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Microscopical Evaluation and TLC Analysis of *Pluchea indica* (L.) Less: Leaf, Stem, and Root

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ABSTRACT

***Pluchea indica* (L.) Less is traditionally utilized to treat postpartum women in Indonesia. The plant has many pharmacological properties, so that it can be further developed as herbal medicine. In that development process, plant authentication is needed to ensure the quality of raw materials. A simple microscopical and thin-layer chromatography (TLC) analysis might be a way to authenticate the plant, but it has never been reported. So, this study evaluates the microscopical and TLC analysis for *P. indica* authentication in standardized herbal medicines production. Plant microscopic observation, fluorescence analysis, and polyphenol screening were conducted. *n*-Hexane, ethyl acetate, and methanol extracts of plant organs were then analyzed by TLC. Here, we reported that in microscopical analysis the *simplicia* of *P. indica* contains trichomes and tannin-containing cells. In addition, chlorogenic acid as a marker was present in TLC analysis by ethyl acetate-water-formic acid-acetic acid (8.5:1.5:1:1, v/v). The results of this evaluation might provide additional information in the identification, authentication, and quality control of *P. indica* as a raw material for herbal medicine.**

1. Introduction

Pluchea indica (L.) Less or Indian camphorweed is widespread in Indonesia and known as beluntas (Susetyarini *et al.* 2020). *P. indica* is traditionally utilized by the Sundanese and the people of the Minahasa region in Indonesia to recover postnatal women (Zumsteg and Weckerle 2007; Roosita *et al.* 2008). Aerial parts and roots of *P. indica* have been reported to be pharmacologically useful for treating various diseases and a promising source of natural medicinal compounds (Chan *et al.* 2022).

Medicinal plants are often prepared and used in dried powder (Mukherjee 2019), known as *simplicia* (Ministry of Health Republic of Indonesia 2017). To be produced into herbal medicine (such as standardized herbal medicines or phytopharmaca), *simplicia* of *P. indica* as herbal raw material initially needs to go through identification and authentification (Ministry of Health Republic of Indonesia 2017). This procedure will provide valuable supporting data for the correct identification of species, botanical quality control, and detection of counterfeiting of laboratory or commercial samples (Kunle *et al.* 2012; Mukherjee 2019). Botanical quality will significantly affect its effectiveness and safety as a starting material for herbal medicine (Muyumba *et al.* 2021).

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Assessment of the macroscopic and microscopic characteristics of plants is the initial stage in the identification and the degree of authenticity of plants as raw materials for herbal medicine (Mukherjee 2019). After that, screening for the chemical content of the plant is an important aspect to evaluate (World Health Organization 1998). Thin-layer chromatography (TLC) methods may be used to evaluate these parameters quickly and easily (Rafi *et al.* 2017). It allows the simultaneous identification of multiple samples on one plate (Zeng *et al.* 2011). TLC fingerprint profiles have been widely used to identify (falsification detection) and standardize extracts or extract formulations (Rafi *et al.* 2017).

Previously, studies that focus on identifying the morphology and anatomical structure of *P. indica* leaves, stems, flowers, and roots have also been carried out using safranin coloration and a scanning electron microscope (SEM) by Susetyarini *et al.* (2020). As the temperature, rainfall, altitude, day length and radiation characteristics, soil conditions, and other environmental factors affect plant growth and development, secondary metabolites are also influenced (Evans 2009). Ultimately, they will affect the extracts for producing herbal drugs (Muyumba *et al.* 2021).

So, this study conducted a simple microscopical evaluation of fresh organs (leaves, stems, and roots) of *P. indica* and their *simplicia*. In addition, the chemical components of the three *P. indica* organ extracts were screened using the TLC method. The information from these investigations can ultimately be applied in the laboratory and for commerce. This is beneficial in preserving the quality, safety, and efficacy of *P. indica* as a source of raw materials for herbal medicine.

2. Materials and Methods

2.1. Chemicals

Solvents *n*-hexane, ethyl acetate, ethanol, methanol, toluene, formic acid, and acetic acid for analysis grade were obtained from Merck (Darmstadt, Germany). The chlorogenic acid (Markherb, Institut Teknologi Bandung (Bandung, Indonesia), quercetin (Sigma-Aldrich Co. (St Louis, USA)), and gallic acid (Sigma-Aldrich Co. (St Louis, USA)) were used as standard. Reagents, chloral hydrate, 2 N HCl, 4 N HNO_3, 4 N H_2SO_4 (in water, v/v), NH_4OH, 5% $AlCl_3$ (in water, v/v), and 5% $FeCl_3$ (in water, v/v), were obtained from Merck (Darmstadt, Germany).

2.2. Plant Collection and Confirmation

Leaves, stems, and roots of *P. indica* were obtained from the Faculty Pharmacy and Sciences garden, Universitas Muhammadiyah Prof. DR. HAMKA, Duren Sawit region, East Jakarta, Indonesia, in December 2022. Organs are harvested in the morning (around 10 a.m.) when the plants are not yet flowering. The age of the plant when the organs were harvested was 5 years. The harvested leaves and stems weigh about 150 g. The harvested root weighs about 15 g. The plant was confirmed by Direktorat Pengelolaan Koleksi Ilmiah, National Research and Innovation Agency (BRIN), Cibinong, Indonesia (No: B-172/II.6.2/R.01.02/2/2023).

2.3. Evaluation of Plant part Characteristics

Macroscopic evaluation was done on fresh leaves, stems, and roots using the eye without tools. Parameters observed included color, odor, taste, and plant shape characteristics following the protocol in the Indonesian Herbal Pharmacopoeia (Ministry of Health Republic of Indonesia 2017). Observations were performed after the material was exposed to air for 15 mins.

2.4. Plant Tissue Characteristics

Fresh leaf, stem, and root are cleaned under running water. Each organ was cut using a razor blade. The incision on the glass slide is added with 1-2 drops of chloral hydrate solution. Microscopic evaluation of the sections was carried out using a microscope (Nikon Eclipse E100, Shanghai, China) at magnification 10 × 10 (World Health Organization 1998).

2.5. *Simplicia* Characteristics

Leaves, stems, and roots were air-dried for 3–5 days indoors (at 27±2°C) with good air circulation. The dried plant organ is then powdered using an electric blender. The powder of dried plant organs, *simplicia*, was then sieved using a sieve mesh of 40 sizes. A small amount of the *simplicia* on the slide was added with 1-2 drops of the chloral hydrate solution. A microscope (Nikon Eclipse E100, Shanghai, China) was used for the microscopic evaluation at magnification 10 × 10 (World Health Organization 1998). Then, the *simplicia* were subjected to Fluorescence analysis. After treating the *simplicia* using various reagents, then the fluorescence was observed in visible and ultraviolet (UV) light (254 and 365 nm) using a UV Box (CAMAG, Germany) (Table 1) (Hanani *et al.* 2019).

Table 1. Fluorescence analysis of *simplicia* of *P. indica* leaves, stems, and roots

Simplicia	Treatments	Visible	UV (254 nm)	UV (365 nm)
Leaves	Water	Colorless	Black	Black
	Ethanol	Light green	Red	Red
	Methanol	Light green	Pale yellow	Yellow
	4 N H_2SO_4	Colorless	Black	Black
	2 N HCl	Colorless	Black	Black
	4 N HNO_3	Yellow	Black	Black
	NH_4OH	Slightly yellow	Light blue	Light blue
Stems	Water	Slightly brown	Black	Slightly blue
	Ethanol	Colorless	Bright pink	Bright pink
	Methanol	Colorless	Pink	Pink
	2 N H_2SO_4	Slightly pink	Black	Black
	2 N HCl	Slightly pink	Black	Black
	4 N HNO_3	Yellow	Black	Black
	NH_4OH	Slightly brown	Black	Light blue
Roots	Water	Slightly brown	Black	Black
	Ethanol	Slightly brown	Yellowish green	Blue
	Methanol	Slightly brown	Bluish-green	Blue
	2 N H_2SO_4	Slightly pink	Black	Black
	2 N HCl	Slightly pink	Black	Black
	4 N HNO_3	Yellow	Black	Black
	NH_4OH	Slightly brown	Light blue	Blue

2.6. Extraction and Chemical Screening

This procedure describes the extraction and chemical compound screening techniques in each organ of *P. indica*. Briefly, each *simplicia* (1 g) was separately extracted using *n*-hexane, ethyl acetate, and methanol with a *simplicia*-to-solvent ratio of 1:10. Extraction was carried out using an ultrasonic bath (Branson 5510, Marshall Scientific LLC., USA) 40 kHz at room temperature (±25-27°C) for 15 min. The filtrate was concentrated using a vacuum rotary evaporator (EYELA, Shanghai, China) with a water bath temperature of 40°C to obtain 5 ml of the extract test solution. Liquid extracts of *n*-hexane (HE), ethyl acetate (EAE), and methanol (ME) were stored in tightly closed containers for analysis using the TLC method. Then, polyphenolic secondary metabolites in each extract, such as phenolics, flavonoids, and tannins, were determined according to standard methods in the Indonesian Herbal Pharmacopoeia (Ministry of Health Republic of Indonesia 2017) and Hanani (2015). The color formed is then recorded and compared with the untreated extract. After that, the chemical components of each extract were determined for their chromatogram patterns using the TLC method. TLC analysis was performed using GF254 aluminium TLC plates from Merck (Darmstadt, Germany). The mobile phase used is adjusted to the extract being analyzed, where toluene-ethyl acetate-acetic acid (9:1:1) for HE, toluene-ethyl acetate-acetic acid (4.65:0.35:1) for EAE, toluene-ethyl acetate-acetic acid (10:5:1) and ethyl acetate-water-formic acid-acetic acid (8.5:1.5:1:1) for ME. Spots were observed in visible and UV at 254 and 365 nm using a UV box (CAMAG, Germany). The spots were visualized using spray reagents, including 10% H_2SO_4 and 5% $FeCl_3$.

3. Results

3.1. Plant Organ Characteristics

The leaves are obovate-shaped (egg-shaped), 3–6 cm long, 2-4 cm broad, pari-pinnatifid with crenate margins, the apex of the leaf is acuminate, and the base is symmetrical reniform. The leaves range in color from light green (bottom side) to green (top side), have a rough texture, are rather hairy on the surface, and are papery. When pressed, the leaves have a specific perfume-like aroma associated with *P. indica*. The stem where the leaves are attached is green and slightly hairy. The main stem is brownish green to grayish brown, branching, strong, stiff, and upright with a woody structure. The taproot with lateral secondary and tertiary roots are yellowish white inside and yellowish brown to brown outside. The leaves, stems, and roots of *P. indica* are presented in Figure 1.

3.2. Plant Tissue Characteristics

Microscopically, the section of *P. indica* leaf showed the presence of lower epidermis, trichome, collenchyma, upper epidermis, palisade, parenchyma, sclerenchyma, xylem, phloem, and parenchyma mesophyll (Figure 2A). *P. indica* has a simple parenchyma type with intercellular spaces. The type of stomata found was an anomocytic type (irregular-celled) (Figure 2B). The stem section shows the presence of the epidermis, pith, trichome, xylem, collenchyma, cambium, phloem, and parenchyma cortex (Figure 3). Macroscopically, the stems are slightly hairy, but unfortunately, in this study, the trichome was difficult to obtain in the stem section. The section of *P. indica* root identified the air cavity, cortex, tannin-containing cells, vascular cylinder, and pith (Figure 4). The epidermis has yellowish-brown tangential walls. Tannin-containing cells are included in the cells of secondary tissues.

Figure 1. (A) Leaves, (B) stems, and (C) roots, of *P. indica*

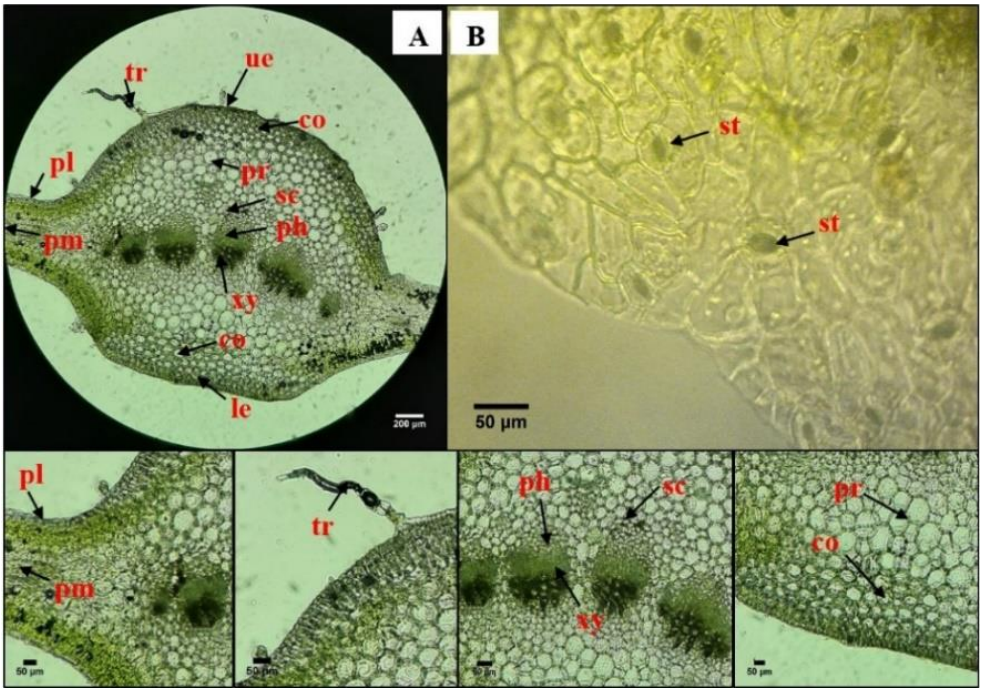

Figure 2. Photomicrographs of *P. indica* leaf. (A) Cross-section of the leaf (10 × 10), (B) lower epidermis of the leaf (10 × 40). le : lower epidermis, tr: trichome, co: collenchyma, ue: upper epidermis, pl: palisade, pr: parenchyma, sc: sclerenchyma, xy: xylem, ph: phloem, pm: parenchyma mesophyll, st: stomata

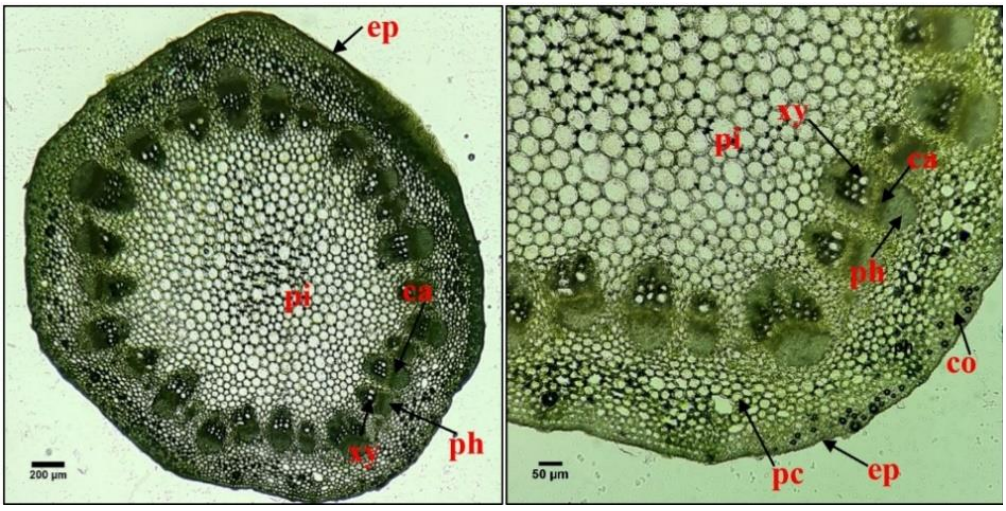

Figure 3. Photomicrographs of *P. indica* stem. Ep: epidermis, pi: pith, xy: xylem, ca: cambium, ph: phloem, pc: parenchyma cortex, co: collenchyma

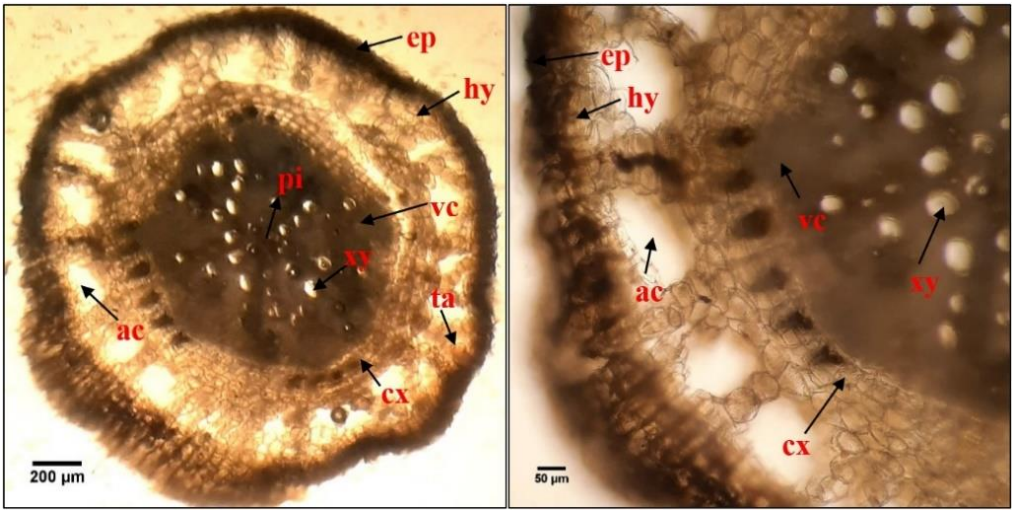

Figure 4. Photomicrographs of *P. indica* root. Ac: air cavity, cx: cortex, ta: tannin-containing cells, vc: vascular cylinder, pi: pith, hy: hypodermis

3.3. *Simplicia* Characteristics

This study evaluated the characteristics of tissue fragments in the *simplicia* of leaves, stems, and roots of *P. indica*. Each *simplicia* of *P. indica* organ has a different odor and taste characteristic. The *simplicia* of leaves have similar aroma as the pressed leaves, the stems have weak aroma, and the roots are odourless. Aftertaste, the *simplicia* of leaves like dry green herbal on the tongue (associated with the "green" flavour typical of dried herbs), and the stems and roots are tasteless. The *simplicia* of *P. indica* leaf is green, stems are brownish green, and roots are yellowish brown.

Specific fragments of *simplicia* of *P. indica* leaves were found in sclereids, vessels with spirals, sclerenchyma, vessels, trichomes, stomas, and starch grains (Figure 5). Stem powder contains trichomes, pitted annular vessels, fiber, spiral vessels, calcium oxalate, tannin-containing cells, and cork cells (Figure 6). Oxalate crystals in the *simplicia* of the *P. indica* stem tend to be similar to monoclinic prismatic crystal types. The *simplicia* of *P. indica* root contains parenchyma cells, tannin-containing cells, fiber, cork cells, and vessels (Figure 7).

After being treated with various reagents, the *simplicia* of *P. indica* leaves, stems, and roots released fluorescence characteristics or colors when exposed to UV (Table 1).

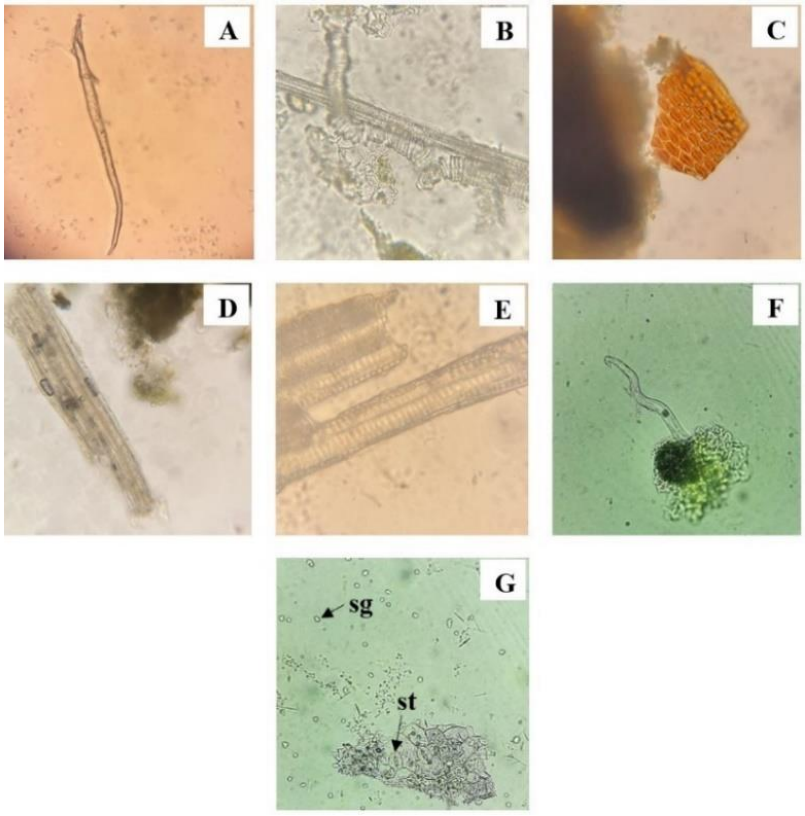

Figure 5. Microscopical of *P. indica* leaves *simplicia*. (A) Sclereid, (B) vessel with spiral type, (C) group of pitted sclereid, (D) sclerenchyma, (E) vessel, (F) trichome, (G) stoma (st) and starch grain (sg)

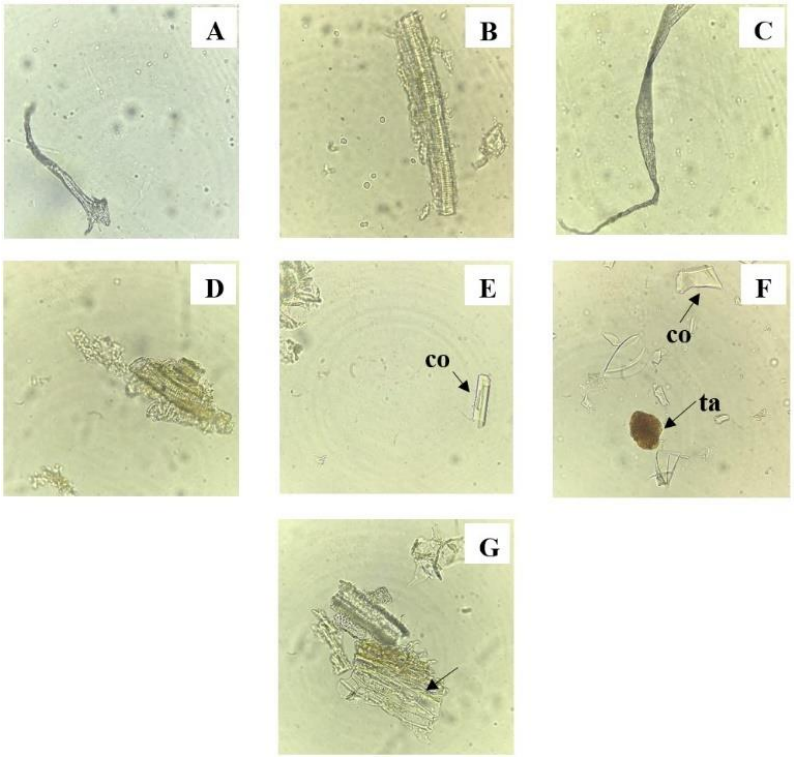

Figure 6. Microscopical of *P. indica* stems *simplicia*. (A) Trichome, (B) pitted annular vessel, (C) fiber, (D) spiral vessel, (E) calcium oxalate (co) crystals, (F) tannin-containing cells (ta) and calcium oxalate (co) square crystal, (G) cork cells

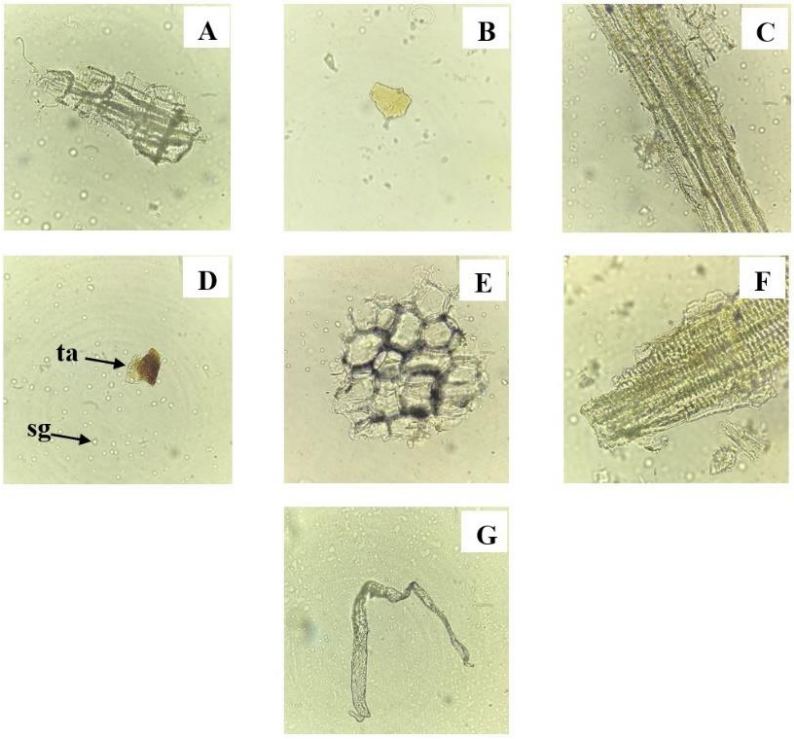

Figure 7. Microscopical of *P. indica* roots *simplicia*. (A) Parenchyma cells, (B) tannin-containing cell, (C) group of fibers, (D) tannin-containing cell (ta) and starch grains (sg), (E) cork cells, (F) vessel, (G) fiber

3.4. Chemical Screening

The results of the polyphenolic screening on *P. indica* leaves, stems, and root extracts are presented in Table 2.

In this study, the TLC method was used to determine the chromatogram pattern of the chemical components of each extract from *simplicia* leaves, stems, and roots of *P. indica*. Based on the TLC analysis in this study, the HE and EAE from the leaves, stems, and roots of *P. indica* have various chemical components. The leaves of *P. indica* have a chromatogram pattern that tends to be similar to the stems (Figure 8). The ME from the three organs of *P. indica* has chemical components that are non-polar to polar, which need to be separated by different mobile phases. The ME from the leaves, stems, and roots of *P. indica* contained several phenolic compounds (around 0.51, 0.80, and 0.84), while HE and EAE did not detect phenolic compounds (Figure 8).

Table 2. Phenolics screening of leaf, stem, and root extracts from *P. indica*

Simplicia	Type of extracts	Constituents		
		Phenolics	Flavonoids	Tannins
Leaf	HE	-	-	-
	EAE	-	-	-
	ME	+	-	+
Stem	HE	-	-	-
	EAE	-	-	-
	ME	+	-	+
Root	HE	-	-	-
	EAE	-	-	-
	ME	+	-	+

HE: *n*-hexane extract, EAE: ethyl acetate extract, ME: methanol extract, (+): detected, (-): not detected

4. Discussion

From the genus *Pluchea*, not only *P. indica* is useful as a medicinal plant, but *P. dioscoridis* and *P. lanceolata* are also often used traditionally for treatment. Morphologically, these species are easily distinguished by the shape of their leaves and flowers (King-Jones 2001). As shown in this study, *P. indica* leaves are obovate-shaped, while *P. dioscoridis* and *P. lanceolata* leaves are lanceolate-shaped (Khan *et al.* 2010; Khafagi *et al.* 2017). Thus, the microscopic characteristics of various types of cells and chemical screening from organs in *P. indica* become very important for the authentication of the plant (World Health Organization 1998).

Microscopy characteristics based on leaf section information show that *P. indica* can be recognized by the presence of trichomes near the upper epidermis on the leaf edges, where it is easy to find. The stem

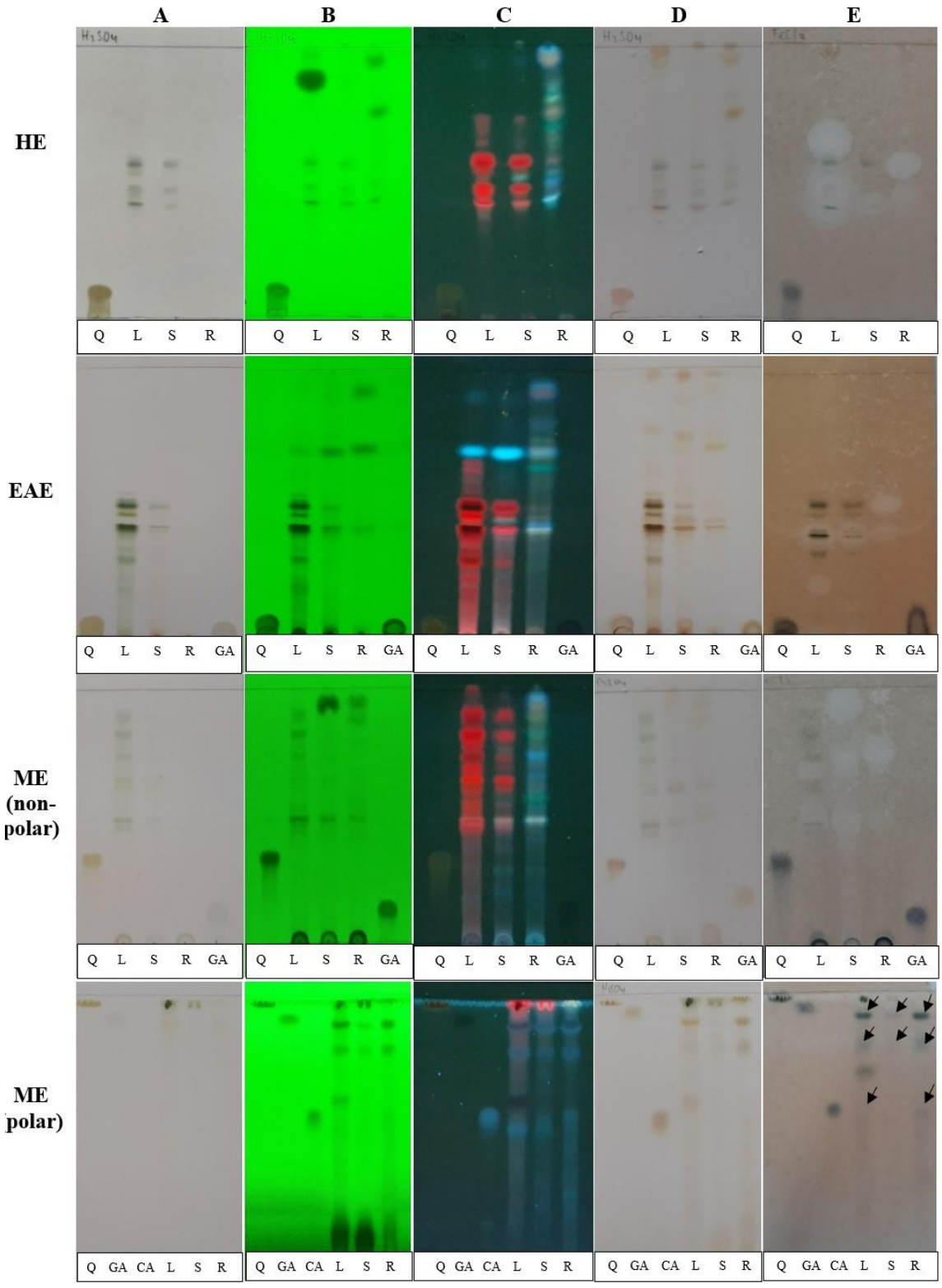

Figure 8. TLC analysis of the extracts from *P. indica*. HE: *n*-hexane extract, EAE: ethyl acetate extract, ME: methanol extract, L: leaves, S: steam, R: root, Q: quercetin, GA: gallic acid, CA: chlorogenic acid, A: visualization under visible light, B: visualization under 254 nm before being treated with a spray reagent, C: visualization under 365 nm before being treated with a spray reagent, D: visualization under visible after being treated with 10% H_2SO_4, E: visualization under visible after being treated with 5% $FeCl_3$, and arrows indicate the presence of phenolic in the sample with blue-black spots

section contains the xylem, phloem, and cambium. Meanwhile, air cavities and tannin-containing cells are easy-to-find features in the root section.

In this study, parenchyma cells were found in *simplicia* of roots, trichomes in both *simplicia* of leaves and stems, and tannin-containing cells are found easily in both *simplicia* of stems and roots. Unbranch (simple) trichomes with glandular unicellular hair types are features in the leaves and stems of the *P. indica* species. The *P. lanceolata* trichomes are glandular uniseriate multicellular hair (Khan 2010), whereas in *P. dioscorides*, the trichomes are glandular with a multicellular stalk and unicellular heads (Gabr 2021). Other findings are that the type of calcium oxalate crystals identified on the stems of *P. indica* are monoclinic prismatic crystals. *P. lanceolata* has rosette-shaped calcium oxalate crystals (Khan *et al.* 2010). There are five types of calcium oxalate crystals: sand, raphide, druse, styloid, and prismatic. However, it is not known with certainty the mechanism controlling the crystal form in plants (Nakata 2002).

Some secondary metabolites are produced in certain tissue plants. The plant cell has segregated biosynthetic sites. Most biosynthetic pathways are processed in the cytoplasm (e.g., alkaloids, furanocoumarins, some terpenes) or chloroplasts (e.g., carotenoids). Hydrophilic compounds are generally stored in vacuoles, apoplast/cell wall, idioblast, or epidermis. Meanwhile, lipophilic compounds are usually stored in resin ducts, glandular hairs (many terpenoids in Asteraceae), or trichomes (volatile compounds, terpenoids, and quinones) (Wink 2010). For example, *P. indica* leaves contain dominant phenolic compounds, including chlorogenic acid (Kongkiatpaiboon *et al.* 2018). Early biosynthesis of chlorogenic acid occurs via the plastidial shikimic pathway. The compound is synthesized in the cytoplasm and chloroplasts (Magaña *et al.* 2021). The chemical is then transported via the xylem and phloem to storage in the vacuoles (Mukherjee 2019).

Furthermore, this study also evaluates fluorescence in *simplicia* of *P. indica* organs after reacting with certain reagents (Mukherjee 2019). In this study, the *simplicia* of leaf and stem treated with ethanol gave a red and bright pink color at 245 nm, while the roots showed a yellowish-green. After being treated with acids, there was a similarity in the fluorescence character of *simplicia* of stem and root, both visible and UV light. Meanwhile, each *simplicia* gave a different fluorescence after being treated with a base. This indicates the possibility of similarities or differences in the chemical components of each *simplicia* that can react with certain reagents. In the past, the first fluorescence observations were made on crude herbs containing important drugs, such as quinine, through UV irradiation. Several pharmacognostic studies reported differences in fluorescence under UV in some crude drugs. Some crude drugs show similar fluorescence in certain reagents, while others show different. Some samples showed fluorescence even without fluorescence after being treated with reagents. The difference in fluorescence from each plant part sample indicates the possibility of various types of compounds (Chase and Pratt 1949). These could be closely related to certain functional groups in plant chemical metabolites (Ul Uza and Dastagir 2022). For example, many crude drugs from leaves give a red fluorescence after being treated with a reagent solution of nitrocellulose in amyl acetate. This is possible because of the chlorophyll component. However, fluorescence analysis has challenges, including a) there are crude drugs whose compounds do not even give fluorescence from some commonly used reagents, and b) there are differences in researchers in determining the fluorescence formed (not all individuals have a normal sense of color). Many factors influence the results of fluorescence analysis, such as identification system (type of reagent, reagent concentration, and pH), temperature, age of harvest of crude medicinal plants, and time of storage of dried crude plants. Thus, to minimize bias, estimation of fluorescence intensity should also be carried out on crude drugs whose compound identities are known as a reference. In addition, to accurately determine the difference in fluorescence intensity quantitatively, the fluorimeter technique can help ensure the results obtained (Chase and Pratt 1949).

Polyphenolic screening was conducted to complete information on herbal material. As a result, phenolic and tannin compounds were detected from all organs' methanol extract (ME). Meanwhile, flavonoids were not detected in all *P. indica* extracts. Polyphenolic compounds were not detected in *n*-hexane extract (HE) and ethyl acetate extracts (EAE) of *P. indica* organs.

In an earlier study by Talia *et al.* (2017), the ethanol extract of *P. indica* leaves obtained from three different growing sites showed a similar TLC chromatogram pattern when using toluene-ethyl acetate (7:3, v/v) as the mobile phase (Talia *et al.* 2017). In this study, the mobile phase of toluene-ethyl acetate-acetic acid (in various ratios) tends to be able to separate HE, EAE, and non-polar components from the ME of *P. indica* organs. Meanwhile, the polar components of the ME extracts can be separated by using a polar mobile phase composed of ethyl acetate-water-formic acid-acetic acid (8.5:1.5:1:1, v/v). With the polar mobile phase, gallic (blue-black spot) and chlorogenic acids (brown-green) are detected as in *P. indica* extracts after being sprayed using a $FeCl_3$ reagent (Waksmundzka-hajnos *et al.* 2008) with R_f values of around 0.93 and 0.51, respectively. Chewchida and Vongsak (2019) reported that ethyl acetate-formic acid-water-toluene (20:2:2:1, v/v) is the best mobile phase for separating chlorogenic acid (R_f = 0.34) in 50% ethanol extract of *P. indica* leaves using High-Performance Thin-Layer Chromatography (HPTLC) method (Chewchida and Vongsak 2019). Hikmawanti *et al.* (2022) reported that with the same mobile phase, chlorogenic acid was also detected in the 50% ethanol extract of *P. indica* stems (R_f = 0.55) (Hikmawanti *et al.* 2022). The polar mobile phase was considered too polar to separate quercetin (R_f > 0.9) in the ME of *P. indica* organs. Previously, quercetin was detected in the methanol-water extract of *P. indica* leaves using the High-Performance Liquid Chromatography (HPLC) method (Andarwulan *et al.* 2010). The ethyl acetate and *n*-hexane extracts from *P. indica* leaves contained smaller amounts of total phenols and flavonoids than the methanol, water, and ethanol extracts (Widyawati *et al.* 2014). In this study, quercetin was in a low concentration in the extracts, so TLC did not detect it (Jesionek *et al.* 2015). The roots predominantly contain terpenoid glycoside compounds, such as plucheoside A, B, C, D, and E (Uchiyama *et al.* 1989, 1991). Studies on the chemical content of polyphenols from *P. indica* stems and roots are still limited.

Eventually, our study suggested that trichomes in leaves and stems, and tannin-containing cells in roots are some of microscopic features of *P. indica simplicia*. Meanwhile, simple TLC analysis to determine the presence of chlorogenic acid as a marker using ethyl acetate-water-formic acid-acetic acid (8.5:1.5:1:1, v/v) can be added to confirm the identification of *P. indica.* This simpel methods might be a help in the laboratory with limited number of instruments for accurately and routinely identifying *P. indica* as a raw material for herbal medicines production. Other modern techniques, such as the analysis of plant molecular markers, both deoxyribonucleic acid (DNA) and protein, and profiling of secondary metabolites with chromatography and capillary electrophoresis techniques coupled with mass spectroscopy, can be applied for the same purpose as this study (Muyumba *et al.* 2021).

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