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Proximate and qualitative analysis of different parts of *Piper sarmentosum*, and quantification of total amides in various extracts

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ABSTRACT
Present study aimed to analyze crude powders and extracts of different parts of *Piper sarmentosum* for proximate, qualitative and quantitative studies to prepare standardized botanical drugs from the plant. Unlike synthetic drugs, standardization of botanical drugs is always challenging for natural product researchers due to inadequacy and unavailability of standards and methods. Standardization of botanical drugs is not just an analytical process which ends with the detection of few constituents rather it embodies a set of analytical, biochemical and biological protocols. Keeping analytical protocols in view, crude powders were analyzed for the content of moisture, total ash, acid insoluble ash, sulphated ash and soluble extractives in water and methanol. These physicochemical properties were found within specified limits. Comparison of Fourier Transform Infrared (FTIR) fingerprints of crude powders of different parts indicated the difference of constituents. Similarly, comparison of ultra violet (UV) profiles of extracts of all the parts exhibited discrimination. Qualitative analysis of aqueous and ethanol extracts by high performance thin layer chromatography (HPTLC) indicated the presence of amides in ethanol extracts of all parts of the plant. Quantitative analysis of extracts indicated that total amide content was significantly higher by colorimetry as compared to UV spectrophotometry. The distribution of amides in different parts was in the order fruit > root > leaf > stem *(P=0.000)*. It is concluded from the study that amide content varies in different parts of the plant and ethanol is a better solvent for their extraction. Additionally, colorimetric method exhibits high content of amides.
Keywords: *Piper sarmentosum*; *Piperaceae*; Standardization; FTIR; HPTLC

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, 3, 4). Additionally, the plant has been investigated for a number of pharmacological activities such as antiamoebic (5), antibacterial (6), antineoplastic (3), neuromuscular blocking (7), hypoglycemic (8), antimalarial (9), antioxidant (10, 11, 12), anti-tuberculosis (13) and antiagiogenic (14). 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 industry.

To maintain consistency in herbal products in different batches is challenging because the chemical constituents of the herbs varies due to various factors. These variations may be minimized by the application of strict quality control protocols right from the beginning, selection of seeds, sowing at suitable time, control of cultivation conditions, collection at suitable age, drying and storage, to compounding and packaging for safe delivery of claimed efficacy to patient. This can goal can be achieved by the

application good agricultural and collection practices and good manufacturing practices. A number of guidelines for good agricultural and collection practices have been suggested by the World Heath Organization (15). These variations can further be minimized by the application of pharmacognostic methods to know which plant, identified by botanical nomenclature and which part of the plant to which percentage is used.

Physicochemical properties and qualitative analysis of the raw material helps in positive identification "that the herb is what it is claimed to be". Keeping in view the medicinal and commercial importance of *Piper sarmentosum*, present study aimed to analyze crude powder and extracts of different parts of the plant for physiochemical properties and qualitative analysis using Fourier Transform Infrared (FTIR). The qualitative analysis of extracts was performed using (UV) spectroscopy and high performance thin layer chromatography (HPTLC).

Phytochemically, *Piper sarmentosum* contains constituents such as alkaloids (amides), pyrones, flavonoids, sterols and neolignans (3, 6, 16, 17). Among these, amides are the most prominent and possess several pharmacological activities. Therefore, present study also aimed to investigate aqueous and ethanol extracts of different parts of the plant qualitatively for the presence of amides and quantitatively for the estimation total amide content.

A number of methods can be used for the determination of amides qualitatively and quantitatively. We performed the qualitative analysis by HPTLC using piperine as a marker and detection was carried out by Dragendorff's reagent. Polarography, ultra violet spectrophotometry, colorimetry and Kjeldahl method can be used for estimation of total amide content. The major limitation of these methods is their non specificity, especially in case of extracts containing diverse nature of constituents (18). In present study, we used two types of analytical methods, Labat reagent and UV spectroscopy, for the estimation of total amide content in various extracts. Labat reagent is specific for alkaloids containing methylene-dioxyphenyl group (19). UV spectrophotometry is still a commonly used method for the estimation of pungent principles in pepper species (20). Therefore, UV analysis was also applied for the estimation of total amide using piperine as a standard and assuming that piperine analogues have same molecular extinction (18).

The aim of present study was to perform physicochemical, qualitative and quantitative analysis on the crude powders and extracts of different parts of *Piper sarmentosum* for standardization and to find the distribution of amide content in various parts of the plant.

MATERIAL AND METHODS

Plant material and extraction

Piper sarmentosum was collected in the month of March from Pulau Balik, Penang, Malaysia. The plant was authenticated by the Prof. Dr. Zhari Ismail, School of Pharmaceutical Sciences, Universiti Sains Malaysia. A specimen voucher (0071/06) was deposited in the Herbal Secretariat of the school. The leaves, stem, root and fruit were separated, dried at 40 ^{o}C and pulverized.

Powdered material of each part (50 g) was extracted twice with 200 ml ethanol and water by reflux for 2 h. The ethanol extracts were dried in vacuo at 40 ^{o}C whereas aqueous extracts were dried in freeze dryer.

Chemicals and solvents

Analytical grade chemicals and solvents procured from Merck include, methanol, ethanol, toluene, ethyl acetate and sulphuric acid. Chemical purchased from Sigma Aldrich include, piperine, potassium bromide and gallic acid.

Instruments

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

Fourier Transform Infrared spectroscopy: FTIR spectra were recorded using FTIR Spectrometer (Thermo Nicolet, USA) equipped with software OMNIC version 6.0 a.

High Performance Thin Layer Chromatography: Analysis was performed on HPTLC comprising of densitometer (CAMAG Model-3 TLC scanner) equipped with winCATS 4 software, semi automatic sampler (Linomat-5) and documentation was carried out by CAMAG PROSTER 3 at 254/366 nm (CAMAG, Berlin, Germany).

Physicochemical analysis

Proximate analysis was performed according to procedures described in the United States Pharmacopoeia (21).

Moisture content

Powdered plant material (2 g) was taken in a tarred silica crucible and dried in an oven at 105 ^{o}C for 30 min, cooled at room temperature in desiccator until constant weight. The powder was then weighed to

calculate the moisture content based on the loss of weight on drying and the results were expressed as a percent of dry powder.

Total ash

Clean dry silica crucible was heated and weighed to a constant weight and 2 g sample was taken in it. The sample was incinerated by gradually increasing the heat to dull redness (675 ± 25 ^{o}C) until free from carbon. The crucible was then kept in desiccator to cool to a constant weight. The content was then weighed to calculate the percentage of total ash with reference to air dried sample.

Acid insoluble Ash

The total ash was boiled in 25 ml dilute HCl for 5 min. The insoluble matter was collected on ash less filter paper and washed with hot distilled water. The filter paper was then dried and ignited in tarred silica crucible until free from carbon. The crucible was allowed to cool in desiccator till a constant weight. The content was weighed to calculate the percentage of acid insoluble ash with reference to air dried sample.

Sulphated Ash

Two gram sample taken in tarred silica crucible was moistened with sulphuric acid and ignited gently. Crucible was then allowed to cool in desiccator and again moistened with sulphuric acid, reignited and weighed. The process was repeated till a constant weight was obtained. The percentage of sulphated ash with reference to air dried sample was calculated.

Alcohol soluble extractives

Five gram powdered drug was macerated with 100 ml of 95% ethanol in a closed flask for 24 h with continuous stirring. The contents were filtered and 25 ml filtrate was evaporated to dryness in china dish and the residue was dried at 105 ^{o}C and weighed. The percentage of alcohol soluble extractives was calculated with reference to air dried sample.

Water soluble extractives

Five gram powdered drug was taken in a closed flask, macerated with 100 ml chloroform water and allowed to stir for 24 h. The contents were filtered and the filtrate was evaporated, dried at 105 ^{o}C and weighed. The percentage of water soluble extractives was calculated with reference to air dried sample.

Qualitative analysis of crude powders by FTIR spectroscopy

The powdered root, stem, leaves and fruit of *Piper sarmentosum* were analyzed in triplicate to get FTIR spectra using KBr discs as: 1 mg of the crude drug powder and 100 mg KBr were ground together and the mixture was transferred to a die. The die was then pressed in hydraulic press to produce discs which were used to get FTIR spectra in mid IR range 4000-400 cm^{-1}. FTIR fingerprint profiles of different parts of the plant were compared with each other for identification.

Qualitative analysis of extracts by HPTLC

The extracts were analyzed qualitatively for amides by HPTLC. Samples were applied on TLC plate (10 X 20 cm Silica gel $60F_{254}$, Merck) by semi automatic TLC sampler using 100 µl syringe as: application/spot 2 µl, band length 6 mm, distance between spots 9 mm, distance from the lower edge 10 mm and first application was at 15 mm. The plate was developed in pre-saturated horizontal chamber with solvent system comprising of toluene: ethyl acetate (7: 3 v/v) and detection was carried out by Dragendorff's reagent. The plates were also visualized under UV at 254 and 366 nm.

Qualitative analysis of extracts by UV Spectrophotometry

Aqueous and ethanol extracts of different parts of *Piper sarmentosum* were dissolved in methanol to prepare stock solution of concentration 5 mg/ml. Working solutions (0.5 mg/ml) were prepared by diluting 1 ml of the stock solution with 10 ml methanol. All the working solutions were scanned from 500-200 nm using methanol as a blank.

Estimation of total amides in extracts

Preparation of sample solutions

The working sample solutions of methanol and aqueous extracts of root, stem leaf and fruit were prepared to a concentration of 5 mg/ml.

Preparation of standard solutions

The stock solution of piperine (1 mg/ml) was prepared in methanol while working standard solutions of 2.5, 5, 10, 30, 50 and 100 µg/ml were prepared by diluting the stock solution in methanol.

Estimation of total amides by Labat reagent

Alcoholic solution of the sample/standard (100 µl), concentrated sulfuric acid (2 µl) and (100 µl) 5% gallic acid solution in methanol were taken in a test tube. The mixture was heated in water bath for 10 min and the absorbance was measured at 660 nm against methanol as a blank. The standard solutions of piperine in different concentrations were used to construct standard curve for the quantification of total amide content. The total amide content (mg equivalents of piperine) was calculated using following equation and expressed as percentage.

Total amides = (C X 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.

Estimation of total amides by UV analysis

The working standard solutions of piperine and various samples were analyzed in triplicate at 343 nm against methanol as a blank. To perform quantification the instrument was programmed in concentration mode.

Statistical analysis

All the samples and standards were analyzed in triplicate and results were expressed as mean ± SD. Amide content in different parts of the plant were analyzed by analysis of variance (one way ANOVA) to compare the means. P value <0.05 was considered significant.

RESULTS

The results of physicochemical properties are presented in (Table 1), which indicate that different parts of *Piper sarmentosum* (root, stem, leaf and fruit) have different content of moisture, total ash, acid soluble ash, sulphated ash, alcohol extractives and water extractives. The moisture content and the ash values were found within the normal recommended limits (moisture contents 6% and ash values 20%). The values of water soluble extractives were higher as compared to alcohol soluble extractives.

FTIR spectra of the powder of different parts of *Piper sarmentosum* shown in (Figure1), indicated bands at 3650 cm^{-1} (amide), 2900-2850 cm^{-1} (C-H), 1634-1500 cm^{-1} (aromatic domain bands), 1725-1705 cm^{-1} (carbonyl) and 1200-1100 cm^{-1} (presence of a strong alkenes). The comparison of FTIR spectra revealed that the constituents in different parts of the plant were different. These spectra may be used as fingerprints to differentiate crude powder of different parts of the plant.

UV profiles of aqueous and methanol extracts of different parts of the plant are given in (Figure 2). Aqueous and ethanol extracts of the root, stem and fruit exhibited absorption maximum at 260 nm while aqueous and ethanol extracts of the leaf exhibited absorption maximum at 260 and 350 nm. The absorption from 350-250 nm is characteristic to aromatic amides and phenolic compounds. The comparison of spectra indicated the similarity and difference in constituents of different extracts.

Both aqueous and ethanol extracts of different parts of the plant were evaluated qualitatively for the presence of amides using HPTLC. Amides were detected in all ethanol extracts using Dragendorff's reagent while amides were not detected in aqueous extracts. It indicates that amides are distributed in all parts of the plant. The absence of amides in aqueous extracts was due to their insolubility in aqueous medium.

The results of the total amide content in aqueous and ethanol extracts of different parts of the plant by UV and colorimetry are presented in (Table 2). In UV analysis, standard curve was constructed at 5 data points and quantification was performed using linear regression equation, $Y= 7.301090e^{-02} + 3.837600\ e^{-02}X$ (R^2 = 0.998849). In colorimetric analysis, standard curve was constructed at 6 data points and quantification was performed using linear regression equation, $Y= -8.9004e^{3} + 6.6807\ e^{3}X$ (R^2 = 0.9963). In colorimetric method, total amide content in ethanol extracts of different parts of the plant were significantly higher as compared UV method *(P=0.000)*. These results also support the work of Genest and co-workers (22). The other possible reason for this discrepancy in results of the two methods is the presence of less analogues of piperine which absorb at 343 nm. In UV analysis, total amide content was significantly different in ethanol extracts of different parts, except ethanol extract of the stem and the root. The distribution of amide content in extracts of different parts was in the order fruit > root > leaf > stem. The effect of solvent on the extraction of amides was also different *(P=0.000)*. The amides were not detected in aqueous extracts. The results indicate that ethanol is a better solvent for the extraction of total amides as compared to water. Additionally, colorimetric method for the estimation of total amide content gives higher values as compared to the UV method.

DISCUSSION

The objective of proximate analysis was to evaluate the raw materials of the plant for physicochemical properties like moisture content, ash values and total extractives in methanol and water. Moisture content plays an important role in the stability of natural products. The moisture content should be minimized in order to prevent chemical degradation as well as microbial contamination. Moisture content is determined by calculating the loss of weight on drying. This method is applicable to materials which do not contain volatile components. Ash values are helpful for determining the quality and purity of powdered crude drugs. The ashing is performed to remove all traces of organic matter to avoid its interference in analysis. Crude drug on incineration normally leaves an ash

Table1: Physciochemical properties of different parts of Piper sarmentosum (PS)

Physicochemical properties (Percentage)	P.S. Root	P.S. Stem	P.S. Leaves	P.S. Fruit
Moisture contents	4.46	6.75	6.70	4.50
Total ash	10.83	9.80	9.12	9.75
Acid insoluble ash	3.54	3.50	3.36	3.60
Sulphated ash	12.65	12.70	12.45	11.90
Alcohol soluble extractives	14.35	15.40	15.90	16.00
Water soluble extractives	16.00	20.00	19.60	18.90

Table 2: Percentage total amides content in ethanol and aqueous extracts of different parts of Piper sarmentosum by UV spectrophotometry and colorimetry (n=3)

Extract	% Total amide (UV analysis)	± SD	% Total amide (Colorimetry)	± SD
Fruit ethanol	22.47	1.35	44.88	0.35
Leaf ethanol	11.65	0.58	9.72	0.19
Stem ethanol	4.59	0.36	19.34	0.13
Root ethanol	5.71	0.39	27.18	0.20
Fruit water	0.00	0.00	0.00	0.00
Leaf water	0.00	0.00	0.00	0.00
Stem water	0.00	0.00	0.00	0.00
Root water	0.00	0.00	0.00	0.00

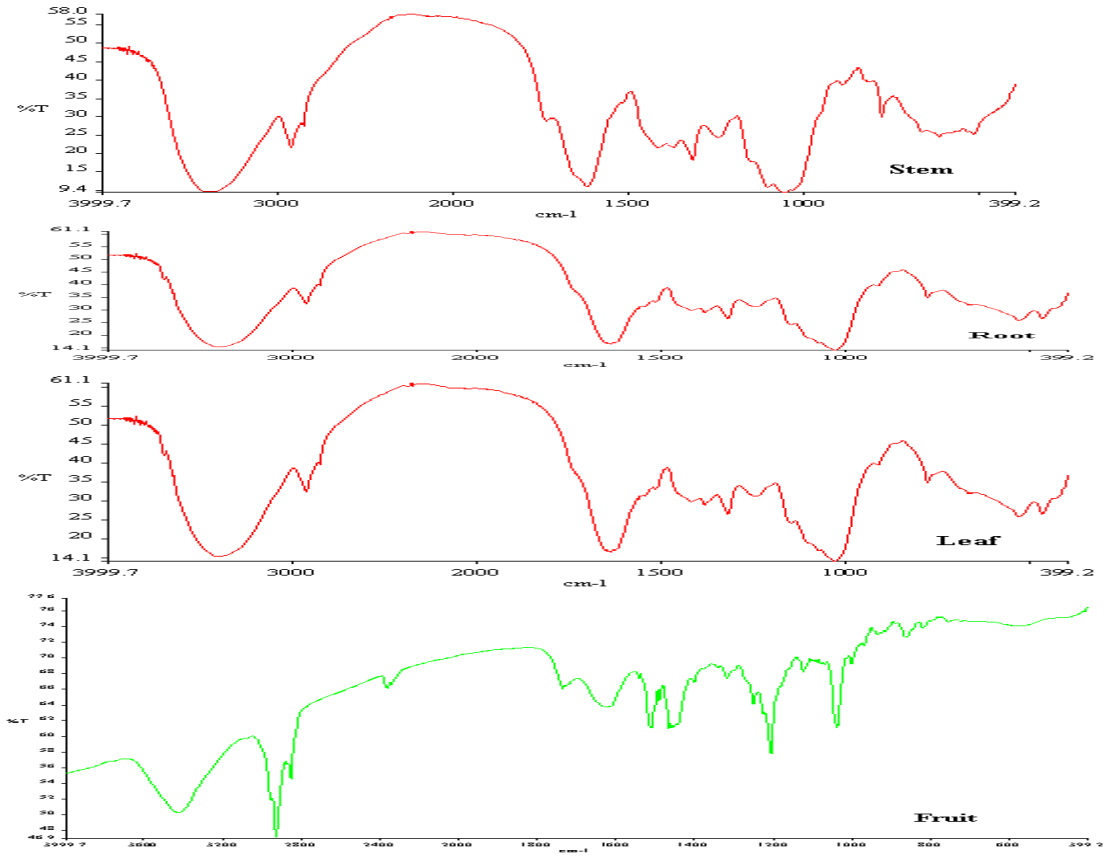

Figure 1: Comparison of FTIR spectra of different parts of Piper sarmentosum in mid-IR range (4000- 400 cm^{-1})

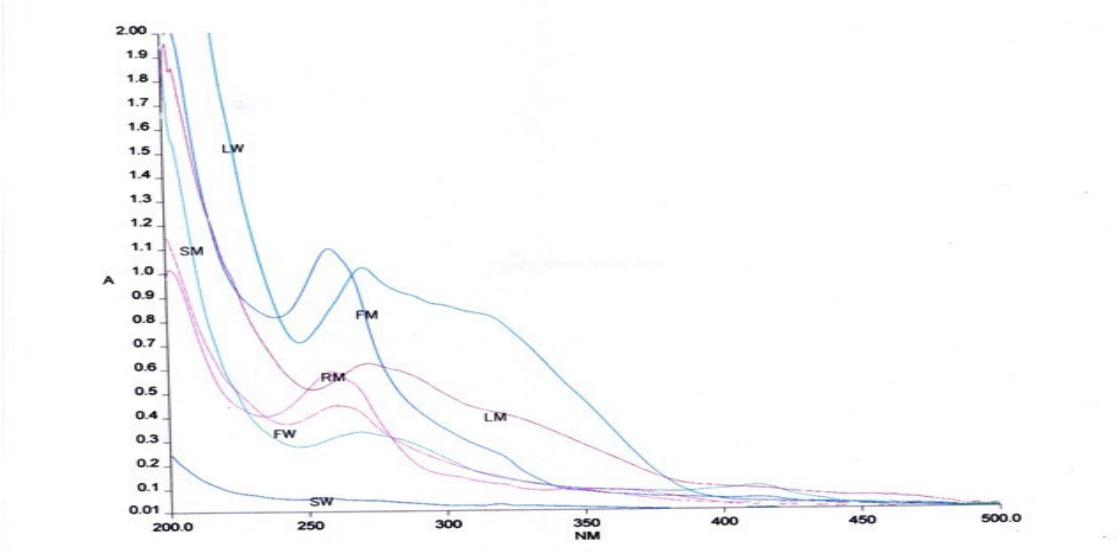

Figure 2: UV spectra of aqueous and ethanol extracts of different parts of Piper sarmentosum in a range (400-200 nm); SW (stem water); SM (stem ethanol); LW (leaf water); LW (leaf water); FW (fruit water); FM (fruit ethanol); RM (root ethanol)

consisting of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. The total ash of a crude drug reflects the care taken in its preparation. If the crude drug has silica or calcium oxalate then acid insoluble ash is performed. Some analysts mix sulphuric acid with powdered crude drug before ashing because sulphated ash is less fusible than the ordinary ash (23).

FTIR profiles are important in quality assessment of herbal materials because often it is not necessary to know the identity of individual constituents that make up fingerprint. Moreover, FTIR spectroscopy is a non destructive technique and offers the analysis of plant material on solid matrix. Fingerprints, characteristic to each material, give quick check of plant material giving reliable indication of some identity or difference.

Ultra violet scanning of the extracts gives characteristic spectra which are of great significance to establish identity by comparing the spectra of a sample with reference. The materials having similar constituents exhibit similar spectra. It means that if spectra are super-imposable then similarity in pharmacological activity may be expected.

Since, extracts are complex mixtures of compound and individual components can not be detected qualitatively and quantitatively in the form of solution. Chromatography offers an easy way to detect individual components due to its separation power. Among different chromatography techniques, high performance thin layer chromatography provides visual comparison of different samples with standards. It is further advantageous and offers the application of many samples and standards together on the same plate for better comparison. The TLC plate can be sprayed with different detecting agents to identify different class of compounds.

From the results of the study it is concluded that ethanol extraction is better to prepare amide enrich extracts from different parts of the plant. For amide-based pharmacological activities, fruit ethanol extracts may be more potent as compared to other parts of the plant. Colorimetry may be a useful method for the estimation of total amide content.

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