Chromatographic and Spectral Fingerprinting of *Polyalthia longifolia*, a Source of Phytochemicals

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Medicinal plants, such as *Polyalthia longifolia* (Indian mast tree), are important therapeutic sources for curing human diseases. In this work *P. longifolia* leaf extract was characterized by chromatographic and spectral fingerprinting techniques, phytochemical and heavy metal analyses, and microscopy. Light microscopy of a transverse section of the leaf of *P. longifolia* revealed the presence of various plant cells. Phytochemical screening results revealed the presence of alkaloids, triterpenoids, tannins, saponin, anthraquinones, and glycosides in the extract. The concentrations of heavy metals determined in the extract were well below the permissible limit. Nine peaks observed in the HPLC spectra showed the presence of various compounds in the extract. The GCMS method used for quantification of (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate (*i.e.*, cycloeucalenol acetate) in the extract was rapid, accurate, precise, linear (R^2 = 0.8752), and robust. The HPTLC analysis showed ten specific peaks for the methanolic extract of *P. longifolia* leaf. Twelve major peaks in the range of 4,000 to 500 cm^{-1} were observed in the FTIR spectra, which represented various specific functional groups in the extract.

Keywords: Fingerprinting techniques; P. longifolia; Heavy metal; Phytochemical; Characterization

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INTRODUCTION

Plant-based therapy has been used as a vital component in traditional medicine systems and also serves as the main source of inspiration for several major pharmaceutical drugs used in the defence against various diseases. The therapeutic potential, including the antioxidant, antimicrobial, and anticarcinogenic properties of higher plants, is due to the presence of secondary metabolites (Canigueral *et al*. 2008; Kaur and Arora 2009). Unsurprisingly, the human population has depended on medicinal plants for protection against diseases since time immemorial. Thus, the search for therapeutic agents and bioactive compounds to fight against emerging and existing diseases is an ongoing process, and medicinal plants have regularly been included as part of this search because they are rich in various bioactive compounds with useful medicinal properties. In consideration of the commercialization of formulations based on medicinal plants, the quality control standards of various medicinal plants that are used in traditional medicine are becoming more important. The characterization of extracts is of great importance to guarantee their quality. As a result, the current study was carried out for the evaluation of chemical components in *Polyalthia longifolia*.

P. longifolia belongs to the family Annonaceae. The tree is native to India and is locally known as Indian mast tree. *P. longifolia* is one of the most important indigenous plants in Indian medicinal literature; it is found throughout Malaysia and widely used in traditional medicine as a febrifuge and a tonic. Almost all parts of this plant are used in Indian medicine for the treatment of various ailments such as uterine disorders, fever, skin disease, diabetes, hypertension, helminthiasis, gonorrhea, and vitiated conditions of vata and pitta, and its significant medicinal properties have been reported in the scientific literature (Rastogi and Mehrotra 1960; Saleem *et al.* 2005; Katkar *et al*. 2010). Herbal Uterine Tonic manufactured by Tan Healthcare, India is an available marketed formulation which contains *P. longifolia.* The leaves possess anticancer potential, in which the bioactive compounds act through different pathways, converging, ultimately, into the activation of apoptosis in cancer cells, which leads to cell cytotoxicity (Verma *et al*. 2008). The stem bark, roots, and leaves have been studied for various biological activities, such as antibacterial, antidiabetic, anti-inflammatory, and antioxidant activity (Goutam *et al*. 2010; Nair *et al*. 2007; Ramakrishna *et al*. 2000; Chang *et al*. 2006). In addition, the bioactive compounds of this plant and its pharmacological activities due to its geographical diversity are relatively new areas for investigation.

Traditional medicinal plants such as *P. longifolia* have been widely used for thousands of years in various traditional medicine systems, such as those of the Ayurvedic, Chinese, Unani, Tibetan, Amazonian, and African cultures. However, one of the characteristics of medicinal plant preparations is that all the herbal medicines are extracted with boiling water (*i.e*., the decoction process) with one or many herbs. This may be the major explanation why the quality control of medicinal plants is extremely important. For this reason, the quantity and quality of the data concerning the safety and efficacy of medicinal plants play an important role in supporting their utilization in the treatment of various diseases. Accordingly, it seems to be essential to identify most of the phytochemical constituents of medicinal plants to ensure the reliability and repeatability of pharmacological and clinical research, to understand their bioactivities and the possible side effects of active compounds, and to enhance product quality control (Yan *et al*. 1999; Raven *et al*. 1999; Xie 2001). Therefore, several modern chromatographic techniques, such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectroscopy (GC–MS), infrared spectroscopy (IR), and high-performance thin layer chromatography (HPTLC), can be useful for this kind of quality control documentation. In this way, the full medicinal plant extract could be considered the active 'compound'. The chromatographic fingerprint of the medicinal plant can be defined as the chromatographic pattern obtained using various modern chromatographic techniques on common phytochemical components of the extract. Normally, this chromatographic fingerprint, which is derived from the fundamental attributes of "integrity" and "fuzziness" or "sameness" and "differences," enables the plant material under investigation to be characterized chemically (Xie 2001; Welsh *et al*. 1996). Therefore, chromatographic fingerprints of the medicinal plant extract can be useful for the authentication and identification of herbal preparations because no chemical characteristic of the phytochemical components of the extract is exactly the same for different sample preparations. Furthermore, the chromatographic fingerprints could demonstrate "variations" between various samples (Xie 2001; Valentão *et al*. 1999). In this study, we focused our investigation on how to construct a good chromatographic fingerprint for efficient evaluation of *P. longifolia* samples for the purpose of quality

control. As a single herbal plant may consist of many phytochemicals, the fingerprints recorded by chromatographic instruments may present a relatively good integral representation of various phytochemicals of *P. longifolia*; this is the main topic of concern in this study. Thus, the characterization of *P. longifolia* extracts helps establish quality assurance for this plant. The present investigation was conducted for the characterization of *P. longifolia* using microscopic characteristics, phytochemical analysis, and heavy metal content analysis with chromatographic and spectral fingerprinting.

EXPERIMENTAL

Chemicals and Reagents

Chemicals and reagents were of analytical grade, purchased from either Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

Plant Sample Collection

The leaves of *P. longifolia* were collected from various areas in Universiti Sains Malaysia in Penang, Malaysia in January 2012 and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a standard sample was deposited (Voucher specimen: USM/HERBARIUM/11306). The leaves were separated and cut into small pieces, which were first washed with tap water and then with distilled water. The leaves were then dried in an oven at 60 °C for 7 days, after which the dried leaves were ground into a fine powder using a grinder. The ground powder was stored in clean airtight bottles that were labelled.

Microscopic Studies and Powder Analysis

The microscopic features of the fresh leaves were determined using the method of Evans (1996). Histomorphological examination of *P. longifolia* leaf specimens was used to identify the histological microstructure. Leaf tissues were fixed in 10% buffered formalin. After fixation, the tissues were dehydrated in a graded series of alcohols, cleared with xylene, and embedded in paraffin wax. Multiple 5-mm microtome sections from the block were mounted on slides, stained with 0.5% methylene blue, and examined under a light microscope. For powder microscopy analysis, powder of *P. longifolia* leaf was mounted on a glass slide and observed under a light microscope (World Health Organization 1992).

Solvent Extraction

The dried sample (approximately 100 g) was added to methanol (300 mL) and soaked for 4 days at room temperature (30 ± 2 °C). The suspension was stirred to allow the leaf powder to fully dissolve in the methanol. Removal of the sample from the solvent was done by filtration through cheesecloth followed by filter paper (Whatman No. 1); the filtrate was concentrated under a vacuum to one-fifth its volume using a rotary evaporator at 60 °C and then sterilized by filtration using a 0.22-mm membrane. The thick paste obtained was further dried in an oven at 40 °C. The resultant extract was kept at 4 °C for further analysis. Methanol was used for extraction to mimic the usage of water by the traditional healers to prepare plant extracts as a decoction. Water and methanol have high

dielectric constants and dipole moments as polar protic solvents. Moreover, the use of methanol makes the process of evaporation easier when compared to water.

Phytochemical Analysis

The leaf extracts of *P. longifolia* were screened for the following phytochemicals: saponin, tannins, steroids, flavonoids, alkaloids, triterpenoids, glycosides, and anthraquinones, using the standard procedures recommended by Evans (1996) and Parekh and Chanda (2007).

High Performance Liquid Chromatography (HPLC) Analysis

The methanolic leaf extracts were further subjected to a reverse-phase HPLC analysis. The HPLC equipment consisted of a Shimadzu LC-6A pump, a Rheodyne injector fitted with a 20-μL loop, and a Shimadzu SPD-10AV UV-VIS with a variable wavelength detector from 190 to 400 nm. The chromatographic analyses were performed on a Novapak C_{18} column (4.6 x 250 mm) at ambient temperature with a mobile phase of methanol/water/formic acid (49:50:1, v/v) at a flow rate of 1 mL min^{-1}. Detection was made at 190 to 400 nm. The data were acquired, stored, and analysed utilising Winchrom software.

Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

The GC/MS analysis was done on a gas chromatograph-mass spectrometer (model Shimadzu 2010) equipped with a DB-5 capillary column (30 m long. 0.25 mm i.d., film thickness 0.25 μm). The column temperature programme was 60 ºC for 0 min, with 10 ºC per min increases to 270 ºC, for which the temperature programme was 30 min. The carrier gas was helium at a flow rate of 1 mL/min with a split ratio of 10:1. The detector and injector temperatures were both maintained at 270 ºC. The quadrupole mass spectrometer was scanned over the range 28 to 400 amu at 1 scan s^{-1}, with an ionising voltage of 70 eV, an ionisation current of 150 mA, and an ion source temperature of 200 ºC. To determine the Kovats index of the components, a mixture of alkenes (C_9-C_{24}) was added to the crude extract before injecting it into the GC-MS equipment and analysed under the same conditions as above. The compounds were identified by a computer search from the commercial libraries of the National Institute of Standards and Technology (NIST) (Torey *et al*. 2010).

Fourier Transform Infrared (FTIR) Analysis

The methanol extracts of *P. longifolia* were mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra were recorded on a Shimadzu FTIR Spectrometer 8000 series, between 4,000 and 500 cm^{-1}. All determinations were performed in triplicate (Torey *et al*. 2010).

HPTLC Finger Print profiles for *P. longifolia* Leaf Extract

TLC condition

A TLC plate of 20 × 10 cm was pre-coated with silica gel 60F254 TLC plates (E. Merck) (0.2-mm thickness) with an aluminum sheet support. The spotting device was a CAMAG Automatic TLC Sample Spotter (Camag Muttenz, Switzerland); the syringe volume was 100 µL; the developing chamber was a CAMAG Glass Twin Trough chamber (20 × 10 cm); and the densitometer consisted of a CAMAG TLC Scanner 3

linked to WINCATS software. The mobile phase was toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 0.2, v/v). The saturation time for the mobile phase was 2 h.

Procedures
The plates were developed in a toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 0.2, v/v) solvent system to a distance of 8 cm. The plates were dried at room temperature in air. The spots on the TLC plates were scanned at UV-Vis wavelength of 365 nm. The R_f values and the colour of the resolved bands were noted, where R_f (retention factor) values describe the distance traveled by the band divided by the distance traveled by the solvent.

Heavy Metal Analysis

Atomic absorption spectrometry (AAS) was used for the determination of the amount or concentration of specific heavy metals. AAS uses the phenomenon that atoms in the ground state absorb light of a specific wavelength that is characteristic of the particular atom when the light passes through an atomic vapour layer of the element to be determined (Ghaedi *et al*. 2008). The determination of lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg) were performed on a Perkin-Elmer 200 atomic absorption spectrophotometer under optimised measurement conditions using hollow cathode lamps according to the standard method in the British Pharmacopoeia (2011).

RESULTS AND DISCUSSION

Microscopy of Leaf

Transverse section

In general, the methods for quality control of medicinal plants involve sensory inspection (such as microscopic examinations) and analytical inspection using instrumental techniques (Choi *et al*. 2002). Accordingly, the current research commenced with the microscopic examination of a transverse section of the *P. longifolia* leaf. The advancement of the microscopy technique could help to achieve the goal of evaluation of the herbal products, particularly as the authentication methods of medicinal plants have been enhanced by the microscopic identification method. The Light Microscopic Method (LMM) for identification of medicinal plants has been applied for many years and has been published by Chinese Pharmacopoeia, as well as in other reference books, such as Chinese Materia Medica, New compendium of Chinese Materia Medica, British Herbal Pharmacopoeia, American Herbal Pharmacopoeia, Japanese Pharmacopoeia, Korea Herbal Pharmacopoeia, and Indian Ayurvedic Pharmacopoeia (Zhao *et al*. 2005). Moreover, the microscopic approach utilizes techniques, such as light microscopy, that facilitate analysis of the characteristics such as the presence or absence of particular cell types, that help to distinguish between the desired plant species and plant parts at the ultrastructural level. Thus, in this research, microscopic evaluation of the leaf material was performed, as the leaf extract was to be used in our subsequent study. A transverse section of the leaf of *P. longifolia* through the midrib was observed under a light microscope, which showed bowl-shaped abaxial parts and a straight adaxial side. Both the adaxial and abaxial epidermal layers were single-layered and thin-walled cubical cells. The epidermal cells were wide, polygonal, and thin-walled; the walls were straight

or slightly wavy. The lower epidermis was stomatiferous. The lower epidermal cells were smaller compared to the upper epidermal cells. The epidermal cells were followed by four to six layers of angular collenchyma cells on both sides. In the midrib region, the vascular bundle was encircled by a schlerenchymatous ring. The bundle sheath, xylem, and phloem were clearly visible (Fig. 1). The light microscopic method offered several advantages over conventional authentication of medicinal plants, including effectiveness, simplicity, and low cost, as well as being widely adopted as an official method in many international herbal pharmacopoeias, as mentioned earlier.

Leaf powder

The powder was observed to be green to brownish in color. The diagnostic features of the powder include xylem elements, pitted vessels, stone cell, parenchyma cells, stomata, fiber of leaf, crystal, and calcium oxalate crystals (Fig. 2)

Phytochemicals Analysis

Phytochemical screening of the *P. longifolia* extracts (Table 1) revealed that the crude extracts contained alkaloids, triterpenoids, tannins, saponin, anthraquinones, and glycosides. The phytochemicals tested are known to exhibit medicinal activity and physiological activity. Alkaloids are an important drug source and have been reported to possess antimicrobial, antioxidant, and cytotoxic activity (Rahman *et al*. 2009). Medicinally, tannins are used in antidiarrhoeal, haemostatic, and antihaemorrhoidal preparations. Saponins are glycosides of triterpenes, steroids, or steroidal alkaloids found in plants. They are useful in lowering cholesterol (hypocholesterolemic property), as antioxidants, and as antidiabetic and anti-inflammatory agents.

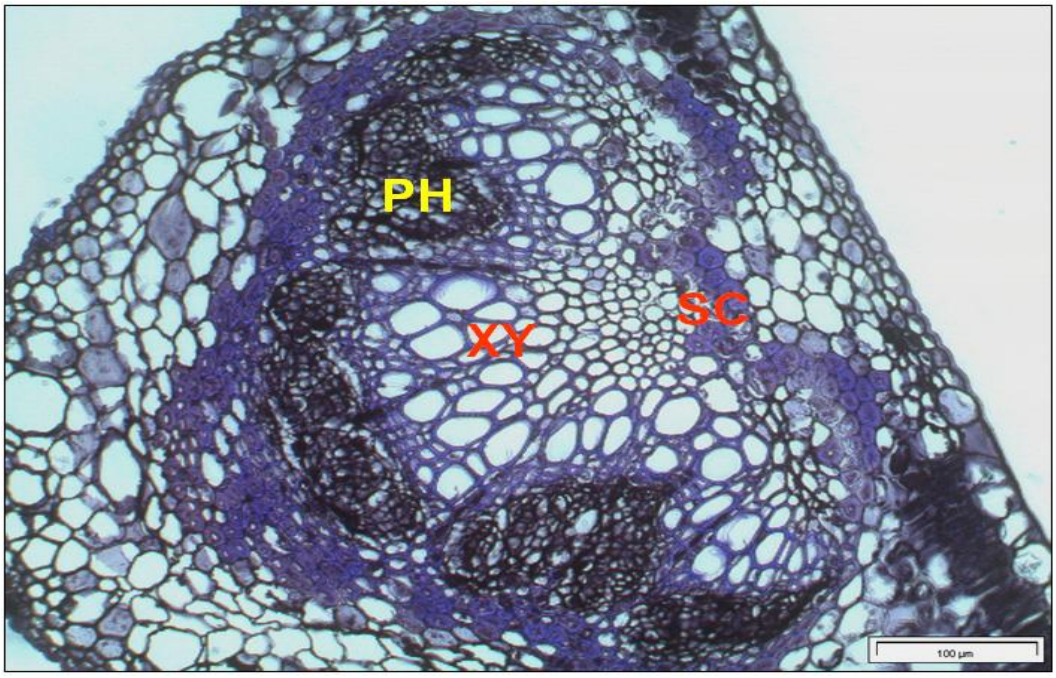

Fig. 1. Transverse section of the leaf of *P. longifolia*. SC: Sclerenchyma cell; XY: xylem; PH: Phloem

Table 1. Phytochemical Analysis of *P. longifolia*

Phytochemicals	*P. longifolia*
Carbohydrates	-
Alkaloids	+
Triterpenoids	+
Tannins	+
Steroids	-
Saponin	+
Flavonoids	-
Anthraquinones	+
Glycosides	+

+ Present; - Absent

Terpenoids are a large and diverse class of naturally occurring organic chemicals found in all classes of living organisms, while steroids display analgesic properties (Rupasinghe *et al*. 2003; Sayyah *et al*. 2004; Malairajan *et al*. 2006; Tamil Selvan *et al*. 2012). Glycosides are drugs used in the treatment of congestive heart failure and cardiac arrhythmia and are found as secondary metabolites in medicinal plants (Edeoga *et al.* 2005). Some anthraquinones have been reported to possess antiosteoporotic activity (Li *et al.* 2009).

This study revealed that the extract of *P. longifolia* contained many important phytochemical constituents with various medicinal properties. The presence of biologically important phytochemicals in the *P. longifolia* extracts may contribute to their reported medicinal value and indicates that it is a potential source for the development of useful drugs.

Heavy Metal Analysis

Another crucial factor relating to the safety of medicinal plants is the presence of contaminants such as heavy metals. The levels of mercury, lead, arsenic, and cadmium in the *P. longifolia* extract were determined using AAS. None of these heavy metals was detected as being above the permissible limits in the extract, with most being well within the acceptable limits (Table 2). According to WHO (2007), the maximum amounts of lead and cadmium in dried plant materials are 10 ppm and 0.3 ppm, respectively, which are based on the acceptable daily intake (ADI) values.

Table 2. Heavy Metal Concentration in *P. longifolia* Leaf Extract

Elements	Concentration (ppm)
Lead (Pb)	< 10
Cadmium (Cd)	< 0.3
Arsenic (As)	< 5.0
Mercury (Hg)	< 0.5

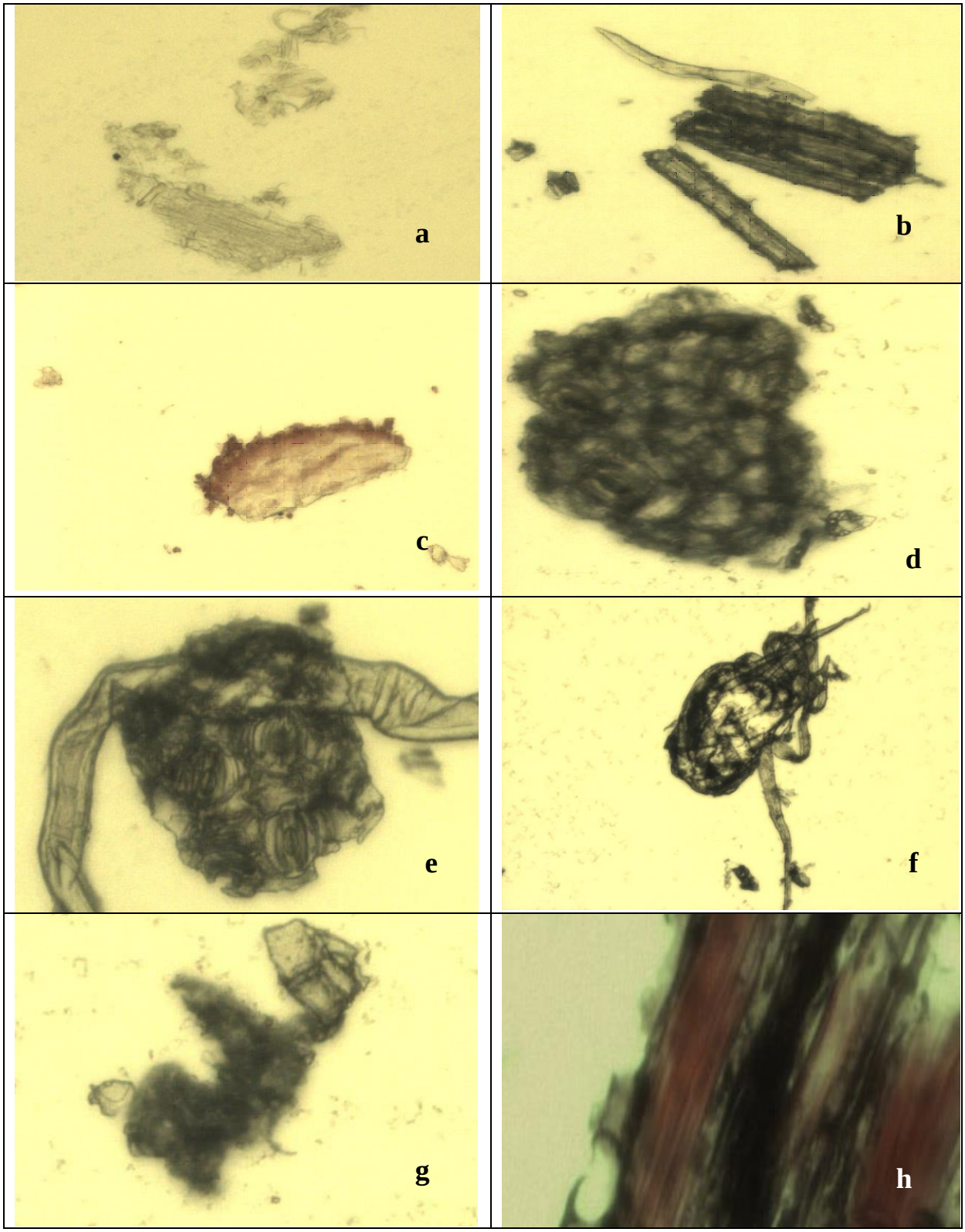

Fig. 2. Powder microscopic features of leaves of *P. longifolia* (x20), (a): Xylem elements, (b): Pitted vessels, (c): Stone cell, (d): Parenchyma cells, (e) Stomata, (f): Fiber of leaf, (g): Crystal, (h): Calcium oxalate crystals

The maximum amounts of toxic metals and non-metals, such as mercury and arsenic, in medicinal plant materials can be given based on the provisional tolerable intake (PTI) values. These values should be established on a regional or national basis, and, for Malaysia, the limits for mercury and arsenic are 0.5 mg/kg and 5 mg/kg, respectively (WHO 2007). Moreover, the findings of this study also prove that the plant material was not contaminated during preparation.

HPLC Analysis

The present study was undertaken with the objective of determining whether samples of prepared *P. longifolia* extract met required standards. The critical determination was done by HPLC. To develop a separation method, various mobile phase combinations were tried. After several preliminary trials, a Symmetry C_{18} reversed-phase column and a gradient elution using a mobile phase consisting of methanol/water/formic acid (49:50:1, v/v) was found to be optimum. To standardize the fingerprint, 10 batches of *P. longifolia* samples from Penang, Malaysia, were analysed with the developed procedure. The peaks that existed in all ten batches of samples were assigned as "common peaks" for the *P. longifolia* extract (Fig. 3).

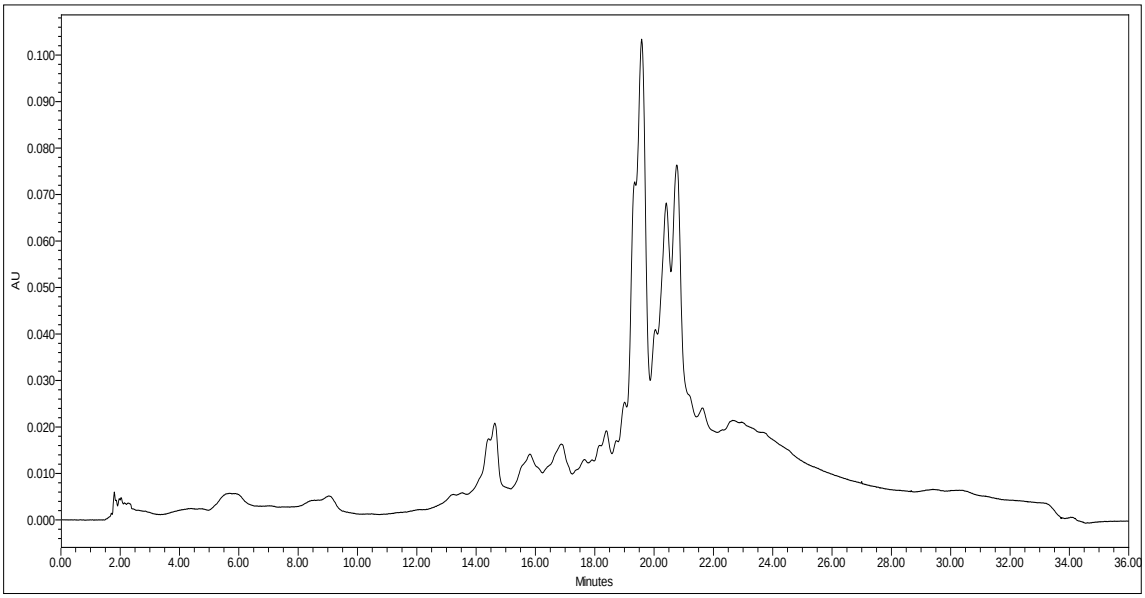

Fig. 3. HPLC fingerprint chromatograms of *P. longifolia* leaf at 190 to 400 nm wavelength in the UV-Vis detector

There were nine "common peaks" in the fingerprint. The whole chromatogram could provide a useful means of identifying and assessing *P. longifolia*. Mok and Chau (2006) recommended the authentication and quality control of herbal medicines using a pattern-based approach because the chemical ingredients of most medicinal plants have been studied; however, it is difficult to determine which compound is effective. Moreover, HPLC is a popular method for the analysis of medicinal plants because it is easy to learn and use and is not limited by the volatility or stability of the extract or compound.

In general, HPLC can be used to analyse almost all compounds in medicinal plants. Thus, in this study, HPLC was also used as one of the characterization methods (Leonard *et al*. 2003; Lin and Chen 2003*;* Cawthray 2003). Furthermore, the USA, China, France, Germany, Malaysia, Britain, India, and the UN's World Health Organization (WHO) have all adopted fingerprinting to evaluate the quality of medicinal plants (Zhang *et al.* 2011).

Characterization of GCMS Quantification of the *P. longifolia* Extract Using Chemical Markers

Generally, chemical markers or bioactive compounds in medicinal plants are employed for evaluating the quality and authenticity of herbal products. In this study, the *P. longifolia* leaf extract was identified as an extract containing constituents documented as being determinant or relevant for efficacy, or as having pharmacological or clinical relevance. In this case, chemically defined constituents (markers) with known therapeutic activity may be used for control purposes (Yadav *et al*. 2009). These markers may be used to monitor good manufacturing practice or as an indication of the content of the extract (Yadav *et al*. 2009). The prevailing compound (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate (*i.e*., cycloeucalenol acetate) in the methanol extract of *P. longifolia*, which has been reported as possessing biological activity (Singariya *et al*. 2012), was used as a chemical marker in this study (Fig. 4). GC-MS is a popular method for the analysis of medicinal plants because it has very good separation ability, which can produce a chemical fingerprint of high quality. Furthermore, with the coupled mass spectroscopy and the corresponding mass spectra database, the qualitative and relatively quantitative composition information of the herb investigated could be provided by GC-MS, which will be extremely useful for elucidating the relationship between chemical constituents in herbal medicine and its pharmacology in further research (Velasco-Negueruela *et al*. 2003a,b).

The GC-MS analytical method for the profiling of the *P. longifolia* leaf extract was standardized. GC-MS for the quantification of (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate was also standardized. Standardization was done by taking the identified marker (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate. By varying the concentration of the extract, 20 µL of the extract solution was injected into the GC-MS system and kept under the same conditions as for the fingerprinting.

A calibration curve was plotted using the area of the peak obtained in the GC-MS chromatogram against the amount of (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate (in the extract) injected. The amount of (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate in the extract was determined by comparing the peak area with the calibration curve. The GC-MS profile and calibration curve of (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate are given in Figs. 4 and 5. These standardized methods enabled the qualitative and quantitative profiling of the *P. longifolia* sterol tested in this study. This method could be applied for the standardization and validation of the *P. longifolia* traditional formulation in terms of (3*β*,4*α*,5*α*,9*β*)-4,14-Dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate as a chemical marker.

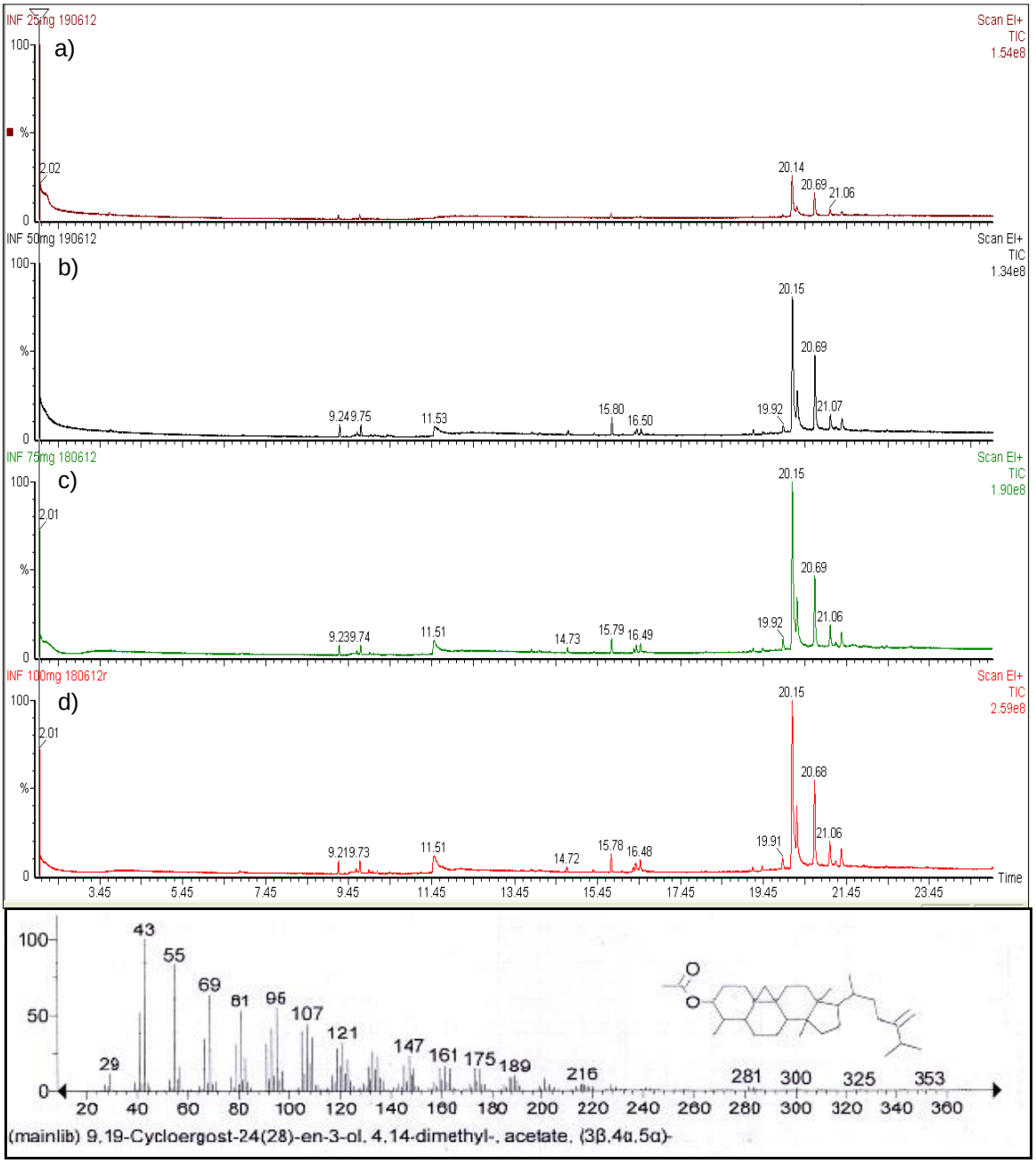

Fig. 4. GC-MS profile of (3*β*,4*α*,5*α*,9*β*)-4,14-Dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate at retention time 20.69 min from *P. longifolia* leaf extract (a=25 mg/mL, b=50 mg/mL, c=75 mg/mL, d=100 mg/mL, concentration of the extract)

High-Performance Thin Layer Chromatography (HPTLC) Fingerprinting

TLC was the preferred common method for medicinal plant investigation before instrumental chromatography methods, such as GC and HPLC, were established. Some major advantages of HPTLC are that it is a more efficient and faster method and the results are more reliable and reproducible.

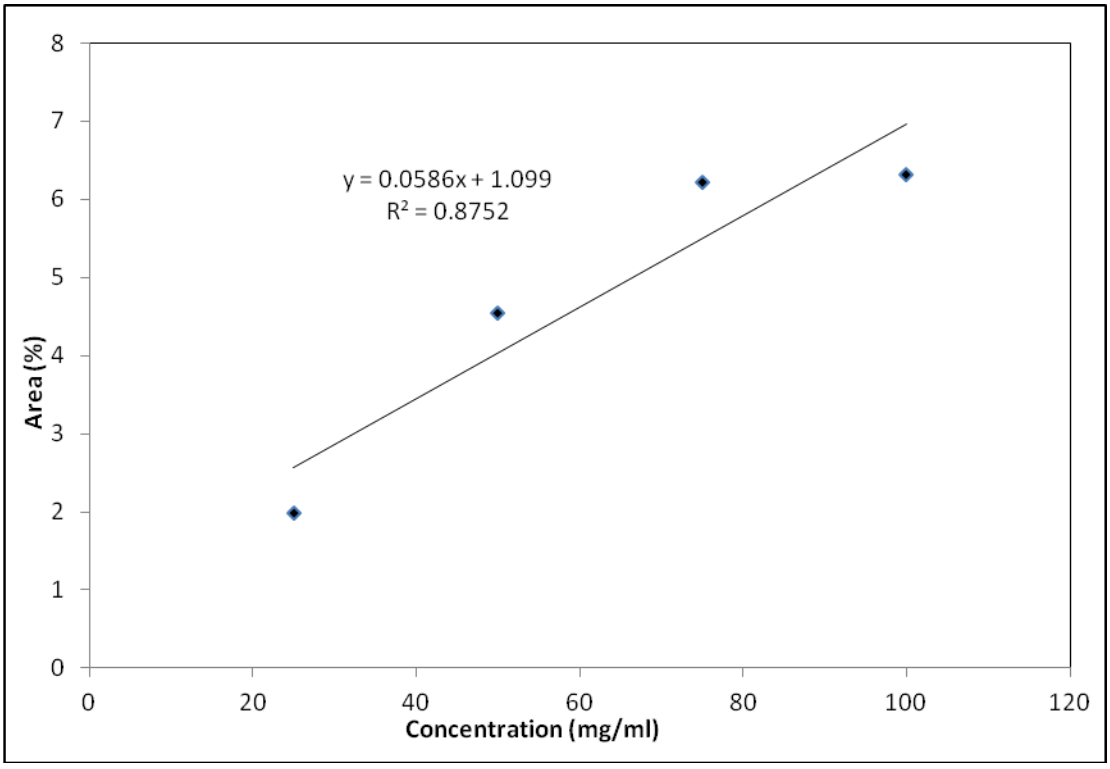

Fig. 5. Calibration curve of (3*β*,4*α*,5*α*,9*β*)-4,14-dimethyl-9,19-cycloergost-24(28)-en-3-yl acetate by GCMS method

In combination with digital scanning profiling, HPTLC also provides accurate and precise R_f values and quantitative analysis of samples by *in situ* scanning densitometry aided by the formation of easily detected derivatives by post-chromatographic chemical reactions as required. In addition, HPTLC also records the separation in the form of a chromatogram with fractions represented as peaks, with defined parameters including absorbance (intensity), R_f, height, and area (Moffat 2001). The HPTLC profile of the *P. longifolia* leaf extract was evaluated for the first time using toluene: ethyl acetate: formic acid: methanol (3:3:0.8: 0.2, v/v) as the eluent system. Figures 6a and 6b illustrate the thin layer chromatography separation of the *P. longifolia* leaf extract visualised under ambient daylight and 365 nm UV light, respectively. The chromatograms indicated the occurrence of at least ten different components in the methanol extract. The chromatogram, as shown in Fig. 5b, revealed the presence of the components in the methanol extract, which are reddish, violet, light blue, and light green in colour, under 365 nm UV light. The HPTLC profile (Fig. 7) convincingly illustrated the presence of ten peaks in the *P. longifolia* leaf extract with R_f values of 0.06, 0.01, 0.14, 0.22, 0.41, 0.75, 0.83, 0.91, 099, and 1.04. The peaks in the HPTLC profile are attributed to the chemical compounds. As in the above work, it has been revealed that the methanolic extract of the *P. longifolia* leaf has ten peaks in the chromatogram, which has been produced by HPTLC. Thus, the developed HPTLC chromatogram of the *P. longifolia* will be specific for the selected solvent system toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 0.2, v/v) and R_f values. Accordingly, the developed HPTLC chromatogram could serve as a better tool for standardization. Hence, HPTLC fingerprinting has proven to be a simple, precise, and accurate method for herbal formulation and can be used further in the quality control of

P. longifolia. Moreover, the developed HPTLC fingerprints will help the manufacturer in the quality control and standardization of *P. longifolia*. Khatoon *et al* (2009) also reported the standardization of *P. longifolia* bark by using HPTLC method with toluene and ethyl acetate (9.3: 0.7) as solvent system. They reported that the *P. longifolia* bark showed some characteristic bands at R_f 0.13 (orange), 0.21, and 0.27 (both fluorescent blue), and 0.38 and 0.49 (both blue) under ultra violet light 366 nm.

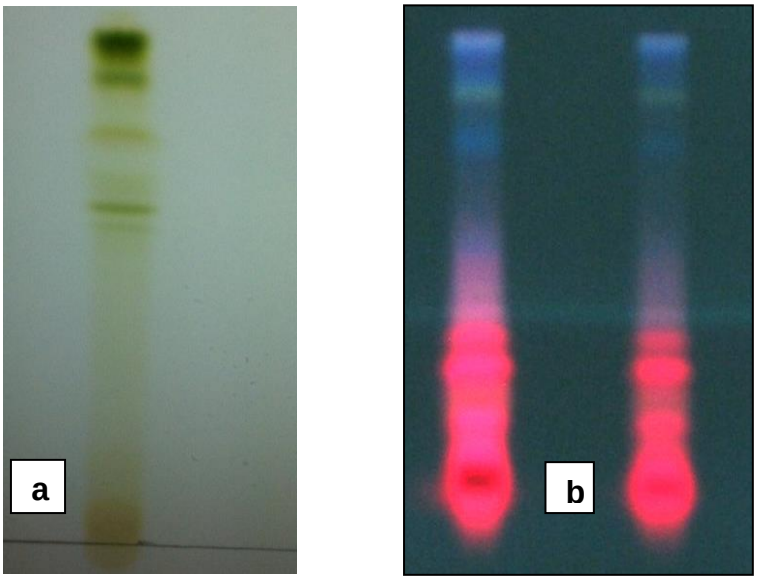

Fig. 6. HPTLC profile of *P. longifolia* leaf extract under daylight (a) and 365 nm UV light (b)

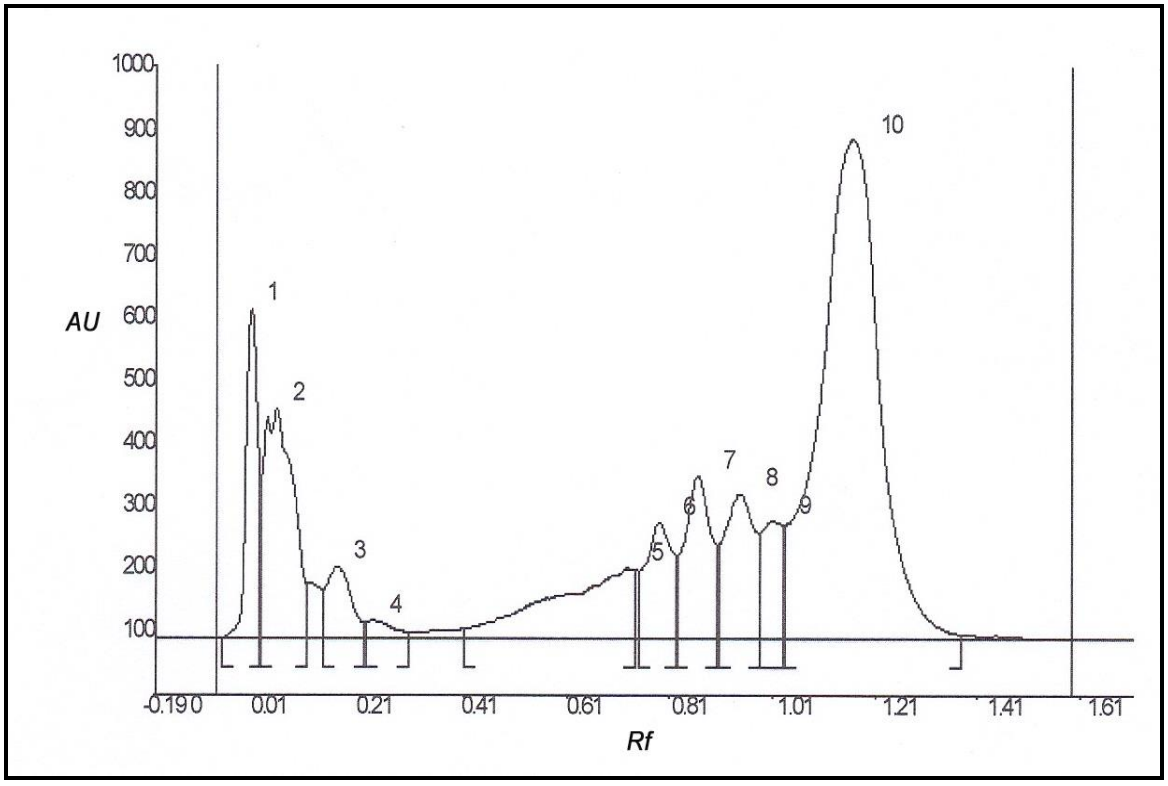

Fig. 7. HPTLC Chromatogram of *P. longifolia* leaf extract

Fourier Transform Infrared (FTIR) Spectral Fingerprinting

The use of FTIR spectral fingerprinting for herbal preparations tends to focus on the identification and assessment of the stability of the chemical constituents' functional groups. The functional group identification is based on the FTIR peaks attributed to the stretching and bending vibrations. The results of the FTIR spectrum of the *P. longifolia* leaf extract are shown in Fig. 8.

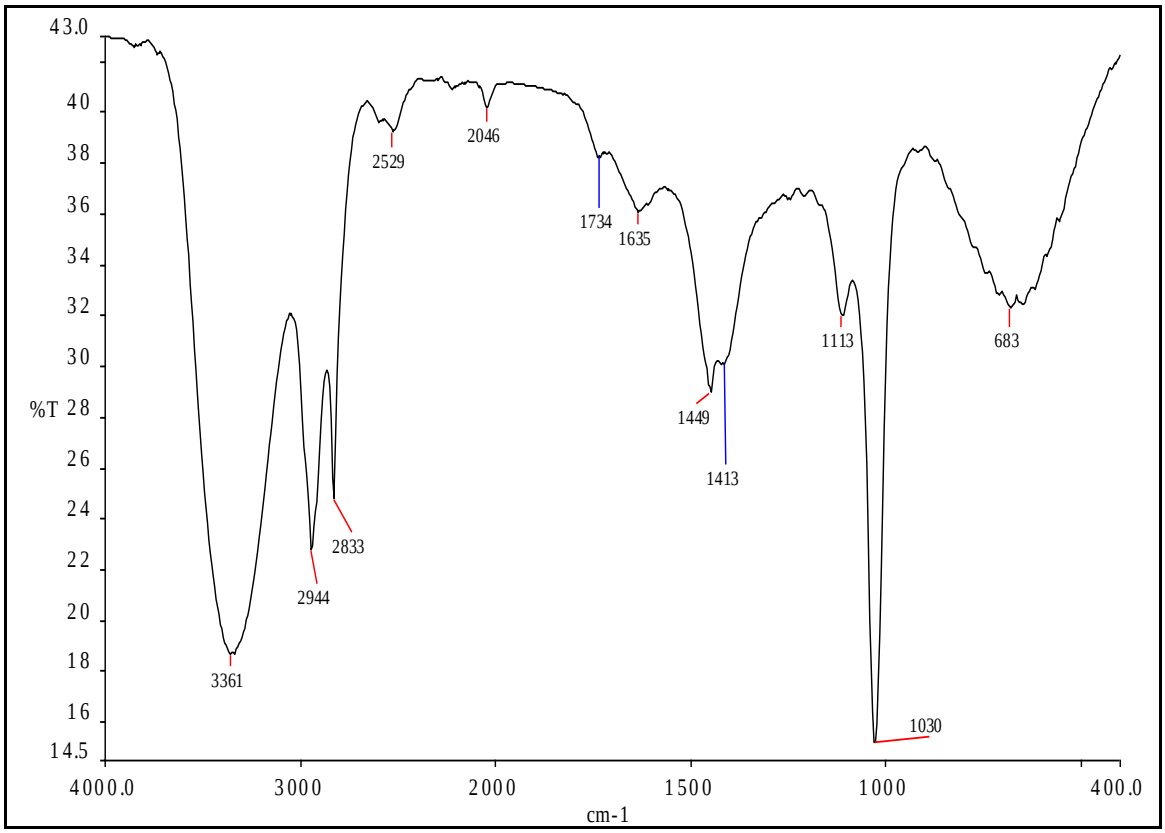

Fig. 8. FT-MIR spectrum of methanolic leaf extract of *P. longifolia*

Twelve areas (marked with wave numbers) were identified in the mid infrared (MIR) domain and the fingerprint region. The results of the functional group analysis using FTIR demonstrated the existence of various characteristic functional groups in the *P. longifolia* extract. IR absorptions below 1000 cm^{-1} corresponded to C-H bending vibrations; the absorptions from 997 to 1130 cm^{-1} were attributed to stretching vibrations of C-O of monosaccharides, oligosaccharides, and carbohydrates, while the absorptions from 1150 to 1270 cm^{-1} corresponded to the stretching vibrations of carbonyl C-O or O-H bendings. The absorptions from 1300 to 1450 cm^{-1} corresponded to stretching vibrations of C-O (amide) and of C-C stretching from the phenyl groups, while the absorptions from 1500 to 1600 cm^{-1} corresponded to the aromatic domain and the N-H bending vibrations. The absorptions in the complex range, from 1600 to 1760 cm^{-1}, corresponded to the bending vibrations of N-H (amino acids), C=O stretching (aldehydes, ketones and esters). The absorptions from 2800 to 2900 cm^{-1} were attributed to C-H stretching vibrations specific to CH_3- and -CH_2- (from lipids and methoxy derivatives), and C-H (aldehydes), as well as from *cis* double bonds. In addition, the absorptions from 3350 to 3600 cm^{-1}

corresponded to the stretching vibrations of the -OH groups (from water, alcohols, phenols, carbohydrates, *etc*.) as well as from amides. Therefore, for future *P. longifolia* methanolic extraction, this FTIR spectrum can be used for comparison. The intuitive evaluation method is to compare the similarities and/or differences in the shape of the FTIR fingerprints. Moreover, the FTIR fingerprint obviously can be used to ensure that the functional groups in the new extract are present in a reproducible manner even though a new extraction has been performed. As a result, the FTIR fingerprints can assist the manufacturer in controlling and ensuring the consistency and the standard quality of the extracts in each phase of the extraction process (Torey *et al*. 2010).

CONCLUSIONS

1. The chromatographic and spectral fingerprinting procedure for the characterization of *P. longifolia* extracts, which can be used as quality control, has been described in this paper.

2. This validated method enables the quality of the *P. longifolia* to be determined and can be used in the pharmaceutical industry as a pharmacognostical tool to identify this medicinally important plant.

3. In addition, this method can be adopted as a chemotaxonomical tool for plant classification. Furthermore, the separation and characterisation of the bioactive compounds from the *P. longifolia* extract will be evaluated and reported in the near future.

4. The developed chromatographic and spectral fingerprints will help the manufacturer with the quality control and determination of compliance with standards for *P. longifolia*.

ACKNOWLEDGMENTS

This project was funded by the Research University Post Graduate Research Grant Scheme (RU-PRGS) (1001/CIPPM/846041) from Universiti Sains Malaysia and UM-MoHE HIR Grant (grant no.: UM-MoHE HIR H-18001-C0020) from the University of Malaya, Malaysia. Subramanion L. Jothy was supported by the MyPhD fellowship from the Ministry of Higher Education, Government of Malaysia, Malaysia.

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Article submitted: April 18, 2013; Peer review completed: August 8, 2013; Revised version received: August 15, 2013; Accepted: August 16, 2013; Published: August 20, 2013.