herbal raw materials, *P. nigrum* L. samples are vulnerable to adulteration and contamination [23]. The development of quality standardization parameters of Thai *P. nigrum* L. is crucial. The geoclimatic variation in quality is another crucial factor that should be carefully considered when developing quality standards.

Therefore, the objective of this study was to compare the metabolomics approach and comparative investigation of *P. nigrum* L. from 15 geographical regions for the identification of the compositional variation of metabolites within the varieties of the same species. Also, information on the 1 activities of *P. nigrum* L. extracts from 15 geographical regions are investigated. This validated scientific orientation will help address the quality-related aspects of medicinal plants or herbal formulations that are used in Thai traditional medicine.

Materials and methods

Chemicals and reagents

A piperine standard was purchased (> 95 % HPLC) from Sigma Aldrich. n-Hexane (AR grade), dichloromethane (AR grade), ethanol (99.9 % absolute denatured, AR grade), toluene (AR grade), sulfuric acid (10 %, AR grade), and hydrochloric acid (37 %, AR grade) were purchased from QRëC (New Zealand). Methanol (AR grade), ethyl acetate (AR grade), acetonitrile (HPLC grade), and formic acid (HPLC grade) were purchased from RCI Labscan (Bangkok, Thailand). Syringe filters (0.45 μm) and membrane filters (0.22 μm) were purchased from Millipore (Bangkok, Thailand).

Plant materials

Fifteen samples of *P. nigrum* L. fruits were collected from various geographical areas in Thailand, which included Chiang Mai (PN-1), Krabi (PN-2), Sa Kaeo (PN-3), Phitsanulok (PN-4), Chiang Rai (PN-5), Phichit (PN-6), Nakhon Ratchasima (PN-7), Lamphun (PN-8), Uthai Thani (PN-9), Udon Thani (PN-10), Nakhon Sawan (PN-11), Ubon Ratchathani (PN-12), Chaiyaphum (PN-13), Lampang (PN-14), and Tak (PN-15) provinces. All sets of crude drugs were authenticated by Ruangrungsi N. Voucher specimens were deposited at the Medicinal Plants Innovation Center of Mae Fah Luang University, Chiang Rai, Thailand. Information about these *P. nigrum* L. samples, including cultivation region, latitude, longitude, and Voucher specimens number, are shown in **Table 1**.

Table 1 Biological activity data of *P.nigrum* L. extracts from 15 different sources throughout Thailand.

Source	Antioxidation		Anti-inflammatory	Anti-cancer	Antibacterial inhibition zone (mm)			
	DPPH assay (µg/mL)	FRAP value[a]	% NO inhibitions	% cell growth inhibition (SW480)	*S. aureus*	*M. luteus*	*E. coli*	*C. albicans*
PN-1	419.97 ± 0.32	0.15 ± 0.01	69.90 ± 3.92	37.97 ± 2.98	NA	NA	NA	NA
PN-2	79.45 ± 2.55	0.25 ± 0.02	43.00 ± 3.60	9.28 ± 1.82	6.5	6.5	NA	NA
PN-3	261.12 ± 8.68	0.11 ± 0.01	70.80 ± 2.08	7.00 ± 2.22	7	7	NA	NA
PN-4	158.20 ± 7.86	0.17 ± 0.01	66.80 ± 1.05	7.48 ± 2.40	7.8	7	NA	NA
PN-5	365.70 ± 5.38	0.24 ± 0.01	56.30 ± 0.75	90.65 ± 0.72	NA	NA	NA	NA
PN-6	158.50 ± 0.24	0.22 ± 0.02	57.00 ± 2.86	58.62 ± 3.03	6.5	NA	NA	NA
PN-7	148.38 ± 3.92	0.25 ± 0.00	44.70 ± 2.26	11.52 ± 0.67	6.5	6.5	NA	NA
PN-8	334.79 ± 0.55	0.20 ± 0.02	28.20 ± 3.12	79.88 ± 0.77	NA	NA	NA	NA
PN-9	216.18 ± 1.06	0.25 ± 0.00	29.40 ± 3.04	58.82 ± 1.61	7.5	6.5	NA	NA
PN-10	198.43 ± 0.96	0.39 ± 0.02	26.30 ± 1.95	9.11 ± 2.55	NA	NA	NA	NA
PN-11	213.54 ± 1.92	0.23 ± 0.02	64.80 ± 2.38	80.98 ± 1.64	NA	NA	NA	NA
PN-12	190.48 ± 3.92	0.34 ± 0.01	46.20 ± 3.13	6.88 ± 2.72	NA	6.5	NA	NA
PN-13	245.33 ± 7.27	0.09 ± 0.03	57.30 ± 2.89	14.82 ± 1.82	6.5	NA	NA	NA
PN-14	207.55 ± 3.80	0.04 ± 0.00	21.30 ± 1.59	58.45 ± 1.58	7	7	NA	NA
PN-15	202.46 ± 0.46	0.33 ± 0.02	60.40 ± 2.52	41.64 ± 2.95	7	NA	NA	NA
Ascorbic acid	1.04 ± 0.04	-	-	-	-	-	-	-
(+)−Catechin	1.24 ± 0.02	0.54 ± 0.003	-	-	-	-	-	-
Ampicillin	-	-	-	-	47	45	25	-
Doxorubicin	-	-	-	76.77 ± 0.36	-	-	-	-
Indomethacin	-	-	66.80 ± 2.41	-	-	-	-	-

[a] mM $FeSO_4$/mg crude extract
NA = not active

Extraction of *P. nigrum* L. fruits

The fruits of *P. nigrum* L. were air dried in the shade at room temperature and pulverized using a mechanical grinder to produce a coarse powder. The powdered samples (50 g) were extractedusing maceration with EtOH under a rotary shaker at 150 rpm for 12 h. This process of extraction was repeated for 3 times to achieve maximum extraction. The extracts were filtered using Whatman filter paper and evaporated under vacuum using a rotary evaporator at 50 °C to yield the dry extract, which was stored at 4 °C. Afterward, the dried extract (10 mg) was suspended in acetonitrile and filtered through 0.22 µm membranes to obtain the stock solution. The stock solution was diluted to provide a 1 mg/mL sample concentration for chromatographic analysis. The percentage yield was calculated and tabulated (Table 1).

UHPLC-QTOF-MS Analysis

The dried extract (10 mg) was suspended in acetonitrile and filtered through 0.22 µm membranes to obtain the stock solution. The stock solution was diluted to provide a 1 mg/mL sample concentration for UHPLC-QTOF-MS analysis. LC-MS/MS was conducted at Mae Fah Luang University, Thailand using an Agilent LC-QTOF 6500 system with an Agilent ZORBAX Eclipse Plus - C18 column (2.1×50 mm, 1.8 µm). The mobile phases were 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) as eluents in gradient mode. The injected volume was 1.0 µL, and the column temperature was maintained at 30 °C. The flow rate was maintained constant throughout the gradient. The column was eluted at a flow rate of 1 mL/min using a linear gradient as follows: 5 % (B) at 0 - 1 min, 5 - 17 % (B) at 1 - 10 min, 17 % (B) at 10 - 13 min, 17 - 100 % (B) at 13 - 20 min, 100 % (B) at 20 - 22 min, 100 - 5 % (B) at 22 - 25 min, and 5 % (B) at 25 - 26 min. The UHPLC system was coupled to a QTOF mass spectrometer (6,500 series; Model-G6545B) that was equipped with a Dual AJS ESI source. The parameters for analysis were set using positive ion mode with spectral acquisition over a mass range from *m/z* 100 - 1,000 and a scan rate of 2

(spectra/s). The MS data were processed using the Mass Hunter vB.0 8.00 software, which provided a list of possible elemental formulae by integrated libraries. Chromatographic peak extraction was performed using the Mass Hunter Profinder software (version 10.0) with molecular feature extraction. The accuracy for the identification of the compounds was established based on the error of less than 5 ppm and MS/MS fragment matching.

MS data interpretation and chemometrics analysis

The Agilent Mass Hunter Qualitative Analysis software, version 8.00, was used for the initial processing of the LC/MS data. Compounds were identified using the molecular feature extractor (MFE) tool in the software. Mass Hunter Profiler Professional (MPP), version 15.1, was used for statistical analysis to profile the samples, such as Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA). The Agilent Metlin Metabolite Personal Compound Database and Library (PCDL) version 5.0 was used to identify the compounds based on MS/MS spectral matching. Only compounds with a minimum peak abundance level of 0.05 were selected. One-way analysis of variance (ANOVA) was used to limit the set to compounds that varied at the $P < 0.05$ level because the sample set produced a list of 20 compounds that were used for statistical analysis.

High performance liquid chromatography (HPLC) analysis and method validation

HPLC analysis was conducted on an Agilent Technology HPLC 1,260 Infinity II system. An Infinity Lab Poroshell 120 EC-C18 column (4.6×150 mm, 4 µm) was used for the separation. The column was maintained at room temperature. The mobile phase consisted of water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The column was eluted at a flow rate of 1 mL/min using the following linear gradient: 52 % (B) at 0 - 7 min, 52 - 65 % (B) at 7 - 8 min, 65 % (B) at 8 - 11 min, 65 - 90 % (B) at 11 - 12 min, 90 % (B) at 12 - 17 min, at 17 - 18 min 50 % (B), and at 18 - 35 min 50 % (B). The solution was filtered through a 0.45 µL syringe filter prior to injection. HPLC method validation was conducted by determining the linearity, specificity, accuracy and precision, the limit of quantification, and the limit of detection based on the International Conference on Harmonization guidelines (ICH Q2 (R1) Guideline). The method specificity was determined by comparing the retention times of the standard and test samples. The sensitivity was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) and calculated based on the equation LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation and S is the slope of the calibration curve. The accuracy of each sample was determined by the recovery method. The sample was spiked with piperine (5, 50 and 100 µg/mL), and the recoveries were calculated by comparing the amounts of those standards with the amount that was originally added. The precision of each sample was evaluated at 2 levels: Repeatability and intermediate precision. The relative standard deviation (RSD) of 9 determinations that covered the specified range (3 concentrations and 3 replicates each) was evaluated and analysed on the first day and 3 consecutive days thereafter. System suitability testing was conducted by using 6 replicates of test concentrations. The variations in the number of theoretical plates, capacity factor, and tailing factor were also calculated from an average of 6 replicates. Statistical analysis was conducted using GraphPad Prism Version 5.0. The results are shown as the mean ± % RSD.

Antioxidant capacities

The method of 2,2 Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and ferric reducing antioxidant powder (FRAP) were the same as in the previous reports [24,25].

Cancer cell (SW480) growth using MTT assay

The percentage of growth inhibition was measured using the MTT assay. SW480 cells were seeded at 4×10^4 cells/well in 96-well plates and incubated at 37 °C and 5 % CO_2 overnight. The cells were treated with 50 µg/mL of samples for 24 h. After 24 h, the cells were washed with PBS and incubated with a 0.5 mM MTT reagent for 4 h. Formazan was detected at 570 nm with a microplate reader. Doxorubicin was used as a positive control.

The percentage growth inhibition can be calculated via the following formula:

% growth inhibition = $[OD_{control} - OD_{sample})/ OD_{control}]\times100$. The results are shown in **Table 1**.

Apoptosis activity using annexin V-FITC/propidium iodide (PI) staining

SW480 cells were seeded 4×10^5 cells/well in 24 well-plates. Cells were treated with samples at concentration 100 µg/mL of for 24 h. Then the cells were stained with Annexin V-FITC/propidium iodide (PI) for 20 min at *P. nigrum* L. room temperature in the dark (Biorad, USA). The apoptotic and necrotic cells were quantified via flow cytometry and analyzed using CyExpert for DeFLeX program.

Anti-inflammatory activity using nitric oxide (NO) assay

RAW 264.7 macrophage cells were used to evaluate the inflammatory activity of *P. nigrum* L. ethanolic extracts. The cells were cultured in DMEM that was supplemented with 10 % FBS. For the nitric oxide assay, the cells were seeded at 4×10^4 cells/well in 96-well plates and incubated at 37 °C and 5 % CO_2 overnight. The cells were cultured with 1 µg/mL LPS for 1 h, which was followed by treatment with *P. nigrum* L. extracts (50 µg/mL) for 24 h. After 24 h, 50 µL of each of the samples was transferred to a new plate. Additionally, each of the samples was mixed with 50 µL of the Griess reagent for 10 min. The nitric oxide was determined at 570 nm with an EZ read 400 microplate reader. Indomethacin was used as a positive control. All samples were prepared in triplicate.

Anti-bacterial activity

The antibacterial activity of *P. nigrum* L. ethanolic extracts was evaluated using 4 bacterial strains: Two strains of gram-positive bacteria (*Staphylococcus aureus* and *Micrococcus luteus*), 1 strain of gram-negative bacteria (*Escherichia coli*), and 1 strain of fungi (*Candida albicans*). Each bacterial strain was subcultured overnight at 37 °C for 3 - 5 h in an incubator. The bacterial growth was harvested using 5 mL of 0.85 % sterile saline water, and its turbidity was adjusted by using McFarland No. 0.5 and diluted to attain a viable cell count of 1.5×10^8 CFU/mL.

The disc diffusion method was used to determine the antibacterial activity of *P. nigrum* L. ethanolic extracts. Each extract of *P. nigrum* L. (100 mg/mL) was dissolved with 10 % DMSO. Then, the samples were loaded over sterile filter paper discs (6 mm in diameter) to obtain a final concentration of 400 µg/disc and dried in a laminar airflow for 24 h before use. Mueller-Hilton agar medium (MHA) was inoculated with the bacterial suspension to attain 1.5×10^7 CFU/mL of the medium. Discs that were loaded with plant extract were placed on top of Mueller-Hilton agar plates. Ampicillin was used as a positive control (1 mg/100 µL), and 10 % DMSO was used as a negative control. The plates were incubated in an incubator at 37 °C for 16 - 18 h to enable extract diffusion. The presence of inhibition zones was measured, recorded, and regarded as an indicator for antibacterial activity [26] (**Table 1**).

Results and discussion

Quantification of piperine via the validated HPLC method

The piperine content from *P. nigrum* L. extracts in the HPLC columns was achieved within the run time of 23 min. The signal appeared at t_R 5.9 min. The content of piperine in the *P. nigrum* L. extracts was determined using a calibration curve that was obtained by plotting the mean peak area (y-axis) versus the concentration (x-axis). **Table 2** lists the piperine contents of samples of *P. nigrum* L. that were collected from various geographical locations. The study showed that piperine was present in *P. nigrum* L. from PN-4 and PN-13 with the highest contents at 0.14 and 0.12 mg/mL, respectively. The lowest contents (0.05 - 0.06 mg/mL) of piperine were found in PN-5, PN-7, PN-11, and PN-14 (**Table 2**).

A chromatogram of standard piperine is presented in **Figure 1**, and chromatograms of the 15 samples are presented in **Figure 2**. HPLC analysis showed that the linearity range of the response of the piperine method was 5 - 100 µg/mL. The correlation coefficient was determined from the calibration curve as > 0.99, which demonstrates that the data are close to the line of best fit. The regression equation was found to be $y = 249.08\times-939.14$ with $R^2 = 0.9875$. The LODs and LOQs were estimated to be 8.97 and 27.20 µg/mL, respectively. The % recovery value (95.35 - 97.23 %) indicated that the method was accurate (**Table 3**). The % RSDs of the intraday and interday precisions were found to be < 2 %, which demonstrated the high repeatability of the method. The number of theoretical plates, capacity factor, and tailing factor were found to be 3,042 (desirable > 2,000), 7.72 (desirable 2 - 10), and 1.25 (desirable < 1.5), respectively, from the mean of 6 determinations of the test concentration.

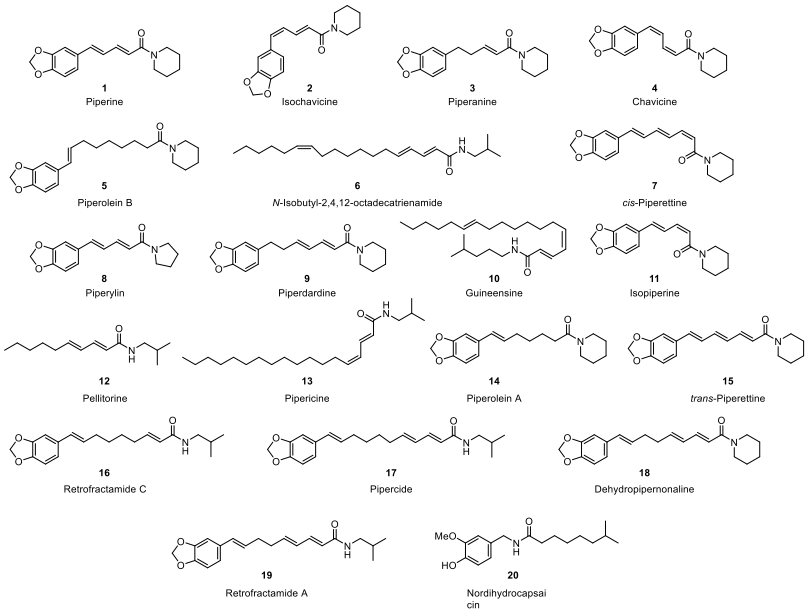

Figure 1 Chemical structures of 20 alkaloid compounds from *P. nigrum* L. for fingerprint study.

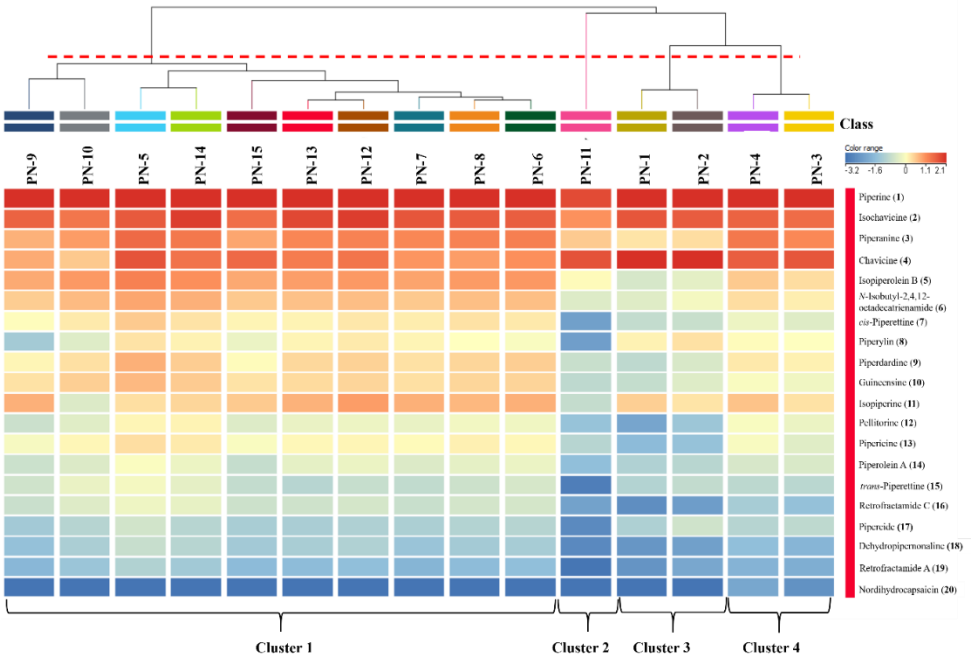

Figure 2 Heat map of 20 major peaks of *P. nigrum* L. from different geographical locations of Thailand.

Identification of metabolites via UHPLC-QTOF-MS analysis

UHPLC-QTOF-MS analysis resulted in the identification of twenty phyto-compounds (**Figure 1**) in each variety of *P. nigrum* L., which include piperine (1), isochavicine (2), piperanine (3), Chavicine (4), piperolein B (5), *N*-isobutyl-2,4,12-octadecatrienamide (6), *cis*-piperettine (7), piperylin (8), piperdardine (9), guineensine (10), isopiperine (11), pellitorine (12), pipericine (13), piperolein A (14), *trans*-piperettine (15), retrofractamide C (16), pipercide (17), dehydropipernonaline (18), retrofractamide A (19), and nordihydrocapsaicin (20). The total ion chromatographs (TICs) of various varieties of *P. nigrum* L. samples are presented in **Figure 3**, and a list of the identified compounds is presented in **Table 4** for all the samples of *P. nigrum* L.

Data extraction

Metabolomic data analysis is a productivity tool for processing multiple samples in profiling analyses, which enables the user to visualize, review, and edit results by compound across many samples. Higher quality results can be obtained based on cross-sample processing. Chromatographic peak extraction was performed with the Profinder software (version B. 08) using molecular feature extraction (MFE) to identify peals and MPP to align masses and retention times, which revealed a set of approximately 20 compounds that were present in all samples of *P. nigrum* L. that were collected from various geographical locations of Thailand. Only compounds with a minimum peak abundance level of 0.05 were selected. One-way analysis of variance (ANOVA) was used to limit the set to compounds that varied at the $p < 0.05$ level because the sample set produced a list of 20 compounds that were used for statistical analysis.

Hierarchical clustering analysis (HCA)

To classify samples, a hierarchical clustering analysis was performed. HCA is an unsupervised classification procedure that involves measuring either the distance or the similarity between objects. It is an ideal technique for the classification of *P. nigrum* L. fruit varieties because it does not require previous information. In this study, a matrix of 15 geographical locations×20 major mass abundances were formed by the relative mass abundances that were extracted using UHPLC-QTOF-MS from 15 mean fingerprint chromatograms of the Piper samples from various geographical locations in Thailand. Each row represents the mass abundance for each sample plant, and each column represents the group. The HCA analysis was classified into 4 groups (cluster 1 - 4). The PN-11 sample was classified as the first group (cluster 2), the PN-1 and PN-2 samples were assigned to the second group (cluster 3), the PN-4 and PN-3 samples were assigned to the third group (cluster 4), and other samples were classified as the 4 group (cluster 1). These results demonstrated that the alkaloid profile of the sample from PN-11 differed from those of the other groups. However, compounds 1 and 4 showed a high concentration in the first group. The alkaloid profiles of the second and third groups showed similarly high contents of compounds 1, 2, 4 and 20. In the 4 group, the samples from PN-5 and PN-14 showed high alkaloid profile concentrations. Therefore, alkaloid derivatives may be regarded as biomarkers for the discrimination of geographic regions of Thailand.

Principal component analysis (PCA)

Chemometrics techniques provide a measure for mining more useful chemical information from the original data. Comprehensive methods and hyphenated techniques that are associated with chemometrics are used to extract useful information and supply various methods for data processing in herbal drug analysis. PCA is the most commonly used technique and was further applied to summarize the multivariate variation for wide data matrices in UHPLC-QTOF-MS fingerprinting analyses. In the analysis, PCA computation was performed on the data array of the UHPLC-QTOF-MS fingerprints. **Figure 3** presents the pairwise comparison of the PC factors of each sample. The first principal component (PC1) contributed 63.53 % of the variability in the data set. The second PC (PC2) accounted for 15.94 % of the variance in the data. PC1 and PC2 described 79.47 % of the variance and reflected significant variability in the data. According to Rafi *et al.* [27], if the cumulative percentage of PC1 and PC2 is greater than 70 %, the score plot offers a good 2-dimensional visualization. The difference of PC1 and PC2 of *P. nigrum* L. from each source demonstrated that their alkaloid profiles had similarities. The differences were affected by their concentrations in PC1 and PC2.

Therefore, PCA was able to separate the 3 groups successfully based on the geographic region. Furthermore, according to the corresponding loading plot (**Figure 4**), compound 4 loading influenced PC1 with negative values and PC2 with positive values. Compounds 3, 6, 9, 10, 12, 13, 15, 16 and 18 had the PC1 with positive and PC2 with negative values, while the loadings of compounds 1, 2, 7, 8, 11, 14, 15, 17, 19 and 20 influenced PC1 and PC2 with positive values. These results suggest that these metabolites are responsible for the discrimination of *P. nigrum* L. from 15 sources geographic regions of Thailand. In the literature we did not find any information or publications on different geographic regions of Thailand. But our results were comparable to those reported for other herbs such as *Zingiber officinale* [28] and *Camellia sinensis* [29].

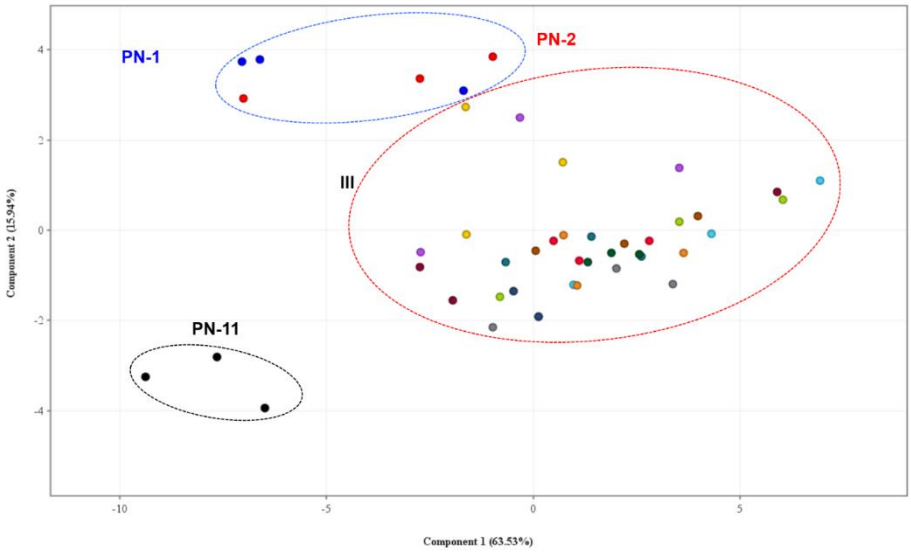

Figure 3 PCA scores plot of the different samples of *P. nigrum* L. from different geographical locations.

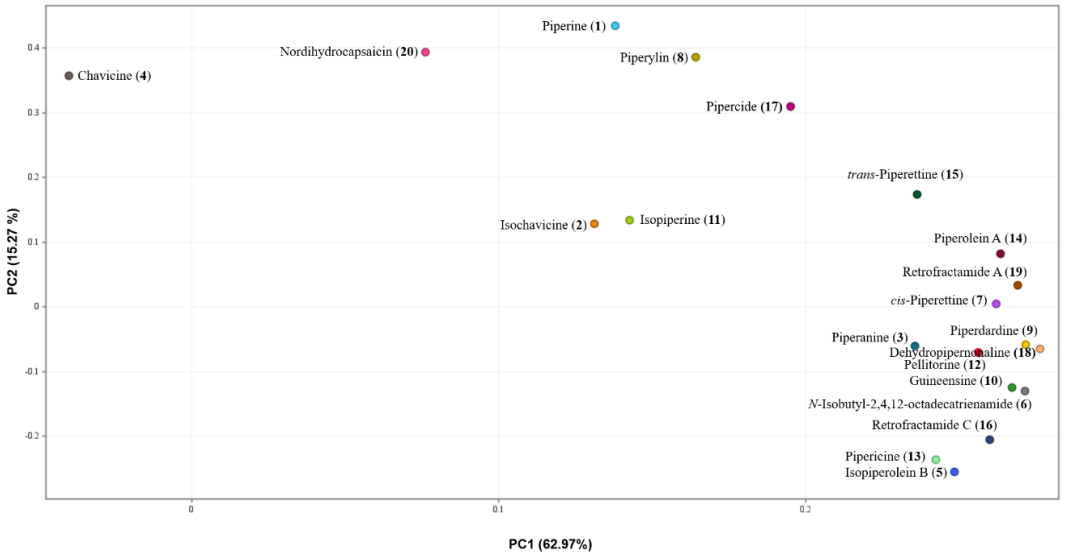

Figure 4 PCA loading plot of the different samples of *P. nigrum* L. from different geographical locations considering PC1 and PC2.

Antioxidant activities in the DPPH assay and FRAP assay

The antioxidant activities, which include free radical scavenging of DPPH in the DPPH assay and the reduction of ferric ions in the FRAP assay, were evaluated. Results relative to the DPPH scavenging activity of the ethanol extract of *P. nigrum* L. showed significant variation among locations. **Table 1** showed IC_{50} of the DPPH scavenging activity varied from 419.97 - 79.45 µg/mL, whereas the FRAP values were 0.15 - 0.39 mM $FeSO_4$/mg of crude extract. The DPPH radical scavenging and the reduction of ferric ion activities of PN-2 exceeded those of other sources. The alkaloid profiles of PN-2 were similar to those of PN-1 in HCA (**Figure 2**), but the antioxidant activities were not related. In addition, the antioxidant activities were not related to the piperine content. The result could be associated to the quantity of chemical composition that is unidentified in our data. Indeed, the antioxidant activity could be attributed to the important content of phenolic compounds [30]. Therefore, the antioxidant activities showed the impact of geographical differences.

Anticancer activity against SW480 cancer cells

The anticancer activity against colorectal cancer cells (SW480) of *P. nigrum* L. extracts was assessed. The results demonstrated that almost all extracts possessed potent anticancer activity (**Table 1**). The percentage of growth inhibition varied from 6.88 - 90.65 %. The samples of PN-5, PN-8, and PN-11 showed potent cell growth inhibition with % inhibition values of 90.65, 79.88 and 80.98 %, respectively. However, the sample PN-4, which had the highest piperine content, showed low inhibition with anticancer activity against SW480 cells (7.48 %). This result suggests that the anticancer activity was not in accordance with the piperine content. The cluster of PN-5 and PN-8 differ with PN-11 in HCA analysis (**Figure 2**). PN-11 on the hand had low concentration of compounds 6-20 which are anti-cancer agents [31,32]. It however, gave a much high inhibition against the SW480 cell line due to synergistic effects of other unidentified metabolite from the *P. nigrum* L. extract.

Apoptosis and necrosis in SW480 cancer cells

Apoptotic and necrotic cells were detected using flow cytometry by staining with Annexin V-FITC/PI. SW480 cells were induced apoptosis and necrosis following exposure to 15 geographical locations of *P. nigrum* L. extracts (PN1-15) (**Figure 4**). Doxorubicin was used as positive control. The late apoptotic and necrotic cells increased more than 80% in samples PN1-4, PN-6, PN-9 to PN-14, except PN-5, PN-7, PN-8, and PN-15 (**Figure 5**). These results might be related to the presence of some metabolite that have been described in extracts of *P. nigrum* L. (**Table 4**), like piperine which has been found as major component in *P. nigrum* L. [33], other constituents, such as alkaloids; piperiogumine, pellitorine, and lignan; kusunokinin which were unidentified in this study, could be involved in the SW480 properties, highlighting the synergistic anticancer activity of the different *P. nigrum* L. component aside from piperine [34].

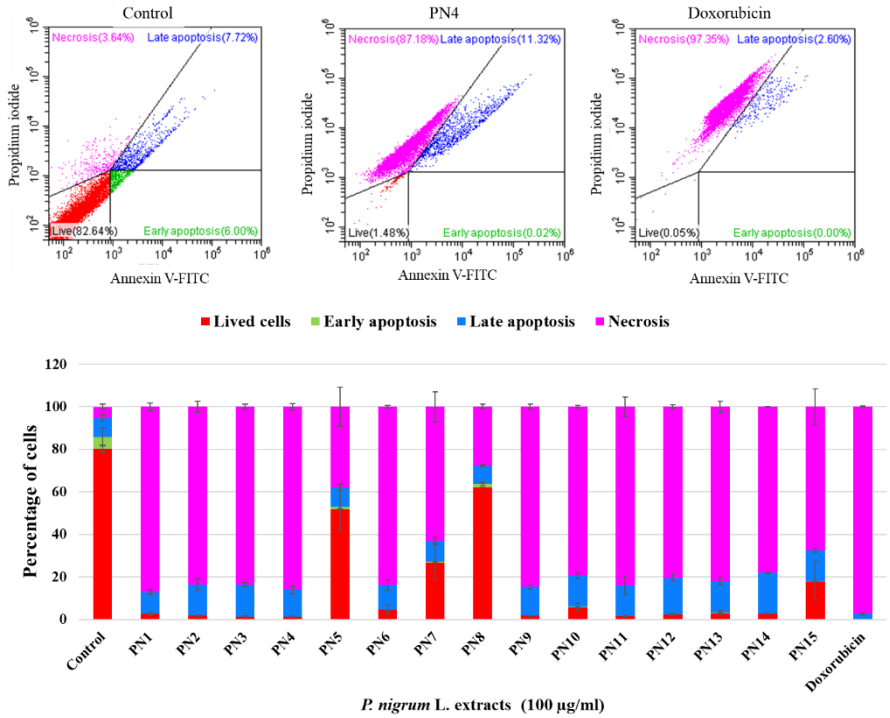

Figure 5 Apoptotic and necrosis effects of the *P. nigrum* L. extract against SW480 Cells were treated for 24 h with the indicated doses of extract. The data shown represent the average of 2 experiments performed in triplicate.

Anti-inflammatory activity

The *P. nigrum* L. extracts from 15 geographical locations were examined for anti-inflammatory effect on RAW 264.7 cells using a nitric oxide (NO) inhibition assay. The extracts from PN-1, PN-3, PN-4, and PN-11 showed high potential for nitric oxide inhibition (**Table 1**). The results demonstrated that the

piperine and their derivatives contents were correlated with the anti-inflammatory effects based on the nitric oxide inhibition potential. The results were related to previous reports [5,35,36].

Antibacterial activity

All samples were screened for antibacterial activity using the disc diffusion method by measuring the inhibition zone diameter. Ampicillin was used as positive control which showed inhibition zone of 47 mm (*S. aureus*), 45 mm (*M. luteus*) and 25 mm (*E. coli*). **Table 1** presents the antibacterial activities of *P. nigrum* L. extracts from 15 sources throughout Thailand. Almost all *P. nigrum* L. extracts inhibited Gram-positive bacteria, which included *S. aureus* and *M. luteus* with zones ranged from 6.5 - 7.8 mm, whereas no extracts were inhibited by Gram-negative bacteria (*E. coli*) and fungal strains (*C. albicans*). The *P. nigrum* L. extract from PN-4 showed the highest inhibition zone (7.8 mm in *S. aureus* and 7 mm in *M. luteus*). The results suggested that *P. nigrum* L. had a low potential for antibacterial activity. These result agree with previous reported that piperine is an excellent antibacterial agent [37,38] and EtOH extract of *P. nigrum* L. showed inactive with *C. albicans* [39]. However, other constituents, which were unidentified in this study, could be involved in the antibacterial properties, interesting that the synergistic antibacterial activity of the different *P. nigrum* L. component aside from piperine [39].

Conclusions

In conclusion, the results reported were the first information on the influence of difference region of *P. nigrum* L. in Thailand on piperine derivative content and biological activities.15 samples of *P. nigrum* L. from various geographic regions of Thailand. UHPLC-QTOF-MS was applied to establish alkaloid profiles of the *P. nigrum* L. samples. PCA and HCA analysis showed that the samples from the PN-1, PN-2 and PN-11 sources differed significantly from those of other sources in terms of compound type and alkaloid concentration profile. Comparative analysis based on a validated HPLC method was used for the standardization of 15 varieties of *P. nigrum* L. with piperine as a standard phytomarker which revealed that PN-4 source possessed a high content of piperine. PN-2 showed the highest antioxidant capacity (DPPH activity) while PN-10, PN-12 and PN-15 showed the highest FRAP activity. PN-11 source showed high inhibition of anti-inflammatory and anticancer properties. Furthermore, PN-3 and PN-4 also showed the highest both anti-inflammatory and antibacterial activity. Almost samples have been shown to induce late apoptosis and necrosis in SW480 cell line. This study will also be useful for the development of quality control parameters through the standardization of varieties of *P. nigrum* L. as raw materials in traditional medicine formulations. The results clearly showed that the chemical profiles and biological activities vary significantly with geographical variations and can beconsidered as a major concern in the quality control of Thai traditional medicine.

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