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Simultaneous quantification of three amides by gas chromatography time of flight mass spectrometry in extracts of *Piper sarmentosum*

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ABSTRACT

Piper sarmentosum is well known due to its culinary and medicinal properties and has a great potential of commercialization. Herbal standardization is a tedious task mainly due to un-availability or inadequacy of methods and standards, and its lacking is the single biggest hindrance in the acceptance of herbal products in main stream of pharmaceuticals. Therefore, present study aimed to develop an analytical method using gas chromatography time of flight mass spectrometry for the simultaneous quantification of three amides, which can be used as pharmacologically active analytical markers to standardize the product made from this plant and others containing these compounds. Three amides, pellitorine (1), sarmentine (2) and sarmentosine (3), isolated and identified previously from fruit of the plant were used as markers to develop and validate the method. Lowest limit of detection (LOD) of 1, 2 and 3 were found to be 0.10, 0.10 and 0.12 ng/ml, respectively, at a signal to noise ratio 3 : 1, whereas the lowest limit of quantification (LOQ) was taken 1.00, 1.00 and 12.00 ng/ml, respectively, at a signal to noise ration 10 : 1. The method was found to be linear (R^2 = 0.9985 to 0.9995) with relative standard deviation (RSD) < 5%. Intraday and inter day accuracy was found to be 97.40-100.00% with precision (RSD < 5%). The percentage recoveries were 97-100% with RSD < 5%. The method was found sensitive and reproducible, and applied successfully to quantify the amides in ethanol and supercritical CO_2 extracts of fruit of *Piper sarmentosum*. The method is found to be simple, fast and easy to perform, and may be helpful for natural product industry as well as natural product scientists to produce standardized extracts and products from the plant.

KEYWORDS

Piper sarmentosum;
GCTOF-MS;
Pellitorine;
Sarmentine;
Sarmentosine.

INTRODUCTION

Piper sarmentosum Roxb. (*Piperaceae)* is cultivated as well as found wild under shady trees in tropical and sub-tropical countries. The plant is popular due to culinary and medicinal properties. Different parts of the plant are used traditionally to cure a number of ailments[1,2]. Additionally, the plant has been investigated for a number of pharmacological activities such as anti-amoebic[3], antibacterial[4], anti-neoplastic[5], neuromus-

cular blocking[6], hypoglycemic[7], anti-malarial[8], anti-oxidant[9-11], anti-tuberculosis[12,13], antiagiogenic[14] and anticancer[15]. Due to these activities, the plant has a great potential of commercialization as medicinal plant. By maintaining consistency in quality, efficacy and safety, the products of the plant may get better and wider acceptance in pharmaceutical market.

In addition to this, extracts of well-defined constituents, standardized, are required for bioassays and clinical trials. The difficulty in standardization of herbal formulations is the un-availability and inadequacy of analytical methods and standards. Herbal products can be standardized by developing analytical methods applying modern analytical techniques and marker compounds of different categories.

The phytochemical constituents of *Piper sarmentosum* are amide alkaloid, pyrones, flavonoids, sterols and neolignans[4,5,16-19]. Among these, amides are the most prominent and possess many pharmacological properties. Keeping it in view, we selected three amides, characteristic of the plat, to be used as markers to develop and validate an analytical method, which may be used to produce standardized products made from this plant and others containing these markers.

Amides are neutral to weakly acidic due to linkage of nitrogen with carboxylic group, and are composed of an acid moiety such as cinnamic acid forming an amide where nitrogen is in a five or six membered ring or an isobutyl chain. Diverse methods have been reported for the quantification of different amides. A gas chromatography/FID method is reported for amides, piplartine, 4-desmethylpiplartine and cenocladamide[20]. UV, HPTLC and HPLC methods are also reported for the quantification and determination of amides in pepper species[21-25]. UV method is non-specific because other constituents present in the extract may interfere to produce false results. HPTLC is also a semi-quantitative technique with high limit of quantification, hence is not suitable to detect minute quantities. HPLC is a good technique due to its resolving power but limit of detection and co-eluting substances are the main concerns. GCTOF-MS is a very sensitive instrument and offer the facility to measure mass in a specific range. Hence, in this study we used GCTOF-MS to quantify three amides in the extracts of *Piper sarmentosum*. According to best of our information, no method has been reported for the simultaneous quantification of pellitorine, sarmentine and sarmentosine using GCTOF-MS.

The aim of this study was to develop a simple method for simultaneous quantification of three amides in *Piper sarmentosum* extracts. So that it can be used to standardize herbal products made from this plant.

EXPERIMENTAL

Plant material

The fruit of the plant was collected from Pulau Pinang, Malaysia in the month of March 2007 and authenticated by Prof. Dr. Zhari Ismail, Herbal Secretariat, School of Pharmaceutical Sciences, Universiti Sains Malaysia, where a voucher specimen was deposited vide reference # 0071/06. The fruit was sliced into small pieces, dried at 40 ^{o}C and pulverized.

Chemicals

HPLC grade methanol and analytical grade ethanol, acetone and hexane were procured from Merck. The standards, pellitorine, sarmentine and sarmentosine, previously isolated from fruit of the plant were used as analytical markers.

Instrumentation

1. Supercritical fluid extraction (SFE)

SC-CO_2 extraction was performed using SFE System (ISCO Inc., Lincoln, NE, USA) consisting of a CO_2 gas cylinder (MOX Gases Bhd, Selangor), a chiller (Yih Der BL-730), supercritical fluid extractor (SFX 220), controller (SFX 200), syringe pump (Model 100DX) and restrictor temperature controller associated with two coaxially heated capillary restrictors.

2. GCTOF-MS

Analysis was performed by gas chromatography system (Hewlett Packard 6890 N Network GC system) equipped with detector LECO Corporation Pegasus III Time of Flight Mass Spectrometer and Injector 7683 series. The column HP-5 (30 m X 0.32 mm ID, 0.25 Vm phase film) was used.

Extraction

Five gram powder of the fruit was extracted with 50 ml ethanol by reflux for 1 h and the procedure was

repeated twice. Fruit powder (5 g) was also extracted with supercritical CO_2 as a blank extraction was performed to clean the valves and supercritical fluid system with acetone and hexane at 60-70 °C and 34.47-41.37 MPa. Then the fruit powder was extracted at 60 °C and operating pressure of 3000 psi. Then both the extracts were filtered through filter paper (Whatman No. 1) using Buchner funnel and dried at 40 °C in vacuo.

Chromatographic conditions

Helium was used as a carrier gas at a constant flow rate of 1.2 ml/min. Temperature of the transfer line, ion source and injector was maintained at 250 °C. Temperature of the oven was kept 80 °C for 5 min and then increased from 80 °C to 280 °C at a rate of 7 °C / min. The voltage of the detector was kept at 1650V. Sample volume was 1.0 µl as split less injection and data acquisition was performed with LECO Chromatof software at a rate of 10 spectra per second (35 to 550 amu).

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

Stock solutions of standards, 1, 2 and 3, were prepared in HPLC grade methanol to a concentration of 1 µg/ml. For LOD and LOQ, working standard solutions were prepared by further diluting the stock solution with methanol to concentration ranging from 0.10-10.00 ng/ml. LOD was noted at S/N ratio of 3 : 1 while LOQ was taken a at S/N ration of 10 : 1. For calibration, stock solution of each of three standards was prepared in methanol to a concentration of 500 µg/ml. The working stock solutions were prepared by diluting the stock solution with methanol to get concentration ranging from 0.20 – 500.00 µg/ml. Calibration curves of the standards were constructed by plotting concentration against peak area and linearity was evaluated by linear regression.

Validation of the method

A quantity of 10 µg of each 1 and 2, and 20 µg of 3 was dissolved in 1 ml methanol to form a mix standard stock solution. From the stock solution a series of working standard solutions were prepared in methanol ranging 10, 20, 50, 100 and 150 ng/ml for compound **(1)** and **(2)** while 20, 40, 100, 200 and 300 ng/ml for compound **(3)**. These working solutions were used to determine recovery, intraday and inter day accuracy and precision of the method. The intraday accuracy and precision were determined for each of the standards by analyzing each working solution six times in a single day, while for inter day accuracy and precision, each of the working standard solutions was analyzed 6 times for 5 consecutive days.

For extraction recovery, pulverized plant material was spiked with working standard solutions of each of the standard. The spiked samples were extracted by a protocol mentioned in extraction. A blank sample, without spiking, was also extracted in the same manner. The extraction recovery value of each of the standard was calculated as a percentage of concentration spiked (true value) and obtained value after extraction over that of an equivalent amount without spike extraction.

Preparation of samples and analysis

The stock solutions of ethanol and supercritical CO_2 extracts of the fruit were prepared in methanol to a concentration of 1.651 and 23.3 mg/ml, respectively. Working solutions were prepared by diluting the stock solutions to 100 times with methanol.

For extraction recovery studies, 200 mg of fruit powder was spiked with mix standard solutions, dried and extracted with 15 ml ethanol by reflux for 1 h, the extraction was repeated twice. The extract was filtered, dried at 40 °C and dissolved in methanol to make solution to a concentration of 1.00 mg/ml. The same quantity of the powder, without spike, was also extracted as a control.

The working samples were filtered by 0.45µm PTFE syringe filter (Whatman, Maidstone, England) and analyzed in triplicate at chromatographic conditions mentioned above. Chromatographic peaks of the samples were identified by comparing the retention time and mass (m/z) with those of the standards and finally quantified using calibration curves.

STATISTICAL ANALYSIS

All the samples and standards were analyzed in triplicate and results were averaged. For intraday and inter-day accuracy the standards were analyzed six times and results were averaged.

RESULTS AND DISCUSSION

The LOD of amide 1, 2 and 3 was found to be 0.10, 0.10 and 0.12 ng/ml, respectively at a signal to noise ratio 3 : 1 while the LOQ was taken as 1.00, 1.00 and 1.20 ng/ml at signal to noise ratio 10 : 1. All the standards showed linearity over the whole range investigated with squared correlation coefficient of 0.9985 or better. The recovery, intraday and inter day accuracy and precision values of the standards are shown in TABLE 1. The recovery values of all the concentrations studied ranged from 97.10 - 100.50%. The intraday accuracy values were 98.16-101.50% while the corresponding precision values expressed as relative standard deviations (RSD), were 0.66 - 5.84%. Inter-day accuracy values of the standards were found to be 97.60-100.79% with corresponding precision (RSD) values 0.86- 4.78%. These results have shown that the method is reliable and reproducible. The chromatograms shown in the Figure 1 indicated that the method had given good separation of the markers. The structures of these markers are shown in Figure 2.

TABLE 1 : Recovery, intraday and inter-day precision and accuracy values of pellitorine (1), sarmentine (2) and sarmentosine (3)

Concentration ng/ml	Recovery (n = 3)		Intraday (n = 6)		Inter-day (n = 6)	
	Mean %	RSD %	Accuracy %	Precision RSD %	Accuracy %	Precision RSD %
Pellitorine						
150	100.00	1.52	100.00	1.87	100.79	1.63
100	99.34	1.13	100.00	0.89	99.43	2.65
50	98.76	3.32	97.98	2.76	98.02	4.26
30	99.42	4.01	101.50	2.05	98.96	2.78
20	98.02	4.21	99.34	3.98	97.87	4.34
Sarmentine						
150	100.03	1.23	99.08	1.45	100.00	1.74
100	99.50	3.26	99.05	0.66	99.64	2.34
50	100.00	2.67	100.12	4.76	100.00	1.39
30	98.32	3.69	100.00	3.56	98.47	4.43
20	98.43	4.17	98.79	5.84	98.72	4.97
Sarmentosine						
300	100.00	1.98	100.00	2.43	100.00	3.16
200	100.50	2.41	100.00	3.51	100.00	0.86
100	99.65	2.90	100.23	0.99	97.60	4.35
60	99.21	3.15	98.16	0.68	98.54	1.69
40	97.10	2.89	99.54	3.52	99.03	4.78

The method was found linear over the whole range of the samples. Robustness of the method was evaluated by making minor changes in gas flow and temperature. It was found that the accuracy of the method is not affected by minor changes in the described chromatographic conditions.

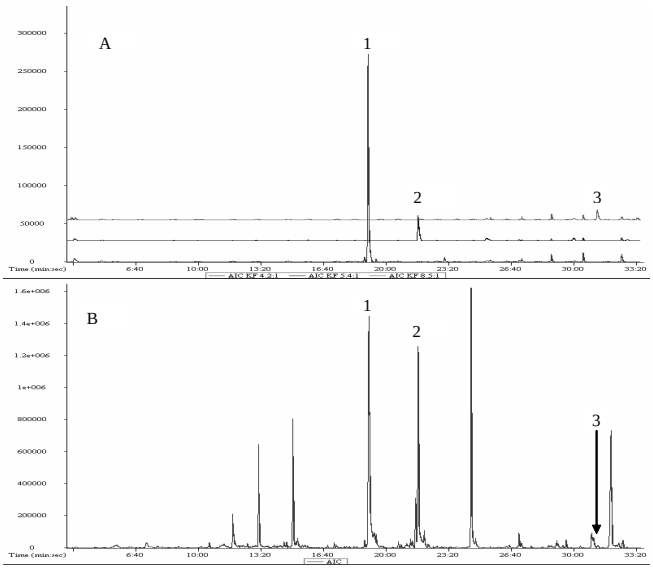

Figure 1 : Ionograms of standards, pellitorin (1), sarmentine (2) and sarmentosine (3) and supercritical CO_2 extract of the fruit of *Piper sarmentosum*, A = standards; B = supercritical CO_2 extract of fruit

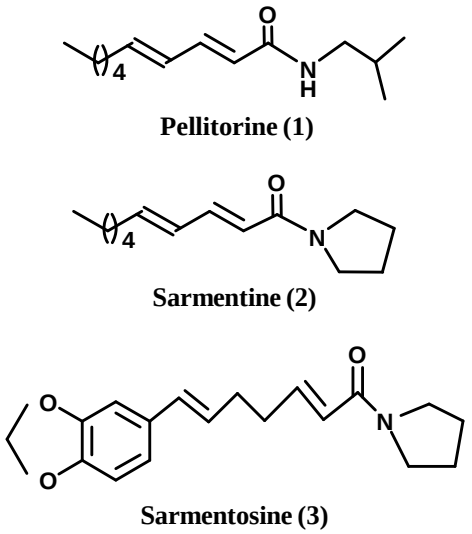

Figure 2 : Chemical structures of the markers used for the development of analytical method

The method was applied for the quantification of amides in ethanol and supercritical CO_2 extracts of fruit of *Piper sarmentosum.* The content of amide 1, 2 and 3 in mg/g of extract is presented in TABLE 2. The content of amides in ethanol extract was found to be lower as compared to supercritical CO_2 extract. It indicates the efficiency of supercritical CO_2 extraction for the

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amides. The relative distribution of the amides, pellitorine, sarmentine and sarmentosine, in fruit of the plant is found to be in a ratio of 1 : 0.54 : 0.25, respectively. These markers are distributed throughout the plant viz fruit, leaves, stem and root. Hence, the extracts of all these parts of the plant can be standardized using this highly sensitive method.

TABLE 2 : Concentration of amides in ethanol and supercritical CO_2 extracts of fruit of *Piper sarmentosum* (n = 3)

Sample	Content of amides (mg/g ± SD)				Relative distribution of amides		
	1	2	3	Total	1	2	3
Ethanol extract	52.10 ± 4.40	1.31 ± 0.02	0.12 ± 0.06	53.530	1	0.025	0.003
SFE extract	63.7 ± 4.00	0.54 ± 0.04	0.96 ± 0.03	65.200	1	0.008	0.015

SFE (supercritical fluid extract)

CONCLUSION

A simple and sensitive GCTOF-MS method for the simultaneous determination of three amides in *Piper sarmentosum* has been developed for the first time. The method is found to be accurate and precise, and can be used to standardize the extracts and natural product made from the extracts of *Piper sarmentosum* using pellitorine, sarmentine and sarmentosine as analytical markers. Moreover, the method may be a value for conducting stability and pharmacokinetic studies of formulations containing these amides.

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