Extraction Efficiency of Water, Ethanol and Supercritical Carbon Dioxide for Amide Content from Fruit of *Piper sarmentosum* using Colorimetry and High Performance Liquid Chromatography

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Summary: Extraction is important for both natural product research and preparation of extracts to be used as raw materials for phytopharmaceuticals. Selection of a suitable solvent as well as type of extraction is prerequisite to prepare extracts enriched with particular type of compounds with peculiar activities. Therefore, the present study aimed to evaluate the extraction efficiency of water, ethanol and supercritical CO_2 for amides from fruit of *Piper sarmentosum* using colorimetry and high performance liquid chromatography (HPLC). The pulverized fruit material was extracted by reflux using water and ethanol, and supercritical CO_2 at 60 °C and operating pressure of 3000, 4000, 6000, 7000 and 8000 psi. The colorimetric analysis indicated that except the water extracts, total amide content in different extracts was not significantly different ($P<0.05$). Similarly, HPLC analysis using pellitorine, sarmentine and sarmentosine as markers indicated that except water extracts, total content of the markers in different extracts was not significantly different ($P<0.05$). These results indicate that extraction efficiency of ethanol for amides is comparable to that of supercritical CO_2. Hence, ethanol may be used to prepare amide enriched extracts without using costly equipment and operating expertise.

Introduction

Herbal products are used in therapeutics either as fresh or dried plant materials and extracts. The extraction is important in phytochemical investigations and preparation of therapeutic products in different dosage forms. In addition to this, extraction is also an essential part of analysis especially if analyte(s) need to be separated from matrix or interfering species. Good separation can be achieved using solvents or solvent systems of varying polarities, and using different extraction techniques. Furthermore, extracts enriched with a particular class of compounds can be prepared using specific solvents. Plant materials are being extracted by peculation, maceration and decoction, both on small as well as larger scale. In natural product industry, ethanol and water are the most commonly used solvents for extraction because of their extractive potential and safety. Recently, supercritical fluid extraction has gotten popularity due to its solubilising efficiency and safety. In the present study, we have used three solvents; water, ethanol and supercritical CO_2 for extraction of amides from fruit of *Piper sarmentosum*.

Piper sarmentosum Roxb. (*Piperaceae)*, a plant of tropical and subtropical region, is well known due to its culinary and medicinal properties. As a traditional medicine, extracts of different parts of the plant are being used to cure various ailments [1-4]. The plant has also shown a number of pharmacological activities such as antiamoebic [5], antibacterial [6], anti-TB [7], anti-neoplastic [3], neuromuscular blocking [8], hypoglycemic [9], anti-malarial [10], antioxidant [11-13], antiangiogenic [14] and cytotoxicity [15]. Based on these activities, nowadays natural product industry has started producing different phytomedicines from extracts of the plant.

Phytochemically, the plant contains constituents such as amide alkaloids, phenylpropanoids, pyrones, flavonoids, sterols and neolignans etc. [16-19]. Among these constituents, amides are most prominent and have both commercial as well as therapeutic importance. The amides are neutral to acidic and cannot be extracted by the protocols used for alkaloids. The amides can

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be extracted with alcohol and supercritical CO_2. Therefore, in this study we have hypothesized that efficiency of supercritical fluid for amides will be better as compared to that of water and ethanol. To check this hypothesis, extracts of fruit of *Piper sarmentosum* were extracted with water, ethanol and supercritical CO_2. The extracts were analyzed using colorimetry for estimation of total amide content, and HPLC for the quantification of pellitorine, sarmentine and sarmentosine. The study may be of a value for natural products scientists and natural product industry to get amide enriched extracts from the plant.

Results and Discussion

The total amide content (mg/g extract) in aqueous, ethanol and supercritical fluid extracts of fruit of *Piper sarmentosum* determined from the linear regression equation, Y = -89004 + 6680.799 X with correlation coefficient 0.9963, are presented in (Table 1). These results clearly indicate that except water extracts, total amide content of the other extracts was not significantly different ($P<0.05$). It is found that water is absolutely inefficient for extraction of amides, whereas ethanol extraction by simple method using reflux has proven equally good to that of supercritical fluid extraction. In supercritical fluid extraction, the effect of pressure on total amide content is also not found to be significant ($P<0.05$).

Table-1: Total amide content and content of pellitorine, sarmentine and sarmentosine in different extracts fruit of *Piper sarmentosum* (n = 3).

Fruit extract	Total Amides (mg/g ± SD)	Contents of amides (mg/g ± SD)			
		Total	A 1	A 2	A 3
Water	0.00 ± 0.00	43.10 ± 3.10	2.20 ± 0.61	0.36 ± 0.03	45.66
Ethanol	448.4 ± 5.98	52.10 ± 4.40	1.31 ± 0.02	0.12 ± 0.06	53.53
SFE 1	442.60 ± 5.85	63.70 ± 4.00	0.54 ± 0.04	0.96 ± 0.04	65.20
SFE 2	439.70 ± 6.23	54.60 ± 5.10	4.30 ± 0.60	1.22 ± 0.08	60.12
SFE 3	447.60 ± 4.97	51.90 ± 5.40	4.40 ± 0.90	0.87 ± 0.03	57.17
SFE 4	434.09 ± 6.21	48.00 ± 7.50	4.00 ± 0.41	0.564 ± 0.051	52.56
SFE 5	439.30 ± 5.67	41.57 ± 4.40	2.66 ± 0.52	0.92 ± 0.19	45.15
SFE 6	443.48 ± 6.73	50.00 ± 5.70	4.20 ± 0.60	0.45 ± 0.03	54.65

A 1(pellitorine); A 2 (sarmentine); A 3 (sarmentosine); SFE 1 (3000 psi), SFE 2 (4000 psi), SFE 3 (5000 psi), SFE 4 (6000 psi), SFE 5 (7000 psi); SFE 6 (8000 psi)

The extracts were analyzed in triplicate by HPLC for the quantification of pellitorine, sarmentine and sarmentosine. The quantification of the markers in different extracts was performed from the calibration curves. The linear regression equations used for the quantification of the markers were given as: pellitorine ($Y= 180.54\ X + 6.9079,\ R^2 = 1$), sarmentine ($Y= 846.2\ X + 12.5760,\ R^2 = 1$) and sarmentosine ($Y= 1444.2\ X + 20.963,\ R^2 = 0.9999$). The detection was carried out at 260 nm because the markers showed maximum absorbance at this wavelength [13, 20]. The chromatograms of the standards and samples are shown in (Figure 1) and (Figure 2), respectively, whereas the content of the markers (mg/gm of extract) in different extracts are given in (Table 1). These results indicate that the markers are not detected in the aqueous extracts, whilst content of the markers are not significantly different in ethanol and different supercritical fluid extracts ($P<0.05$). It is evident from the results of this study that extraction efficiency of ethanol with reference to amides is equivalent to that of supercritical CO_2.

Like other piper species, *Piper sarmentosum* contains amides in addition to other compounds. These amides have medicinal, ecological and commercial importance. These compounds are derivatives of an acid such as cinnamic acid forming an amide where nitrogen is in a five or a six member ring or isopropyl moiety [21, 22]. In contrary to alkaloids, basic in nature, these compounds are neutral to weakly acidic because nitrogen is in an amide linkage, hence cannot be isolated by standard acid or base extraction methods that are commonly used for alkaloid extraction.

Extraction of a particular class of compounds can be achieved by selecting a suitable solvent. Water and ethanol being non-toxic are the most commonly used solvents for the extraction of medicinal plants in industry. Supercritical fluids are also getting popularity in extraction due to a number of advantages. Supercritical fluid of particular interest is CO_2 because it has low critical temperature (304 K) and moderate pressure (71 atm). Furthermore, it is non-inflammable, non-toxic, environmental friendly and miscible with variety of organic solvents, and can easily be removed to dry or concentrate the extracts. Despite these benefits, supercritical fluid extraction needs costly equipment and special trained personnel.

The contents of amides can be estimated by a number of methods, but in the present study we have used Labat Reagent method. In this method,

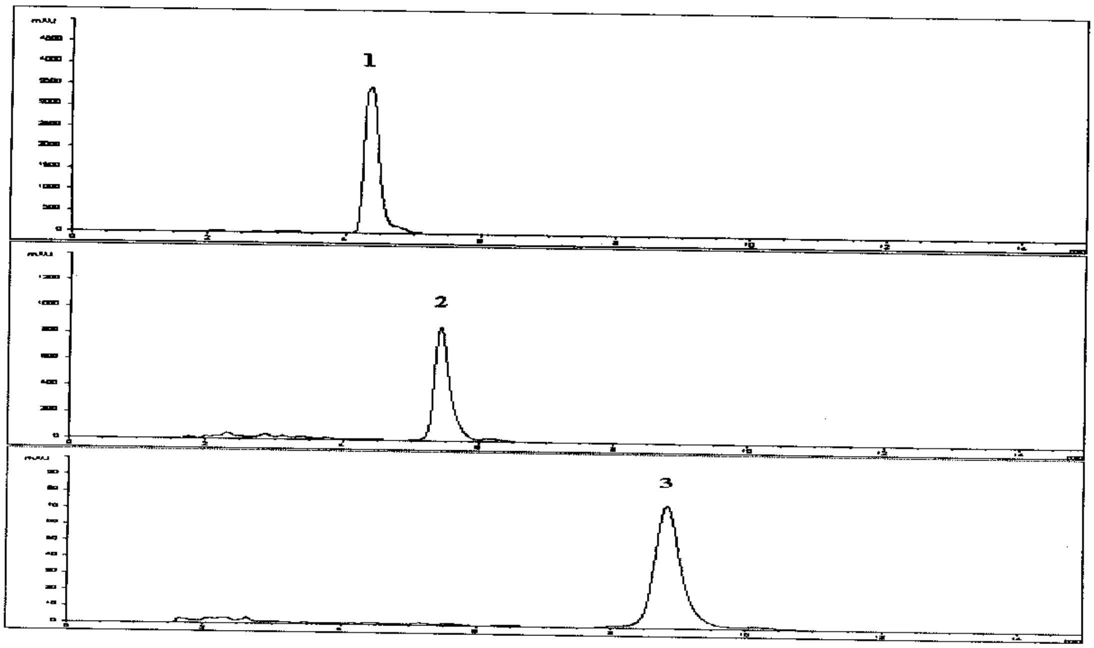

Fig. 1: HPLC chromatograms of standards, pellitorine (1), sarmentine (2) and sarmentosine (3), at 260 nm.

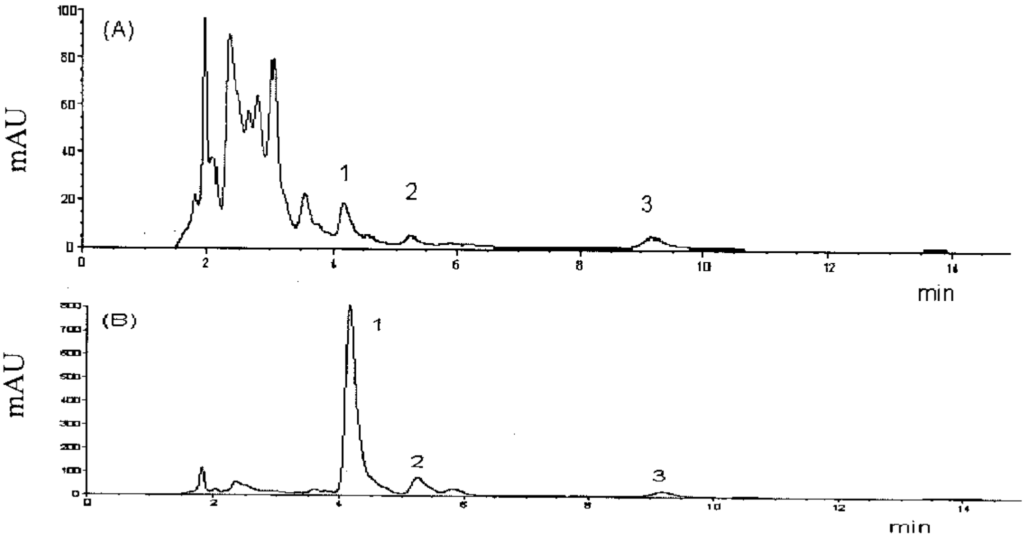

Fig. 2: HPLC chromatograms of different extracts of *Piper sarmentosum* at 260 nm; A (fruit ethanol extract); B (fruit supercritical fluid extract at 5000 psi), 1 (pellitorine); 2 (sarmentine), 3 (sarmentosine).

methylene dioxyphenyl group containing compounds produce green color in the presence of sulphuric acid and gallic acid, which changes to blue on heating [23]. This blue colored compound exhibits maximum absorbance at 660 nm. Most of the amides in *Piper sarmentosum* are having methylene dioxypheyl moiety and therefore, can be estimated by Labat reagent using piperine as a standard. For the quantification of pellitorine, sarmentine and sarmentosine in extracts, HPLC has been used due to its high resolving efficiency. The method used for quantification of the markers has given optimum separation the compounds.

In the present study, we have investigated three methods of extraction to answer the question about the superiority of supercritical fluid extraction. The methods of extraction were compared on the basis of total amide content and the content of the three markers. Water proved to be inefficient for the extraction of amides because we could not detect any amide by colorimetry as well as HPLC, whereas ethanol and supercritical fluid (CO_2) exhibited comparable efficiencies to one another for extraction of amides. Supercritical fluid extraction carried out at different operating pressure, indicated that pressure had no significant effect on amide extraction. In comparison to supercritical fluid extraction, ethanol extraction needed neither expertise nor costly equipment.

The results of this study signify that ethanol may be used as a solvent for the preparation of refined and enriched extracts having high content of amides from *Piper sarmentosum*. These findings may be important for natural product industry to prepare amide enriched extracts for formulation of phytomedicines from the plant.

Experimental

Plant Material

Fruit of the plant was collected in March, 2006 from the Botanical Garden of the School of Pharmaceutical Sciences, Universiti Sains Malaysia and authenticated by Prof. Dr. Zhari Ismail, Herbal Secretariat, School of Pharmaceutical Sciences, Universiti Sains Malaysia where a voucher specimen was deposited vide reference No. 0071/06. The fruit was cleaned, cut into small pieces and dried at 40 °C to bed pulverized.

Extraction

Five gram powder of the fruit was extracted separately with 50 ml ethanol and water by reflux for 1 h. Both the extracts were filtered through filter paper (Whatman No. 1) using Buchner funnel. The ethanol extract was dried at 40 °C in vacuo while the aqueous extracts were dried in freeze dryer.

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 powder was extracted at 60 °C and operating pressure of 3000, 4000, 6000, 7000 and 8000 psi.

Chemicals

The chemicals and solvents of analytical/HPLC grade procured from Merck included ethanol, methanol, acetonitrile, hexane, acetone and sulphuric acid. Piperine and gallic acid were obtained from Sigma Aldrich. Pellitorine, sarmentine and sarmentosine isolated previously from the fruit of *Piper sarmentosum* were used (13).

Instruments

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 pumps (Model 100DX) and restrictor temperature controller associated with two coaxially heated capillary restrictors.

Ultra Violet/Visible Spectroscopy

The ultra violet and visible analysis was performed using UV/Visible spectrophotometer (PerkinElmer Lambda 45, Shelton, CT, USA).

High Performance Liquid Chromatography

The samples were analyzed using HPLC system (1100 series, Agilent Technologies, Waldronn, Germany) equipped with degasser (G1379 A), quaternary pump (G1311 A), auto sampler

(G1313 A), column oven (G1316 A) and UV detector (G 1314 A).

Preparation of Sample Solutions

Sample solutions of aqueous and ethanol extracts of fruit of *Piper sarmentosum* were prepared in methanol to a concentration of (1.50 mg/ml) whilst sample solutions of supercritical fluid extracts (SFE) were prepared in methanol to a concentration as: SFE1 (23.3 mg/ml), SFE 2 (2.8 mg/ml), SFE 3 (2.3 mg/ml), SFE 4 (3.2 mg/ml), SFE 5 (8.15 mg/ml) and SFE 6 (3.9 mg/ml).

Preparation of Standard Solutions

Stock solution of piperine was prepared in methanol to a concentration of 1.00 mg/ml. Then a series of working standard solutions of concentrations 0.25, 0.50, 1.00, 1.50, 2.50, 5.00, 10.00 and 15.00 µg/ml were prepared by diluting the stock solution with methanol.

Stock solutions of pellitorine, sarmentine and sarmentosine were prepared in methanol to a concentration of 50.00 µg/ml. Then a series of working standard solutions were prepared to cover the whole range of samples as: 0.01, 0.10, 0.50, 1.00 and 1.50 µg/ml for pellitorine and sarmentine, while 0.08, 0.80, 4.00, 8.00 and 1.20 µg/ml for sarmentosine by diluting the stock solution with mobile phase.

All the samples and the standards were filtered by 0.45 µm PTFE (polytetrafluoroethylene) syringe filter (Whatman, Maidstone, England).

Estimation of Total Amide Content

Total amide content was estimated by a method [23], briefly described as: alcoholic solution of sample/standard (100 µl), concentrated sulfuric acid (2 µl) and 5 % gallic acid solution in methanol (100 µl) were taken in a test tube. The mixture was heated for 10 min in water bath and absorbance was measured at 660 nm against a blank containing equivalent amount of methanol in place of sample/standard. Piperine was used as a standard and amide content was calculated from calibration curve of the standard. Total amide content in the extracts was determined using the following equation, and expressed as mg equivalents of piperine.

$$\text{Total amides} = (C \times V)/W$$

Where C (µg/ml) is concentration of piperine equivalents obtained from the calibration curve, V is the final volume of the extract in ml and W is the weight of sample in grams.

Chromatographic Conditions

The samples/ standards (15 µl) were eluted by an isocratic mobile phase comprising of methanol: water: acetonitrile (80: 15: 5 v/v) at a flow rate of 1 ml/min using column (Hiber Rt 250-4, LiChrosorb RP 18, 10µm, Agilent Technologies) that was maintained at 25 °C. The elution time was 15 min and detection was carried out at 260 nm. The detector was operated in a sensitivity range of 0.005 AUFS (absorbance units full scale), output of 15 mV and data acquisition was performed by ChemStation version A. 08.03.

Each sample/standard was analyzed in triplicate and the marker compounds were quantified from their respective calibration curves.

Statistical Analysis

All the samples were analyzed in triplicate and results were expressed as mean ± standard deviation. Data was analysed by analysis of variance (ANOVA) followed by multiple comparison with Bonferoni test using SPSS 12.0 (SPSS Inc. Chicago, IL). P values < 0.05 were regarded as significant.

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