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Original Article

Comparative study of three *Marantodes pumilum* varieties by microscopy, spectroscopy and chromatography

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ABSTRACT

Marantodes pumilum (Blume) Kuntze (synonym: *Labisia pumila* (Blume) Fern.-Vill), Primulaceae, is well known for its traditional use as a post-partum medication among women in Malaysia. Three varieties of *M. pumilum*, var. *alata* Scheff., var. *pumila* and var. *lanceolata* (Scheff.) Mez. are commonly used. Nowadays, *M. pumilum* powder or extracts are commercially available as herbal supplements and beverages. Authentication of the variety is an important component of product quality control. Thus, the present work was aimed to compare the three varieties using microscopic, spectroscopic and chromatographic techniques. Microscopic anatomical examination and powder microscopy were performed on fresh and dried plant materials, respectively. Fingerprint profiles of the varieties were obtained using attenuated total reflectance-Fourier transform infrared spectrophotometer, high performance thin layer chromatography and high performance liquid chromatography. The microscopic examination showed presence of anisocytic stomata, scale and capitate glandular trichome in all varieties. The type of stomata and trichomes, outline structure of stem and leaf margin, petiole and midrib, organization of vascular system, areolar venation, pattern of anticlinal walls, the distribution of secretory canals and cell inclusion as well as the measurement of selected structures could be used to distinguish and identify each variety of *M. pumilum*. In addition, spectroscopic and chromatographic fingerprint analyses of the three varieties exhibited distinguishable profiles based on the intensity of certain peaks or bands. The findings from this study will provide systematic identification for these varieties.

Introduction

Marantodes pumilum (Blume) Kuntze belongs to the family Primulaceae. It was previously known as *Labisia pumila* (Blume) Fern.-Vill. or *L. pumila* (Blume) Mez. and classified under genus *Labisia* of the family Myrsinaceae (The Plant List, 2013). According to the classification of Angiosperm Phylogeny Group III (APG III), the apomorphies of Myrsinaceae and Primulaceae are closely related and are very difficult to recognize. The limits of the two families have been substantially changed, whereas the limits of Primulaceae were extended based on the numerous recognizable synapomorphies (Mabberley, 2008).

M. pumilum is locally known as Kacip Fatimah in Malaysia (Burkill, 1935) and is one of the most popular Malaysian medicinal herbs that has been used by the Malay women for more than 400 years (Choi et al., 2010). The water decoctions of the root and the whole plant have been utilized to induce and facilitate labor during delivery, to help with delayed fertility and to regain body strength after delivery (Zakaria and Mohd, 1994). Other folkloric uses include curing of flatulence, dysentery, dysmenorrhea, gonorrhea and bone sickness (Burkill, 1935; Quattrocchi, 2012).

Several scientific studies have shown that *M. pumilum* possesses antibacterial (Karimi et al., 2011), antioxidant (Norhaiza et al., 2009), antifungal and anti-inflammatory (Karimi et al., 2013) properties. It has also been reported to reduce the risk of cardiovascular diseases (Ayida et al., 2008), protect skin cells from photo-aging caused by UVB irradiation (Choi et al., 2010) and have anti-carcinogenic activity (Pihie et al., 2011). *M. pumilum* was found to contain mainly triterpenoid saponins and alkenated phenolics (Avula et al., 2011), methyl gallate (Hisham et al., 2011), flavonoids and phenolic compounds (Karimi and Jaafar, 2011), dialkenated benzoquinones, dialkenated dibenzofuran and dexyloprimulanin

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(Ali and Khan, 2011), alkyl resorcinols and dimeric benzoquinone derivatives (Al-Mekhlafi et al., 2012). Recently, many commercial products are available *such as* health supplements in a form of capsule containing *M. pumilum* powder or extract, and beverages (Singh et al., 2009). Hence, there is an increasing demand for the supply of *M. pumilum* in pharmaceutical and food industries. Accurate authentication of the raw material is very important as a first step of a quality control process.

According to Sunarno (2005), there are eight varieties of *M. pumilum*. However, in Malaysia three varieties are well-known, that is, *M. pumilum* var. *alata* Scheff., *M. pumilum* var. *pumila* and *M. pumilum* var. *lanceolata* (Scheff.) Mez. (Stone, 1989). The first two varieties are commonly used in traditional preparations and research. However, the close macromorphological characteristics of the two varieties made physical identification rather difficult. Thus, an effective method of identification needs to be developed in order to help with the authentication of the varieties. Hence, the present study was aimed to compare the leaf and stem parts of the three varieties based on microscopic study and chemical profiling using attenuated total reflectance-Fourier transform infrared spectrophotometer (ATR-FTIR), high performance thin layer chromatographic (HPTLC) and high performance liquid chromatographic (HPLC) techniques.

Materials and methods

Plant materials

Three varieties of *Marantodes pumilum* (Blume) Kuntze, Primulaceae (Fig. 1) were collected from the Bujang Melaka Forest Reserve, Kampar in August 2012. The plants were authenticated by Emeritus Professor Dato' Dr. Abdul Latiff Mohamad and voucher specimens of *M. pumilum* var. *alata* (UKMB 30006/SM 2622), var. *pumila* (UKMB 30007/SM s.n.) and var. *lanceolata* (UKMB 30008/SM s.n.) were deposited in the Herbarium of Universiti Kebangsaan Malaysia. The plant materials were garbled, washed, separated into the leaf and stem parts, air-dried under shade and then coarsely powdered using a rotary grinder. The powdered materials were individually stored in an airtight container until further investigation.

Preparation of extracts

The powdered materials were individually macerated with dichloromethane (Merck, Germany) in a ratio of 1:5. The extracts were filtered and the solvents were evaporated under vacuum. The steps were repeated three times. The dried extracts were stored in a refrigerator at 4 °C until further analyses.

Microscopic analysis

For anatomical study, fresh leaves, petioles, and stems were fixed in AA (1:3) of acetic acid (30%) and ethanol (70%). Fixation, embedding, sectioning, epidermal mechanical scrapping and staining were done according to the procedures by Johansen (1940) and Sass (1951) with suitable modification. Stem and petiole transverse section of the specimens were made with a sliding microtome at 20–30 μm thickness and stained in 1% safranin in 50% alcohol and 1% alcian green in 100 ml purified water with three drops of acetic acid. Sections of the leaf marginal and lamina were made from the middle and marginal parts of the leaf lamina using a Reichert sliding microtome. All slides were mounted in Euparal after dehydration using alcohol series of 50, 70, 95, and 100%, successively. Photomicrographs of the sections were taken by using either a LeitzDiaplan polarizing microscope fitted with a JVCCCD camera or a Reichert Polyvar 2 microscope fitted with a digital camera. The images were processed using Analysis DocuSoftware imaging system. All slides were deposited in the Microtechnique Laboratory, Universiti Kebangsaan Malaysia, Malaysia. On the other hand, histochemical analysis was done on the powdered plant materials to observe presence of certain microscopic structures using the respective specific chemical reagents: lignified cell wall (phloroglucinol/HCl), calcium oxalate (chloral hydrate), starch (N/50 iodine), tannin (ferric chloride) and suberized cell wall (Sudan red TS) (WHO, 2011). The measurement of length and/or width of selected structures were obtained using LeitzDiaplan polarizing microscope fitted with camera. The mean and standard deviation were calculated.

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectrophotometric profiling

One dimensional (1D-ATR-FTIR) spectra of every crude powdered leaves and stems were collected in the range of 4000–650 cm^{-1} with a resolution of 4 cm^{-1} for 16 scans using an ATR-FTIR spectrophotometer (Spectrum 100 FTIR spectrophotometer (Perkin Elmer Ltd., USA), universal ATR (UATR) DiComp™ crystal of a diamond ATR with zinc selenide focusing element (Perkin Elmer Ltd., USA) and equipped with the Perkin Elmer Spectrum Express software). The presented spectrum was an average of eight repeated experiments ($n = 8$).

High performance thin layer chromatographic (HPTLC) profiling

HPTLC was performed for all the dichloromethane extracts (10 mg/ml) using pre-coated glass plates with silica gel (Merck, GF_{254}, 10 × 10 cm, 0.2 mm thickness) at room temperature. Toluene–acetone (9:1) and chloroform–methanol (9:1) were selected as mobile phase systems. The extracts were applied onto the plate using a semi-automatic applicator (CAMAG Linomat 5). Images of separated bands were captured using a digital camera TLC visualizer connected to winCATS software (CAMAG) under day light, UV at 254 nm and at 366 nm, as well as after dipping with a mixture of *p*-anisaldehyde and sulphuric acid reagent and followed by heating at 100 °C for 10 min. Densitometric analysis was also performed for the separated bands.

High performance liquid chromatographic (HPLC) profiling

HPLC was carried out according to the method described by Avula et al. (2011) with slight modification. HPLC (Waters 2535) equipped with an analytical reversed phase C-18 (4.6 mm × 250 mm, 5 μm; XBridge, Waters, Ireland) and photodiode array detector (Waters 2998) were used. DCM extracts (10 mg/ml) in HPLC-grade methanol were injected (20 μl) three times. The fractions were separated using a gradient system of acetonitrile (solvent A) and deionized water (adjusted to pH 2.5 with orthophosphoric acid; solvent B) at a flow rate of 1.0 ml/min. The column was initially equilibrated with solvent A (100%) for 15 min and then followed by a linear gradient elution: 0 min, 70% solvent A: 30% solvent B increased in the next 30 min to 100% solvent B by employing a slightly concave gradient profile (Waters curve type 7). Separated components were detected at 210 nm.

Results and discussion

Microscopic analysis: anatomical study of the leaf and stem

Observations were made on the adaxial and abaxial of leaf surfaces of the three taxa. Findings showed that there are common and variation in leaf epidermis anatomical characteristics. All taxa exhibited polygonal shaped cells on the adaxial surfaces and polygonal or have irregular contour on the abaxial surfaces

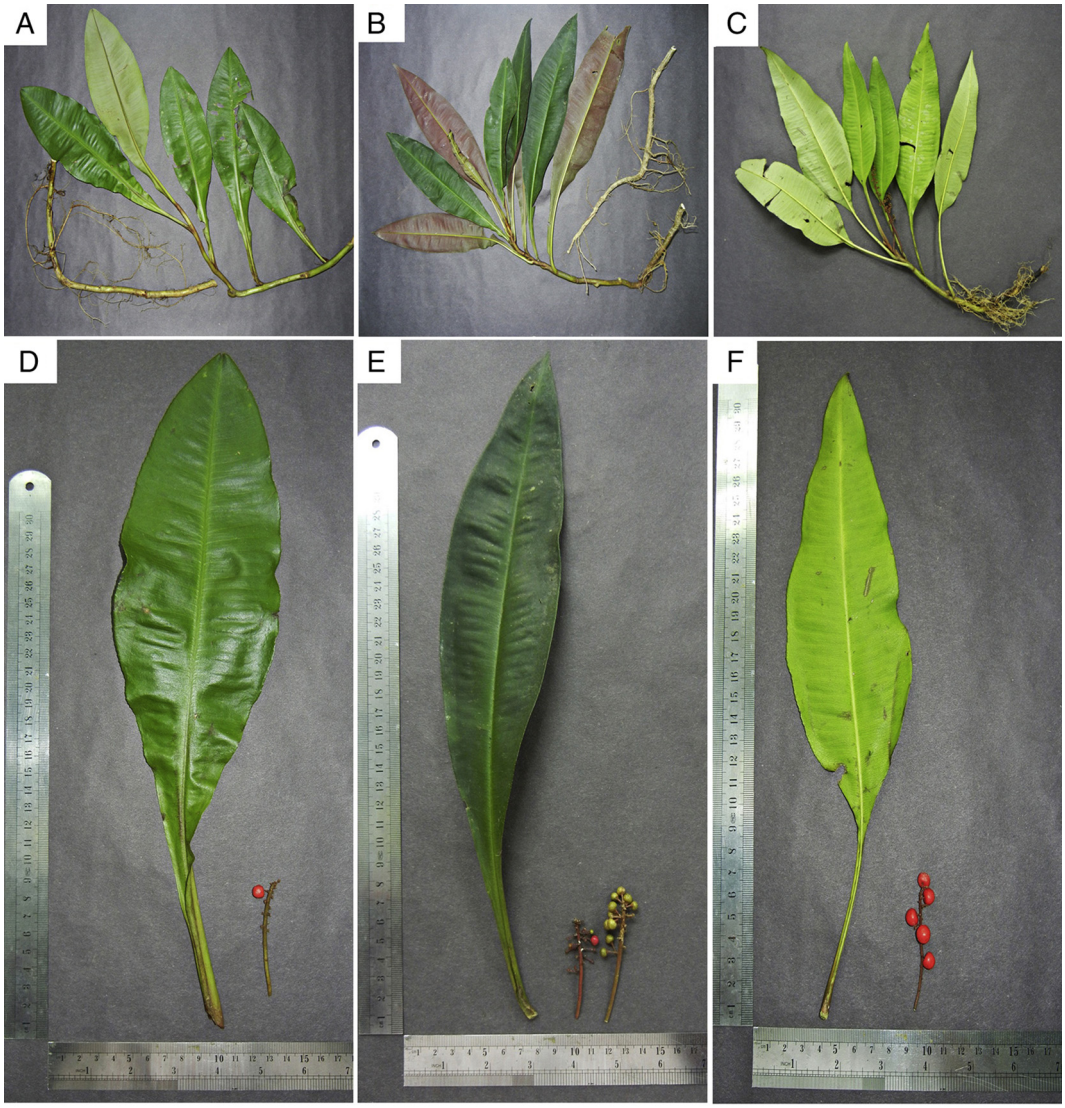

Fig. 1. The whole plant: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila.* C. *M. pumilum* var. *lanceolata.* Macroscopic characteristic of leaf and fruit: D. *M. pumilum* var. *alata.* E. *M. pumilum* var. *pumila.* F. *M. pumilum* var. *lanceolata.*

(Fig. 2A–F). The anticlinal walls of all *M. pumilum* varieties were straight to wavy on the abaxial and straight to curve on the adaxial epidermis of *M. pumilum* var. *alata* and var. *pumila* (Fig. 2D–F). The stomata were present only on the abaxial epidermis of all taxa and this character was known as hypostomatic leaf (Fig. 2G–I). Variation in the leaf epidermis anatomical characteristics could be seen in the type of stomata. Anisocytic stomata were found in all varieties, while staurocytic and diacytic stomata were present only in *M. pumilum* var. *alata* and *M. pumilum* var. *pumila*, respectively. Therefore, *M. pumilum* var. *lanceolata* could be differentiated based on the absence of staurocytic and diacytic stomata. The distribution patterns of stomata based on their orientation and dispersion were found to be stable and hence could be taxonomically useful (Metcalfe and Chalk, 1950; Rajagopal, 1979). According to Luna et al. (2013), anisocytic stomata were only found on abaxial surface of species *Stylogyne* (Myrsinoideae-Primulaceae). Trichomes were found randomly distributed on the abaxial epidermis of all varieties, but only on the adaxial epidermis of *M. pumilum* var. *pumila*. Two types of trichome were clearly found, namely as scale and capitate glandular (Fig. 2D–F). The presence or absence of these

Table 1
Variation in the leaf epidermis anatomical characteristics in *Marantodes pumilum* varieties.

Taxa	Type of trichome		Type of stomata		Pattern of anticlinal walls	
	Adaxial epidermis	Abaxial epidermis	Adaxial epidermis	Abaxial epidermis	Adaxial epidermis	Abaxial epidermis
M. pumilum var. *alata*	Absence	Scale, Capitate glandular	Absence	Anisocyatic, Staurocyctic	Straight to curved	Straight to wavy
M. pumilum var. *pumila*	Simple, 2-armed, Scale	Scale	Absence	Anisocyatic, Diacytic	Straight to curved	Straight to wavy
M. pumilum var. *lanceolata*	Absence	Scale	Absence	Anisocyatic	Straight to wavy	Straight to wavy

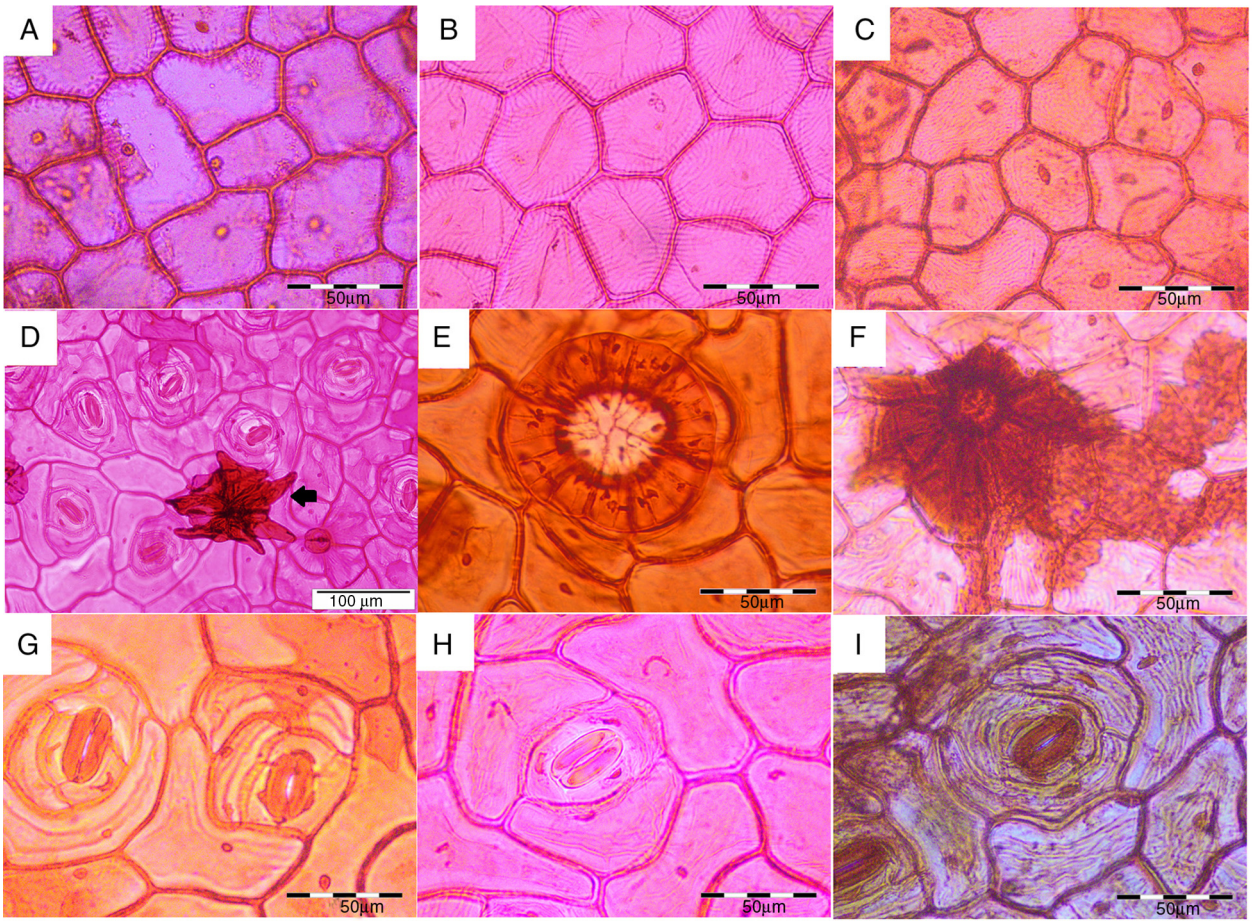

Fig. 2. Adaxial leaf surface with polygonal shaped cells: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila*. C. *M. pumilum* var. *lanceolata.* Scale bar = 50 µm. Abaxial leaf surface showing presence of scale and capitate glandular trichomes: D. *M. pumilum* var. *alata.* Scale bar = 100 µm. E. *M. pumilum* var. *pumila.* F. *M. pumilum* var. *lanceolata.* Scale bar = 50 µm. Higher magnification of stomata: G. *M. pumilum* var. *alata.* H. *M. pumilum* var. *pumila.* I. *M. pumilum* var. *lanceolata.* Scale bar = 50 µm.

characteristics can be used to distinguish between varieties. The significant variation in the leaf epidermis anatomical characteristic that could be used in taxa differentiation is presented in Table 1.

All *M. pumilum* varieties had incomplete marginal venation and as for the areolar venation, the closed system with free ending veinlets was observed only in *M. pumilum* var. *lanceolata.* Meanwhile, both of the *M. pumilum* var. *alata* and var. *pumila* exhibited closed and a few opened system with minor free ending veinlets (Fig. 3). Therefore, the type of areolar venation can be used to distinguish between *M. pumilum* varieties. The anatomical characteristic of leaf venations of three varieties is given in Table 2.

The three varieties of *M. pumilum* shared similar leaf lamina anatomical characteristics with homogeneous mesophyll and had large intercellular spaces. The main vascular bundles in the leaf lamina were found in the middle with no connection to adaxial as well as abaxial epidermis, and surrounded by sclerenchymatous sheath. Solitary crystals (calcium oxalate) were present abundantly in the main vascular bundles (Fig. 4A–C). However, the three varieties could be differentiated by the marginal direction: tapering outline with recurved downwards (30–45°) in *M. pumilum* var. *alata* and rounded outline with recurved upwards (10–30°) in *M. pumilum* var. *pumila* and *var. lanceolata* (Fig. 4D–F). The summary of the anatomical characteristics of leaf lamina and marginal direction between the taxa is displayed in Table 3.

In transverse sections, the outline of midrib *M. pumilum* var. *alata* was slightly convex-shaped at adaxial side and U-shaped at abaxial side, while the midrib outline of the other two varieties was flat-shaped at adaxial side and 3/4 of circle at abaxial side (Fig. 5A–C). Furthermore, the outline of petiole of *M. pumilum* var. *alata* and var. *pumila* showed the presence of wing at both of the left and right of adaxial side and 3/4 of circle shape at abaxial side, whereas *M. pumilum* var. *lanceolata* was oval in shape (Fig. 5D–F). Moreover, both of midrib and petiole of the three varieties exhibited the presence of collenchyma cells under the epidermis layer. On the other hand, the clustered sclerenchyma cells were found outside of phloem of vascular bundles. The trichomes were found in two types; scale and capitate glandular. The main vascular tissue arrangement of midrib and petiole was observed as open and closed systems, respectively, and consists of main, medullary and additional vascular bundles. The details of organization vascular system of the midrib and petiole in all taxa studied are described in Tables 4 and 5, respectively. In addition, brachyscelereids were distributed under an epidermis of petiole of *M. pumilum* var. *alata* and

Table 2
Leaf venation anatomical characteristics in *Marantodes pumilum* varieties.

Taxa	Marginal venation	Areolar venation
M. pumilum var. *alata*	Incomplete	Closed, a few opened, minority free ending veinlets
M. pumilum var. *pumila*	Incomplete	Closed, a few opened, minority free ending veinlets
M. pumilum var. *lanceolata*	Incomplete	Closed, free ending veinlets

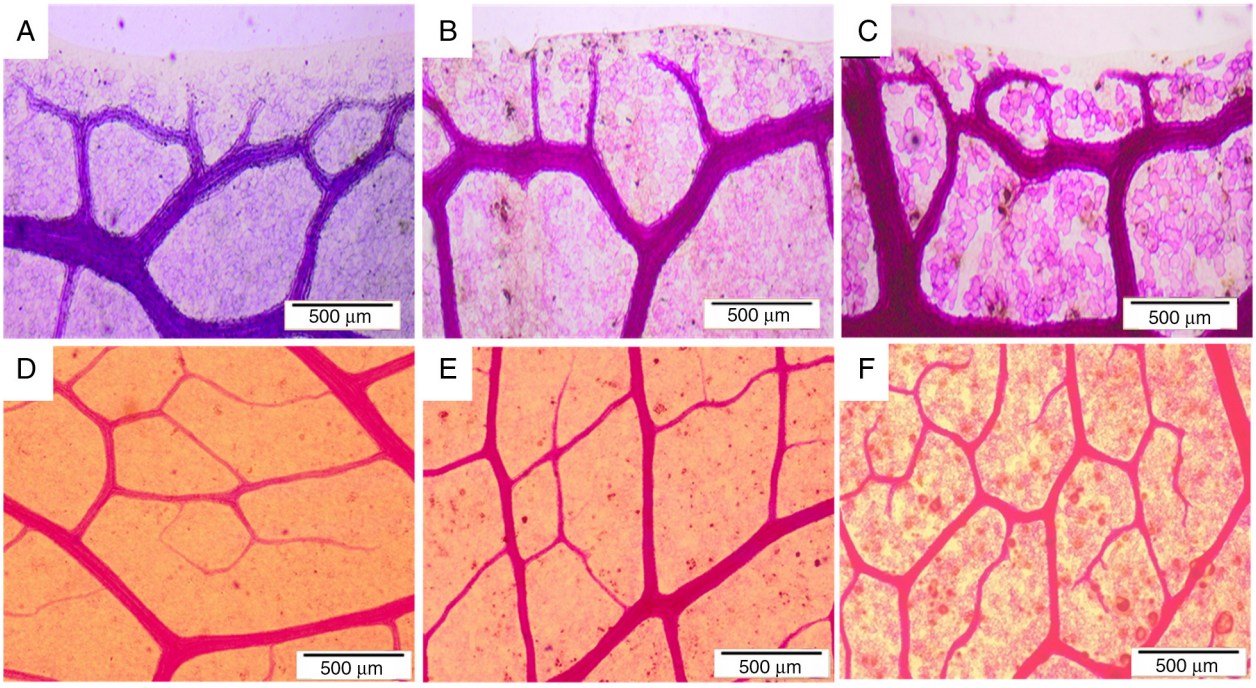

Fig. 3. Incomplete marginal venation: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila.* C. *M. pumilum* var. *lanceolata.* Closed and free ending veinlets: D. *M. pumilum* var. *alata.* E. *M. pumilum* var. *pumila.* F. *M. pumilum* var. *lanceolata.* Scale bar = 500 µm.

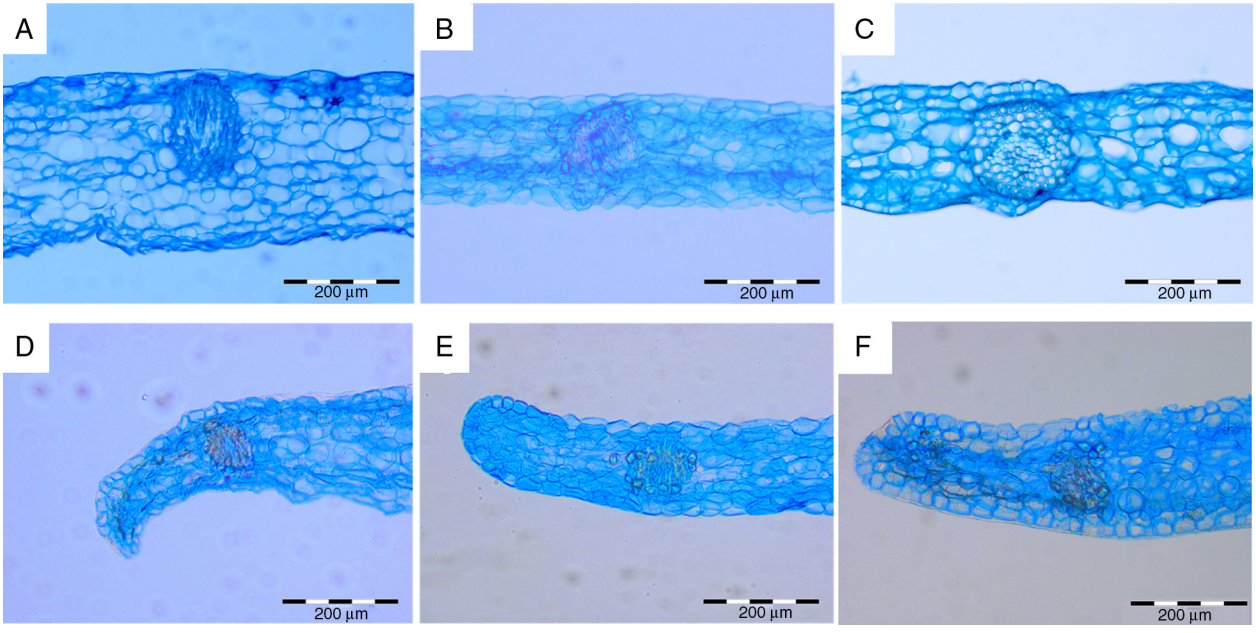

Fig. 4. Transverse section of leaf lamina: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila.* C. *M. pumilum* var. *lanceolata.* Transverse section of leaf margins: D. *M. pumilum* var. *alata.* E. *M. pumilum* var. *pumila.* F. *M. pumilum* var. *lanceolata.* Scale bar = 200 µm.

var. *pumila*, but absent in *M. pumilum* var. *lanceolata.* Previous study by Luna et al. (2013) reported that the presence of brachysclereids in the petiole was also an efficient character to distinguish *Stylogyne* species (Myrsinaceae). Besides, the presence or absence and the distribution of this feature in the plants have been used by some authors to differentiate close related species (Costa et al., 2010). The presence of druses and solitary crystals (calcium oxalate) were also observed in transverse sections of midrib and petiole in the

Table 3
Variation in the leaf lamina and marginal anatomical characteristics in *Marantodes pumilum* varieties.

Taxa	Main vascular bundle in the leaf lamina Location	Marginal outline	Marginal direction
M. pumilum var. *alata*	Close to the adaxial epidermis	Tapering	30–45° downwards
M. pumilum var. *pumila*	Equidistant to abaxial and adaxial epidermis	Rounded	10–30° upwards
M. pumilum var. *lanceolata*	Equidistant to abaxial and adaxial epidermis	Rounded	10–30° upwards

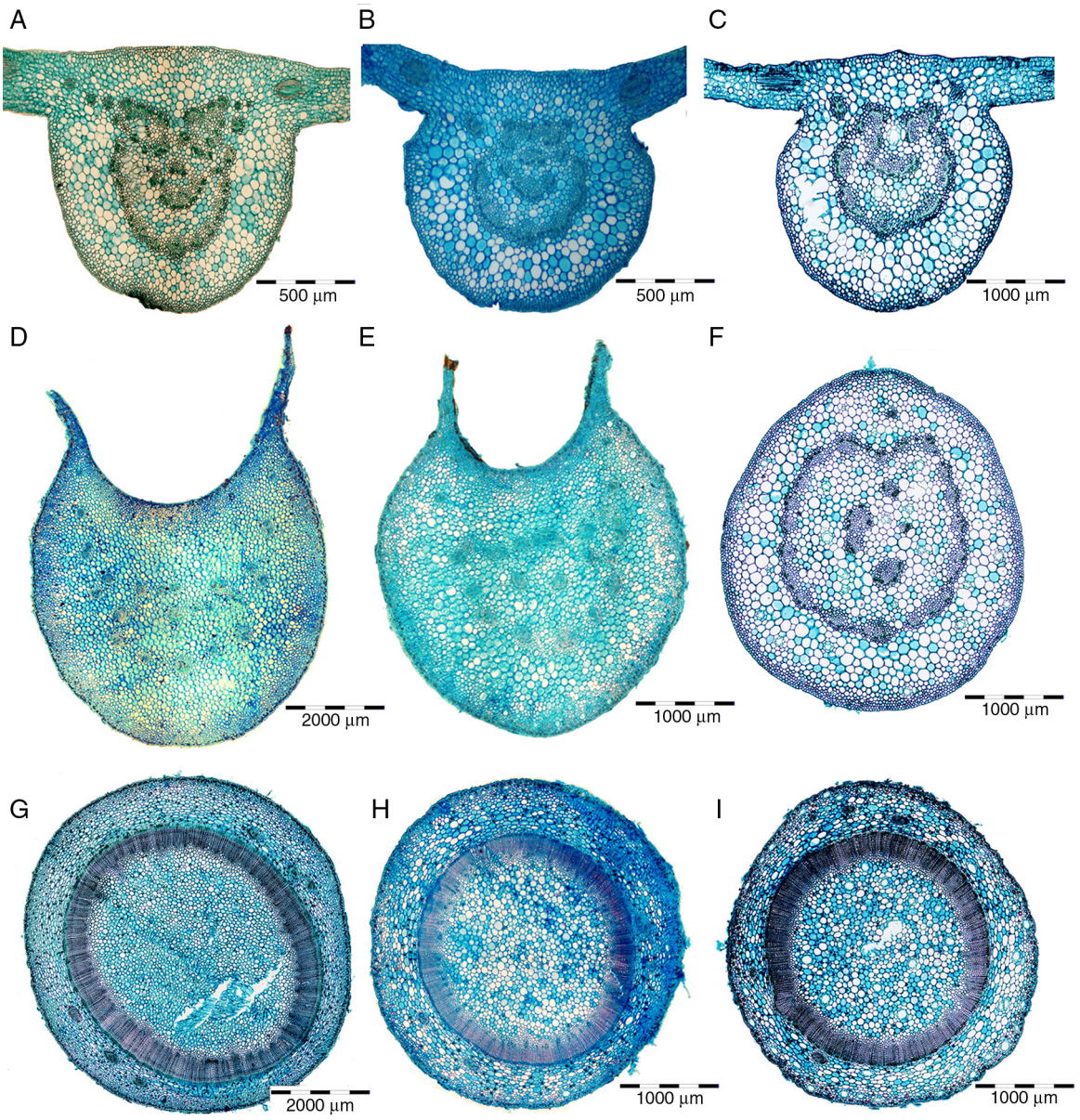

Fig. 5. Transverse section of midrib: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila.* C. *M. pumilum* var. *lanceolata.* Transverse section of petiole: D. *M. pumilum* var. *alata.* E. *M. pumilum* var. *pumila.* F. *M. pumilum* var. *lanceolata.* Transverse section of stem: G. *M. pumilum* var. *alata.* H. *M. pumilum* var. *pumila.* I. *M. pumilum* var. *lanceolata.*

Table 4
Midrib anatomical characteristics of *Marantodes pumilum* varieties.

Taxa	Outline		Vascular tissues			Cells inclusion	Type of trichome
	Adaxial	Abaxial	Main vb	Medullary vb	Additional vb		
M. pumilum var. *alata*	Slightly convex	U-shaped	Opened system, U-shape at the abx side (separated several vb)	Consists of 2 O-shaped vb at the adx side, U-shaped vb at the abx side with 1 vb in the middle	Several vb at the left and right of adx side	Solitary crystal CO (rectangular), druses, scattered starch grains	Scale, Capitate glandular
M. pumilum var. *pumila*	Flat/straight	3/4 of circle	Opened system, 3/4 of circle at abx side (separated several vb)	Consists of a stack of vb at the adx side, U-shaped vb at the abx side with 1 vb in the middle	One vb at the left and right of adx side	Solitary crystal CO (cubic), clustered starch grains	Scale, Capitate glandular
M. pumilum var. *lanceolata*	Flat/straight	3/4 of circle	Opened system, 3/4 of circle at abx side (separated several vb)	Consists of 2 vb at the adx side, U-shaped vb at the abx side	One vb at the left and right of adx side	Solitary crystal CO (cubic), druses, scattered starch grains	Scale

abx, abaxial; adx, adaxial; vb, vascular bundle; CO, calcium oxalate.

Table 5
Petiole anatomical characteristics of *Marantodes pumilum* varieties.

Taxa	Outline	Vascular tissue			Cell inclusions	Type of trichome
		Main vb	Medullary vb	Additional vb		
M. pumilum var. *alata*	Wing presence at the left and right of adx side, 3/4 of oval at abx side	Opened system with separated several vb, U-shaped at abx side	Opened system, consists of single vb	Consists of 3–4 vb at the right and left of adx side	Brachyscelereids, solitary crystals CO (cubic), druses	Scale
M. pumilum var. *pumila*	Wing presence at the left and right of adx side, 3/4 of oval at abx side	Closed system with separated several vb, flat at the adx side and U-shaped at abx side	Opened system, consists of two vb	Consists of 3–4 vb at the right and left of adx side	Brachyscelereids, Starch grains, solitary crystals CO (cubic), druses	Scale, Capitate glandular
M. pumilum var. *lanceolata*	Oval	Closed system with separated several vb, O-shaped	Closed system, O-shaped with separated several vb	Consist of single vb at the middle of adx side	Starch grains, solitary crystals CO (cubic), druses	Scale

abx, abaxial; adx, adaxial; vb, vascular bundle; CO, calcium oxalate.

three varieties of *M. pumilum*. The solitary crystals (calcium oxalate) present in rectangular and/or cubic shape were sparsely found in the three varieties. The druses were present in the midrib of *M. pumilum* var. *alata* and var. *lanceolata* and in the petiole of three varieties. Previous study by Große (1908), reported that leaf druses were widely distributed in the Myrsinaceae and that the calcium oxalate in the leaf of *Heberdenia bahamansis* was in the form of solitary, rhomboid crystal but it occurred as druses in *H. pendulifora* (Myrsinaceae). The starch grains were sparsely present in transverse sections of midrib and petiole of all varieties except for petiole of *M. pumilum* var. *alata*. However, they were found abundantly in clustered form in parenchyma cells of the midrib of *M. pumilum* var. *pumila.* According to Pandey and Chadha (1993), starch granules were generally found in group with varying sizes and shapes. Therefore, the anatomical characteristic of the outline structure, shape and arrangement of vascular bundles in midrib and petiole as well as the presence and absence of selected features could be used to differentiate the three varieties of *M. pumilum*.

Transverse sections of the stem outline of three varieties *M. pumilum* were circular in shape (Fig. 5G–I). The three varieties of *M. pumilum* have several common features in the stem anatomical characteristic. In this study, the trichomes were divided into two types: scale and capitate glandular. The cortex of all taxa was consisted of collenchyma cells alongside of the epidermis layer and followed by parenchyma cells. Clustered sclerenchyma cells were present outside the phloem of vascular bundles. Most druses, calcium oxalate and brachyscelereids were widely distributed along the epidermis in all varieties. The starch grains were present in all varieties and found abundantly in clustered form in parenchyma cells of the stem of *M. pumilum* var. *pumila.* However, all taxa were observed to exhibit slight difference in some characteristics in which *M. pumilum* var. *alata* and var. *pumila* consisted of six-separated additional vascular bundles, while *M. pumilum* var. *lanceolata* consisted of five-separated vascular bundles in the parenchyma cortex. The central part of the stem of *M. pumilum* var. *alata* had relatively wide pith compared to *M. pumilum* var. *pumila* and var. *lanceolata.* Several secretory canals occurred in the pith of the three varieties and in the cortex of *M. pumilum* var. *lanceolata* and var. *pumila*. Hence, these characteristics could be used to distinguish the three varieties of *M. pumilum*. In addition, the three varieties of *M. pumilum* showed the presence of cells inclusion. Table 6 lists the characteristics of stem transverse section of *M. pumilum* varieties.

The observation on cells inclusion in all taxa studied showed the presence and absence of solitary crystal (calcium oxalate), brachyscelereids, starch grains, druses and secretory canals in transverse sections of midrib, petiole and stem. The anatomical characteristics are displayed in Fig. 6.

All of the taxa in this study had an anatomical characteristic that is similar to other Myrsinaceae (Metcalfe and Chalk, 1950). The most characters that have systematic significance were the outline structure, configuration of vascular system, distribution of secretory canal, cell inclusion, type of stomata and trichome in *M. pumilum* varieties. Hence, the analysis of anatomy characteristic in this study may be potentially used to identify and differentiate these varieties.

Table 6
Variation in the stem anatomical characteristics of *Marantodes pumilum* varieties.

Taxa	Outline	Parenchyma cortex	Num. of additional vb in cortex	Pith	Cell inclusions	Type of trichome	Secretory canals
M. pumilum var. *alata*	Circular	Ca. 10–20	6	Relatively wide	Brachyscelereids, solitary crystals CO, druses, starch grains	Scale	Presence in pith parenchyma
M. pumilum var. *pumila*	Circular	Ca. 8–10	6	Relatively medium	Brachyscelereids, Starch grains, solitary crystals CO, druses, starch grains	Scale, Capitate glandular	Presence in pith and parenchyma cortex
M. pumilum var. *lanceolata*	Circular	Ca. 8–10	5	Relatively medium	Brachyscelereids, Starch grains, solitary crystals CO, druses, starch grains	Scale, Capitate glandular	Presence in pith and parenchyma cortex

vb, vascular bundle; Ca., is about (±); CO, calcium oxalate.

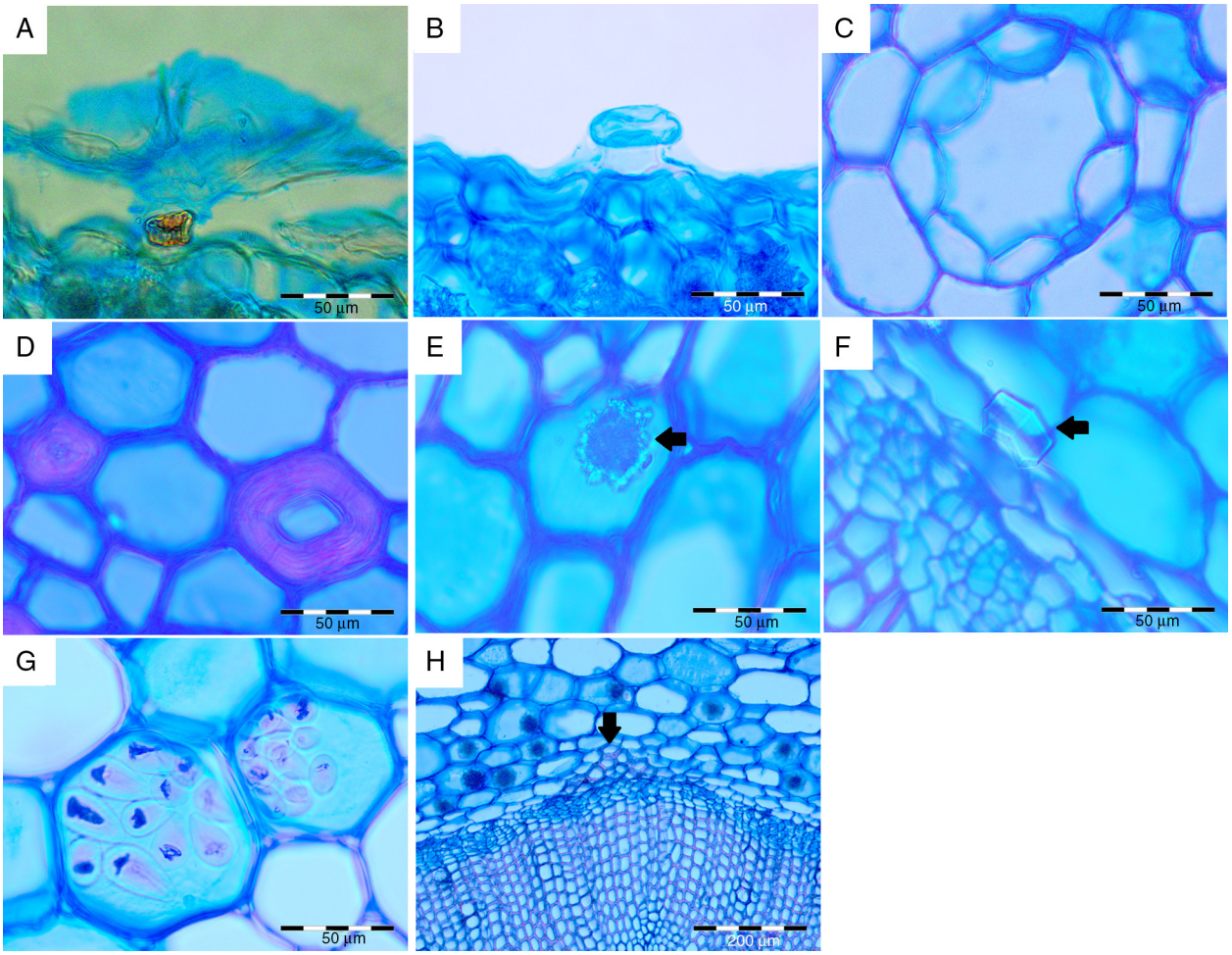

Fig. 6. Microscopic analysis: A. Scale trichome in petiole of *M. pumilum* var. *alata*. B. Capitate glandular trichome in petiole of *M. pumilum* var. *pumila*. C. Secretory cell in midrib of *M. pumilum* var. *lanceolata* D. Brachysclereid in stem of *M. pumilum* var. *lanceolata*. E. Druse in stem of *M. pumilum* var. *lanceolata* (arrow). F. Solitary crystal (calcium oxalate) in stem of *M. pumilum* var. *lanceolata* (arrow). G. Starch in midrib of *M. pumilum* var. *pumila*. Scale bar = 50 µm. H. Cluster of sclerenchyma cells (arrow) observed outside the phloem of vascular bundles of *M. pumilum* var. *lanceolata* stem. Scale bar = 200 µm.

The general anatomical investigation of the leaf and stem of three varieties of *M. pumilum* showed the presence of capitates glandular trichome, consisting of the short stalk and quite spherical glands and scale trichome, which were already mentioned by Lestern (1977), Stone (1989), Metcalfe and Chalk (1950) that these two types of trichome appeared to be common throughout the Myrsinaceae. The trichome characteristic was used by Otegui and Maldonado (1998) to characterize *Myrsine* species, in which types of trichome were identified to differentiate three *Primula* species (Fico et al., 2007). During the revision of *Deppa* (Rubiaceae) by Lorence and Dwyer (1988), they stated that the type, size and distribution of trichomes as important characters in the identification of plant species.

The presence of secretory canal is an outstanding feature in Primulaceae (Judd et al., 2009) Among *M. pumilum* varieties, the secretory canal was widely distributed in the midrib, petiole and stem of *M. pumilum* var. *lanceolata* compared to *M. pumilum* var. *pumila* and var. *alata*. Muntoreanu et al. (2011) revealed that the secretory cavities as distinctive characters for species segregation. According to Teixeira et al. (2000), the micromorphology and localization of secretory tissue also has been used by many authors in other families as useful features to taxonomy as in *Lonchocarpus* Kunth (Fabaceae).

A study by Otegui and Maldonado (1998), mentioned that secretory structure in *Myrsine laetrvirens* (Myrsinaceae) produced benzoquinone substance. Similarly, Tuntiwachwuttikul et al. (1997) and Luna et al. (2014) reported that the chemical compositions *such as* terpenoids, lipids, phenolics, hydroxybenzoquinone derivatives and lipophilic substances were synthesized from secretory tissues of several Primulaceae species. The secretory chemicals are involved in a wide range of pharmacological activities *such as* anti-oxidant (Sumino et al., 2002), anti-inflammatory (Ahmad et al., 2011), anthelmintic (Challam et al., 2010) and antinociceptive (Hess et al., 2010). Hence, these plants may have potential as a source of bioactive natural products.

The outline observation and configuration of vascular system in leaf (midrib, petiole, lamina and margin) and stem of *M. pumilum* varieties gave significant anatomical characters which could be used to identify and discriminate among the taxa studied. The shape and arrangement of the vascular system could be used in the differentiation of species (Fahn, 1990) and according to Reis et al. (2004), these features were proved to be useful for taxonomy. Noraini et al. (2008) stated that petiole outline could be used for identification of some species in genus *Coelostegia*. Previous study by Luna et al. (2013) noted that the configuration of the vascular system in the petiole was used to differentiate *Stylogyne* species (Myrsinoideae).

The microscopic measurement of selected structures of three varieties is tabulated in Table 7. Results showed significant differences of size in lamina, midrib, stem, druse and stomata within three varieties.

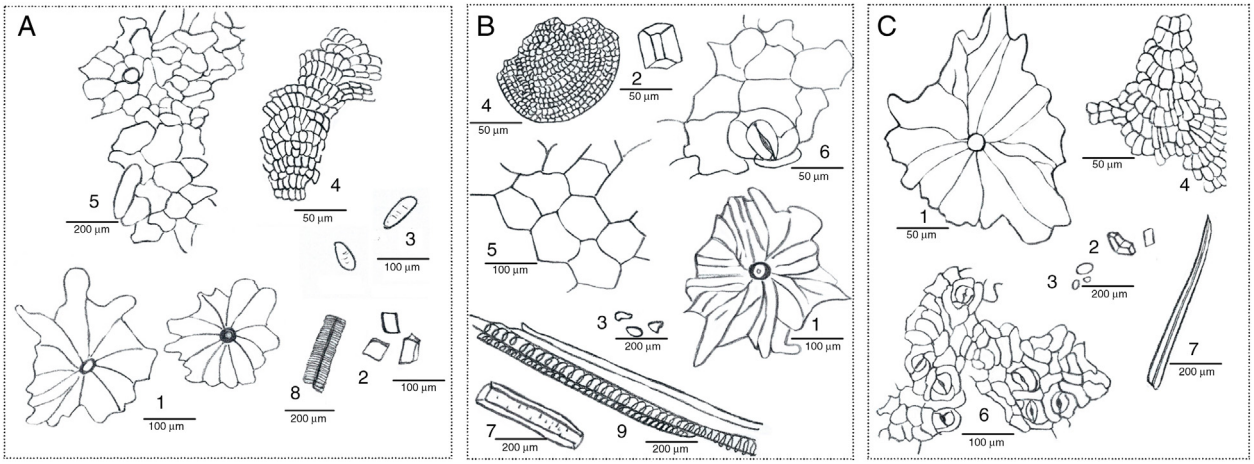

Fig. 7. Powdered characteristics of leaf: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila.* C. *M. pumilum* var. *lanceolata.* 1, Scale trichome; 2, Solitary crystal (calcium oxalate); 3, Starch grains; 4, Fragment of parenchyma; 5, Epidermis; 6, Stomata; 7, Fiber; 8, Vessel; 9, Fragment of vessel.

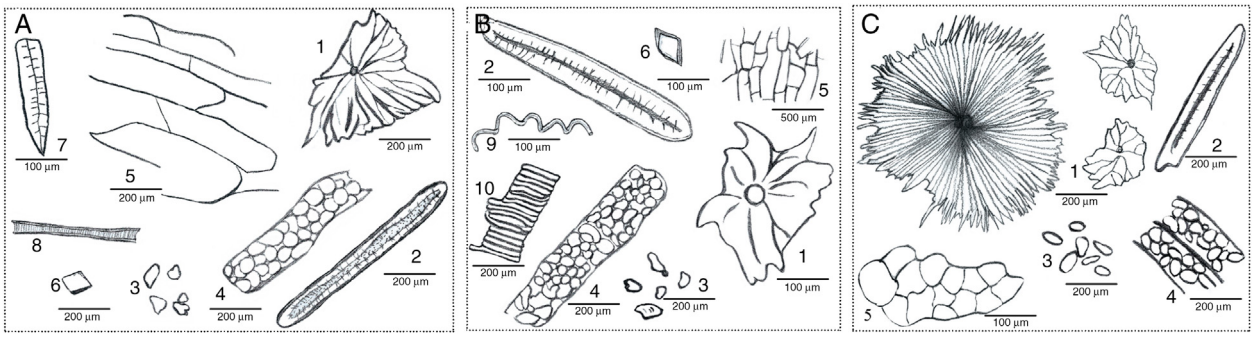

Fig. 8. Powdered characteristics of stem: A. *Marantodes pumilum* var. *alata.* B. *M. pumilum* var. *pumila.* C. *M. pumilum* var. *lanceolata.* 1, Scale trichome; 2, Sclereid; 3, Starch grains; 4, Cluster of starch grains in cell; 5, Epidermis; 6, Solitary crystal (calcium oxalate); 7, Sclereid; 8, Vessel; 9, Xylem (spiral); 10, Parenchyma.

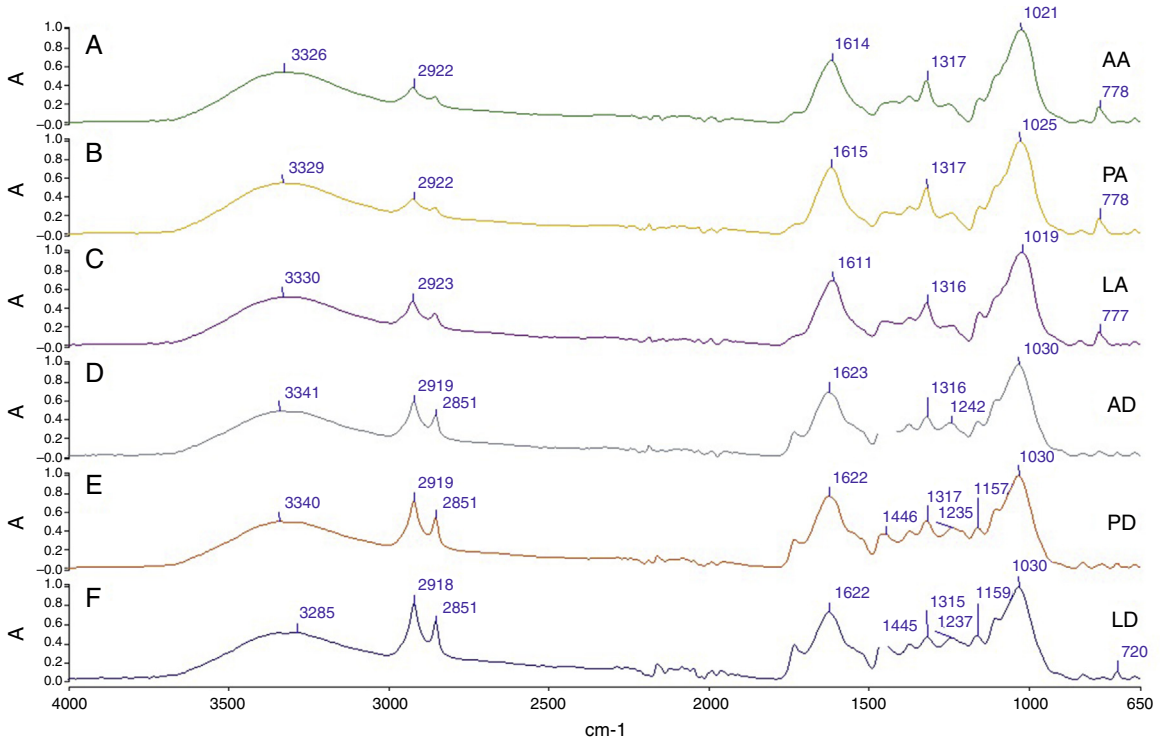

Fig. 9. Zero order spectra of one-dimensional ATR-FTIR spectrophotometry of the crude powdered stems and leaves of *Marantodes pumilum* varieties (A–F). AA, *M. pumilum* var. *alata* stems; PA, *M. pumilum* var. *pumila* stems; LA, *M. pumilum* var. *lanceolata* stems; AD, *M. pumilum* var. *alata* leaves; PD, *M. pumilum* var. *pumila* leaves; LD, *M. pumilum* var. *lanceolata* leaves.

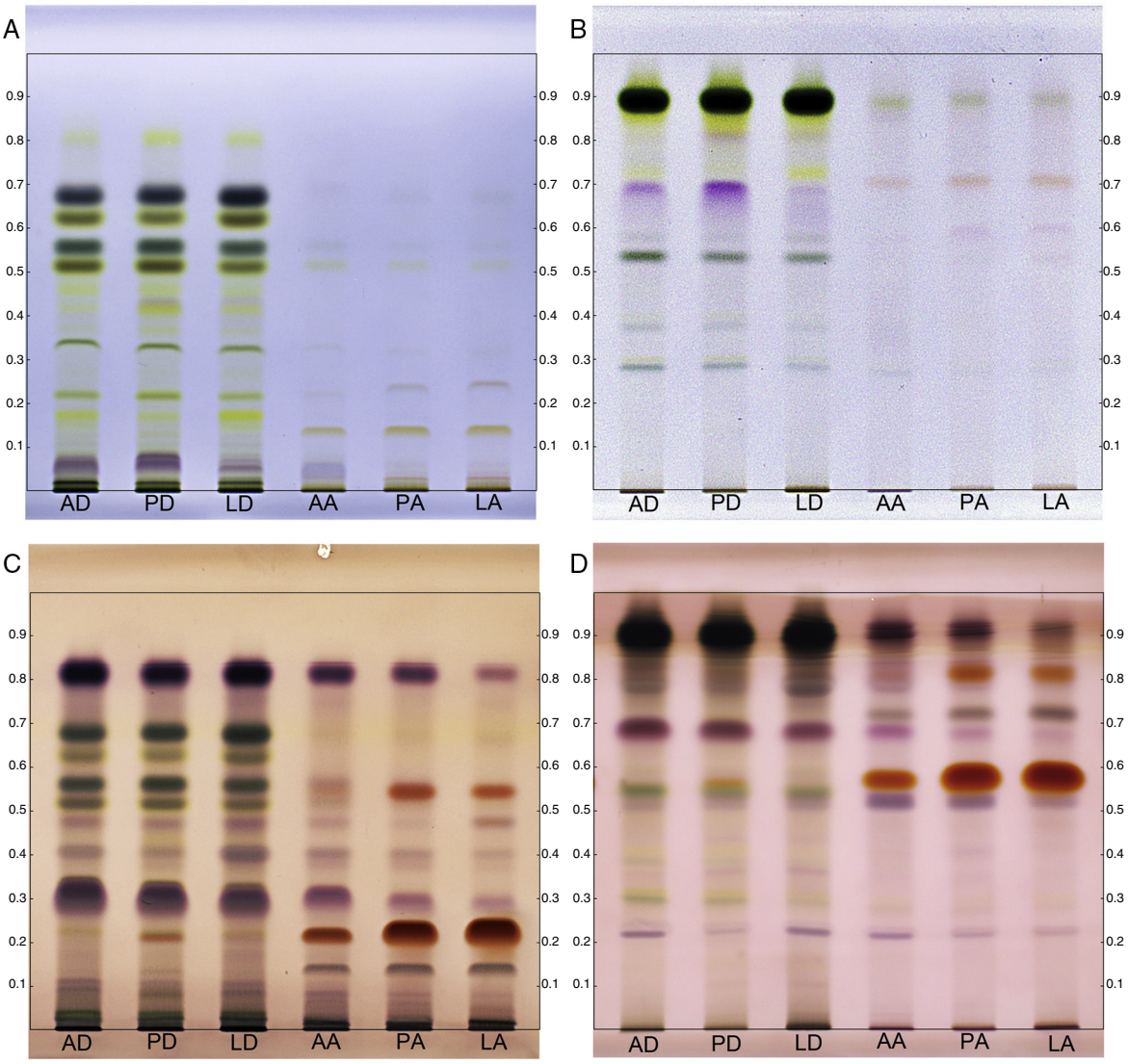

Fig. 10. HPTLC fingerprint chromatograms of dichloromethane extracts of *Marantodes pumilum* varieties developed using toluene–acetone (9:1) and chloroform–methanol (9:1) and observed under daylight before and after derivatisation. AD, *M. pumilum* var. *alata* leaves; PD, *M. pumilum* var. *pumila* leaves; LD, *M. pumilum* var. *lanceolata* leaves; AA, *M. pumilum* var. *alata* stems; PA, *M. pumilum* var. *pumila* stems; LA, *M. pumilum* var. *lanceolata* stems.

Microscopic analysis: histochemistry of powdered leaves and stem

From the analysis, the dried powdered leaf and stem of *M. pumilum* varieties have similarities as well as differences in microscopic features. Both of the leaf (Fig. 7A–C) and stem (Fig. 8A–C) of the three varieties showed the presence of solitary crystal (calcium oxalates), scale trichomes, starch grains, vessels, fibers, epidermis and parenchyma. Starch grains were found to be more accumulated in the stem compared to the leaf of the three varieties and formed as oval and irregular shape with various sizes. They were found scattered and occurred as a group inside the parenchymatous cell. The solitary crystal (calcium oxalate) and scale trichomes were distributed in the three varieties of *M. pumilum*. The presence of calcium oxalates in these varieties seems to be more accumulated in the stem parts as druses compared to the leaf. On the other hand, the common characteristic of leaf part is the presence of mesophyll and anisocytic stomata on the abaxial epidermis. Brachysclereids occurred abundantly in stem part of the plants. They were found singly or in groups as elongated sclereids with pointed ends and lignified cell walls.

Table 7
Selected micrometric measurement for the anatomic studies of *Marantodes pumilum* varieties.

Feature	Micrometric measurement (mm)		
	M. pumilum var. *alata*	*M. pumilum* var. *pumila*	*M. pumilum* var. *lanceolata*
TS stem	6.534 × 6.673	3.718 × 3.633	4.596 × 4.864
TS lamina	0.100–0.140	0.070–0.090	0.075–0.011
TS midrib	2.331 × 2.498	1.488 × 1.709	1.854 × 1.838
Druses (stem)	0.020–0.035	0.036–0.052	0.040–0.044
Stomata (leaf)	0.035 × 0.021	0.038 × 0.021	0.037 × 0.022

Results are presented as mean, $n = 3$.
Standard deviation < ±8.

Histochemical screening of the dried powdered leaf and stem of *M. pumilum* varieties also showed the presence of cell contents, including lignified and suberized cell wall, calcium oxalate, starch granules, as well as tannins. The cell contents in three varieties were detected using specific reagent and presented in Table 8. Histochemistry analysis is one of the microscopic identification techniques. Plant organelles and structures are colorless and difficult to be observed using naked eye (Peterson et al., 2008). Thus, histochemical test would selectively stain microscopic features to confirm their presence and identity.

Table 8
Histochemical analysis of *Marantodes pumilum* varieties.

Taxa	Plant part	Cell contents				
		Lignified cell wall	Calcium oxalate	Starch	Tannins	Suberized cell wall
M. pumilum var. *alata*	Stem	+	+	+	+	+
	Leaf	+	+	+	+	+
M. pumilum var. *pumila*	Stem	+	+	+	+	+
	Leaf	+	+	+	+	+
M. pumilum var. *lanceolata*	Stem	+	+	+	+	+
	Leaf	+	+	+	+	+

+, Present.

ATR-FTIR spectrophotometric profiling

Fig. 9 shows the spectra for crude powdered materials of all varieties. The assignments of functional groups for all the peaks are summarized in Table 9. The stem samples in Fig. 9A–C displayed generally similar profile with the presence of three absorbance peaks at 2923–2922 (C—H stretching), 1317–1316 (O—H bending) and 778–777 (C—H out-of-plane bending) cm^{-1}. However, the absorption peaks at 3330–3326 (O—H stretching), 1614–1611 (C═C stretching) and 1025–1019 (C—O stretching) cm^{-1} could be used to distinguish stem of *M. pumilum* var. *alata*, var. *pumila* and var. *lanceolata*. The leaf samples in Fig. 9D–F exhibited similar absorption peaks at 2919, 2851, 1623–1622 and 1030 cm^{-1}, but could be clearly differentiated based on the peaks at 3341–3285 (O—H stretching), 1242–1235 (C—O stretching) and 1159–1157 (C—O stretching) cm^{-1}. Interestingly, the stem samples showed obviously different profiles from the leaves. The profile variation between varieties and plant parts suggests that they may contain different phytochemicals. Thus, the chemical fingerprinting technique could be used to determine the identity and to control the quality of raw materials containing the *M. pumilum* varieties. This is the first study to report on the distinctive variation of *M. pumilum* varieties based on their plant parts using ATR-FTIR spectrophotometric method. Previous study by Abdullah et al. (2012) on characterization and identification of *M. pumilum* by multi-steps infrared spectroscopy analyzed only the leaf part using FTIR with KBr disk method. In contrary, our study used a simple and non-destructive analytical method without the use of KBr disk so that

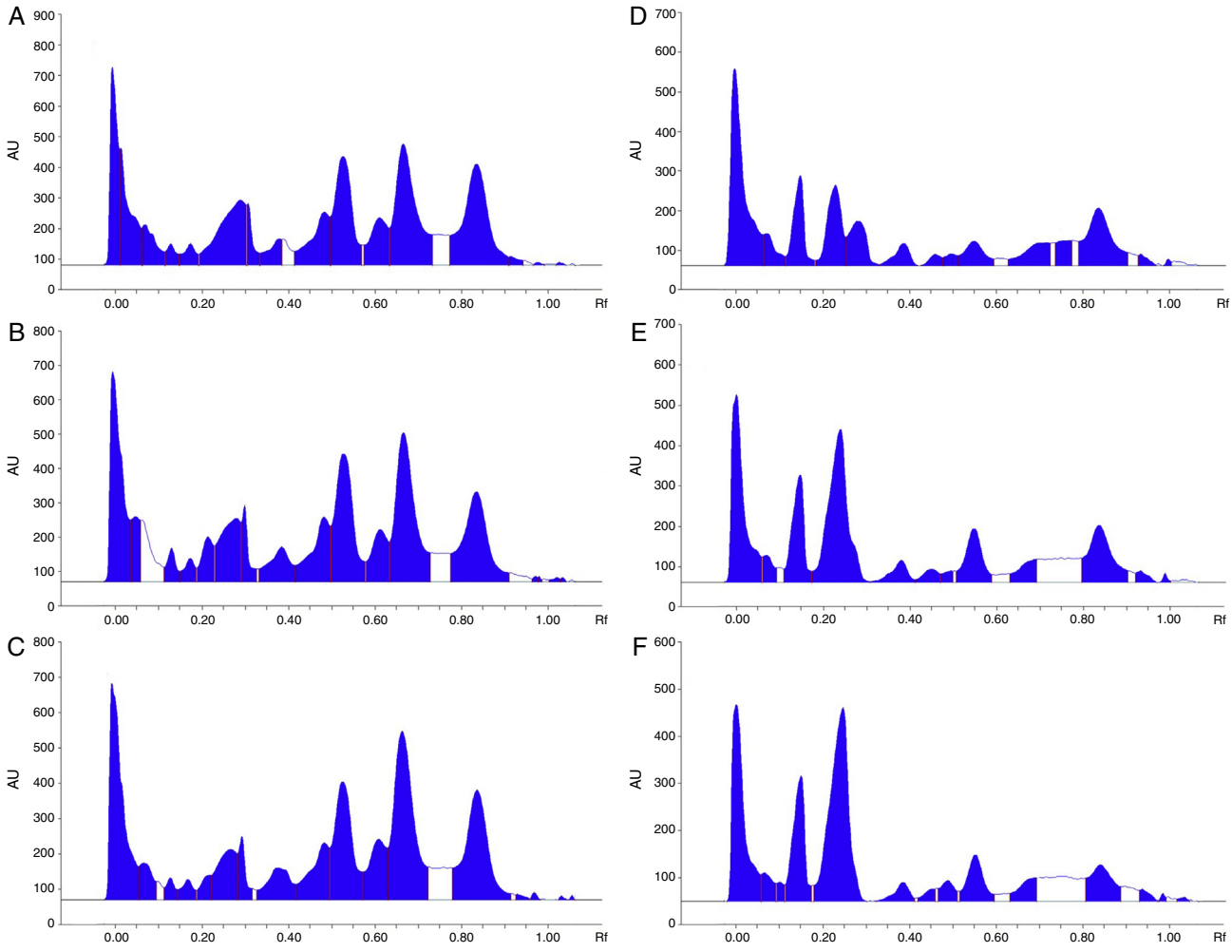

Fig. 11. HPTLC-densitometric chromatograms of dichloromethane extracts of leaves: A. *Marantodes pumilum* var. *alata*. B. *M. pumilum* var. *pumila*. C. *M. pumilum* var. *lanceolata;* and stems: D. *M. pumilum* var. *alata*. E. *M. pumilum* var. *pumila*. F. *M. pumilum* var. *lanceolata*. Toluene–acetone (9:1) was used as the mobile phase.

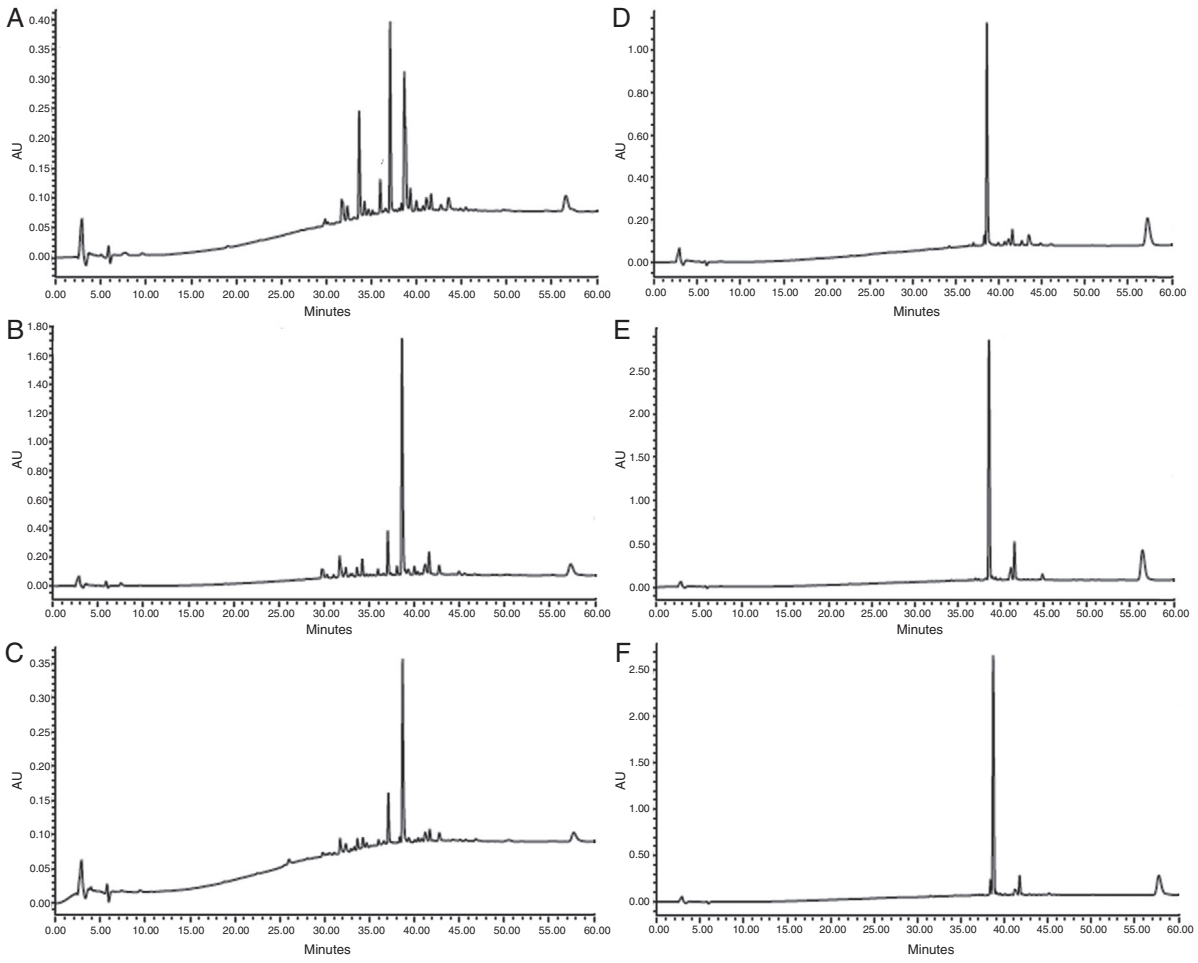

Fig. 12. The HPLC fingerprint chromatograms of dichloromethane extracts of leaves: A. *Marantodes pumilum* var. *alata*. B. *M. pumilum* var. *pumila*. C. *M. pumilum* var. *lanceolata*; and stem: D. *M. pumilum* var. *alata*. E. *M. pumilum* var. *pumila*. F. *M. pumilum* var. *lanceolata*. Wavelength, 210 nm; mobile phase, acetonitrile (A); water, pH 2.5 (B); gradient elution: 0 min, 70% A: 30% B increased in the next 30 min to 100%; flow rate, 1.00 ml/min.

to eliminate IR sample preparation step and that the samples could be recovered.

HPTLC profiling

In this study, combination of toluene and acetone (9:1) and chloroform and methanol (9:1) as mobile phases of HPTLC analysis of *M. pumilum* varieties resulted in well-separated, compact and symmetrical bands. HPTLC fingerprint profiles of the leaves and stems of *M. pumilum* var. *alata*, var. *pumila* and var. *lanceolata* dichloromethane extracts are shown in Fig. 10. HPTLC fingerprint profiles for leaf extracts were obviously distinguished from the stem due to the presence of several green chlorophyll pigment bands at R_f 0.20–0.70 (Fig. 10A and B) and purple bands at R_f 0.69 (Fig. 10B). *M. pumilum* var. *pumila* leaves could be visually differentiated from the other two varieties based of the presence of an orange band at either R_f 0.22 (Fig. 10C) or R_f 0.56 (Fig. 10D). Similar profiles of separated bands of stem extracts could only be observable after derivatization with *p*-anisaldehyde/H_2SO_4. The stem extract of *M. pumilum* var. *alata* had the least intense orange band at either R_f 0.22 and R_f 0.56 (Fig. 10C) or R_f 0.56 and R_f 0.85 (Fig. 10D), respectively.

Generally, HPTLC profiles of the *M. pumilum* leaves and stem were somewhat analogous with varied intensity, suggesting presence of similar phytochemicals content of different concentration. This is evidenced from the HPTLC-densitometric chromatograms shown in Fig. 11. Two peaks at R_f 0.29 and 0.85 present in all extracts could be used as fingerprint markers for *M. pumilum*. The peaks are indicated as major compounds for the leaf extracts in the order of *M. pumilum* var. *alata* > var. *pumila* > var. *lanceolata* (Fig. 11A–C). On the other hand, a peak at R_f 0.22 appeared as a major compound of the stem of *M. pumilum* varieties in the order of var. *lanceolata* > var. *pumila* > var. *alata* (Fig. 11D–F). Therefore, these compounds could be suggested as chemical markers for the three *M. pumilum* varieties and could be used for quality control and identification of different parts of the plant materials. In addition, the results also provide information on the amount and nature of compounds in each extract (Joshi, 2012). According to Giri et al. (2010), separation of chemical substance using HPTLC can be used

Table 9
Typical infrared absorption frequencies of chemical functional groups according to Pavia et al. (2009).

Frequency range (cm^{-1})	Assignment of functional groups
3400–3200	O—H stretching (H-bonded, alcohols and phenols)
3000–2850	C—H stretching (CH_3, CH_2 and CH, alkanes)
1725–1705	C=O stretching (ketones)
1680–1600	C=C stretching (alkenes)
1600 and 1475	C=C stretching (aromatic rings)
1450–1375	C—H bending (CH_3 and CH_2 deformation, alkanes)
1430–1330	O—H bending (alcohols and phenols)
1300–1000	C—O stretching (alcohols and phenols)
900–650	C—H out-of-plane bending (aromatic rings)
850–780	C—H out-of-plane bending (alkenes)
770–650	O—H out-of-plane bending (alcohols and phenols)

to facilitate development of validation and standardization processes, as well as determination of class of compounds and isolation of pure compounds. Hence, plant chemical fingerprinting is helpful in establishing the identity and quality of herbal drugs.

HPLC profiling

HPLC fingerprint chromatograms of the dichloromethane extracts for the leaves and stems of *M. pumilum* varieties showed a number of peaks of different intensity at the retention times (R_t) between 30 and 60 min (Fig. 12). The peaks were detected at a wavelength of 210 nm according to the previous report for alkenated phenolic and triterpenoid saponins present in *M. pumilum* (Avula et al., 2011). Generally, four distinctive peaks at R_t 33.367, 37.072, 38.658 and 57.211 min could be used as chemical markers for the *M. pumilum* varieties. The leaf and stem extracts could be distinguished based on the absence of two peaks at R_t 33.367 and 37.072 min in the latter extract. Phytochemical composition of the four peaks for *M. pumilum* leaf extracts was found in the order of var. *pumila* > var. *alata* > var. *lanceolata*; whereas the two peaks for stem extracts was in the order of var. *pumila* > var. *lanceolata* > var. *alata*.

The HPTLC and HPLC methods performed in this study are mutually complementary and both methods have their own advantages and limitations for the analysis of *M. pumilum* varieties. HPTLC is an efficient method in terms of solvent usage, development time and resolving power; as well as improved quality of sorbents and consistency in plate manufacture; while automated sample application allows for improved separation, band resolution and reproducibility of results. HPTLC is also used as a preliminary screening approach to HPLC (Reich and Schibli, 2007). For HPLC, the gradient system of mobile phase is one of greatest advantages over other chromatographic methods because the solvent system can be manipulated depending on the chemical nature of compounds, allowing easy separation of complex mixture of phytochemical compounds with a wide range of polarity (Fallon et al., 1987). From this study, it is found that HPLC fingerprint is a better profiling technique than HPTLC because it is able to specifically distinguish between leaf and stem extracts of *M. pumilum* varieties.

Conclusion

Results from this study provide important information and data for systematic identification and authentication of the different plant part and varieties of *M. pumilum*. Transverse section of the fresh plant materials would give the most accurate identification of the three varieties based on the characteristic differences of anatomical features in terms of type of stomata and trichomes, outline structure of leaf margin, petiole and midrib, organization of vascular system, areolar venation, pattern of anticlinal walls of the epidermis, as well as the distribution of secretory canals and cell inclusion of the stem. However, ATR-FTIR, HPTLC and HPLC techniques could be utilized to distinguish the powdered plant materials of *M. pumilum* varieties based on the presence or absence of certain phytochemical constituents, manifested as peaks or bands from the chemical fingerprint profiles. Proper identification and authentication of raw plant materials to be used in the product manufacturing would ensure well-defined and consistent quality, safety and efficacy of herbal preparations and products.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Author's contribution

NAA is a M.Sc. candidate who conducted the experimental work and drafted the manuscript. JAJ is the project leader responsible for the research design and editing of the manuscript. NT helped with the microscopic analysis and reviewed the microscopic section of the manuscript. NUMH conducted the ATR-FTIR spectrophotometric analysis. MRAR, CWS and KM provided technical assistance for the microscopic and chromatographic experiments. KH and JJ are project team members who contributed ideas for the research design. All authors have read the final draft and approved submission of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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