© 2023 Journal of Pharmacy & Pharmacognosy Research, 11 (3), 523-536, 2023
ISSN 0719-4250
https://jppres.com

DOI: https://doi.org/10.56499/jppres23.1613_11.3.523

Original Article

Pharmacognostic evaluation and antioxidant capacity of *Tetracera macrophylla* Hook. F. & Thoms twigs

[Evaluación farmacognóstica y capacidad antioxidante de las ramas de *Tetracera macrophylla* Hook. F. & Thoms]

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Abstract

Context: *Tetracera macrophylla* Hook. f. & Thoms is used empirically in traditional medicine, specifically the twig, to treat dysentery, diarrhea, diabetes, and tuberculosis symptoms. The plant contains phenolic and flavonoid compounds that offer potential as antioxidants.

Aims: To evaluate the pharmacognostic characteristics of *T. macrophylla*, phytochemical, total phenolic and flavonoid contents, and the antioxidant capacity twig of *T. macrophylla* from n-hexane, ethyl acetate, and methanol extracts.

Methods: Pharmacognostic evaluation was carried out by macroscopic-microscopic observation leaf and twig of *the Tetracera macrophylla*, and phytochemical analysis was carried out using several chemical reagents. The chromatographic profile was analyzed by thin-layer chromatography and qualitative identification of compounds by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). The total phenolic (TPC) and flavonoid contents (TFC) were determined colorimetrically. The antioxidant capacity was determined *in vitro* by the FRAP and DPPH methods.

Results: The microscopic analysis of leaf powder showed the presence of raphide crystals, uniseriate trichomes, and parasitic stomata, while the twig powder showed the presence of raphide crystals. The primary phenolic compounds in the active extracts were identified as 6-hydroxy-2-(2-phenylethyl) chromone, d-catechin, isorhamnetin, and epicatechin gallate. The highest TPC and TFC were found in the ethyl acetate extract. The ethyl acetate and methanol extract showed a higher antioxidant capacity than the hexane extract. The correlation matrix between TPC, TFC, and antioxidant capacity showed a robust correlation.

Conclusions: The pharmacognostic evaluation of *T. macrophylla* revealed the presence of characteristic morphological features of the plant. A microscopic study of the leaf showed the presence of uniseriate trichomes and paracytic stomata on the abaxial epidermis. Powder analysis of twigs showed the presence of irregular starch grains, raphide crystals, and stone cells. The TPC and TFC in twigs of *T. macrophylla* were robustly correlated to antioxidant capacity with the FRAP method. Meanwhile, the TPC and TFC gave a moderate correlation with DPPH methods. The ethyl acetate and methanol extracts of *T. macrophylla* twigs showed a higher antioxidant capacity than the n-hexane extract.

Keywords: antioxidant; authenticity; phytochemical; *Tetracera macrophylla*.

Resumen

Contexto: *Tetracera macrophylla* Hook. f. & Thoms se utiliza empíricamente en la medicina tradicional, específicamente la ramita para tratar la disentería, la diarrea, la diabetes y los síntomas de la tuberculosis. La planta contiene compuestos fenólicos y flavonoides que ofrecen potencial como antioxidantes.

Objetivos: Evaluar las características farmacognósticas de *T. macrophylla*, fitoquímicos, contenido total de fenólicos y flavonoides y la capacidad antioxidante ramita *de T. macrophylla* de extractos en n-hexano, acetato de etilo metanol.

Métodos: La evaluación farmacognóstica se llevó a cabo mediante observación macroscópica-microscópica de la hoja y ramita de la *T. macrophylla*, y el análisis fitoquímico se realizó utilizando varios reactivos químicos. El perfil cromatográfico se analizó mediante cromatografía en capa fina y la identificación cualitativa de los compuestos mediante cromatografía líquida-espectrometría de masas/espectrometría de masas (LC-MS/MS). El contenido total de fenoles (TPC) y flavonoides (TFC) se determinó colorimétricamente. La capacidad antioxidante se determinó *in vitro* mediante los métodos FRAP y DPPH.

Resultados: El análisis microscópico del polvo de hoja mostró la presencia de cristales de rafidios, tricomas uniseriados y estomas parásitos, mientras que el polvo de ramita mostró la presencia de cristales de rafidios. Los compuestos fenólicos primarios en los extractos activos se identificaron como 6-hidroxi-2-(2-feniletil) cromona, d-catequina, isorhamnetina y galato de epicatequina. TPC y el TFC más elevados se encontraron en el extracto de acetato de etilo. Los extractos de acetato de etilo y metanol mostraron una mayor capacidad antioxidante que el extracto de hexano. La matriz de correlación entre TPC, TFC y capacidad antioxidante mostró una correlación sólida.

Conclusiones: La evaluación farmacognóstica de *T. macrophylla* reveló la presencia de rasgos morfológicos característicos de la planta. Un estudio microscópico de la hoja mostró la presencia de tricomas uniseriados y estomas paracíticos en la epidermis abaxial. El análisis del polvo de las ramitas mostró la presencia de granos de almidón irregulares, cristales de rafidios y células pétreas. El TPC y el TFC en ramitas de *T. macrophylla* se correlacionaron sólidamente con la capacidad antioxidante mediante el método FRAP. Mientras tanto, el TPC y el TFC dieron una correlación moderada con el método DPPH. Los extractos de acetato de etilo y metanol de las ramitas de *T. macrophylla* mostraron una mayor capacidad antioxidante que el extracto de n-hexano.

Palabras Clave: antioxidante; autenticidad; fitoquímica; *Tetracera macrophylla*.

ARTICLE INFO
Received: February 20, 2023.
Accepted: June 10, 2023.
Available Online: July 2, 2023.

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INTRODUCTION

Pharmacognostic evaluation is an effort to obtain quality natural medicinal raw materials. The extracts as raw materials for traditional medicines must meet quality, safety, and efficacy requirements. Macro-microscopic characterization of plants, phytochemical analysis, and compound content in extracts are ways to achieve this goal. Medicinal plants have secretory structures that produce metabolites. Metabolite production in plants is variable. Metabolites such as phenolics, flavonoids, steroids, and terpenoids can be identified qualitatively using thin-layer chromatography (TLC) and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Tetracera macrophylla Hook. f. & Thoms belongs to the *Dilleniaceae* family and is a woody climber found widely across many Asian countries and some parts of Africa (Mazlun et al., 2021). A decoction from the stems of *T. macrophylla* is efficacious in treating throat problems (Mazlun et al., 2021). The roots and leaves are mixed to treat itching, and a decoction of the roots can treat diarrhea and dysentery (Lima et al., 2014; Ong et al., 2011; Quattrocchi, 2012). Many of these pharmacological properties involve antioxidant mechanisms. To date, there has been no evaluation of the macros-microscopic characteristics of *T. macrophylla*. Microscopic characterization is important in evaluating specific fragments to identify and standardize raw materials. Specific fragments can distinguish plants with organoleptic or macroscopic similarities, such as plants belonging to the same genus. Fig. 1 contains images of the leaves, flowers, twigs, and roots of *T. macrophylla*, which were determined for their characteristics.

T. macrophylla has a high phenol and flavonoid content, which has potential radical scavenging activity (Roheem et al., 2020). Antioxidants are compounds that can reduce free radical attacks and inhibit oxidation. An imbalance between the number of free radicals and endogenous antioxidants causes oxidative stress. Oxidative stress will trigger cell damage and cause degenerative metabolic diseases. Many studies have shown that antioxidant compounds can reduce the risk of chronic diseases such as diabetes, hypertension, cancer, and coronary heart disease (Sharifi-Rad et al., 2020; Zanwar et al., 2014). This study will assist in authenticating genuine plant material and analyzing its phytochemicals and antioxidant capacity.

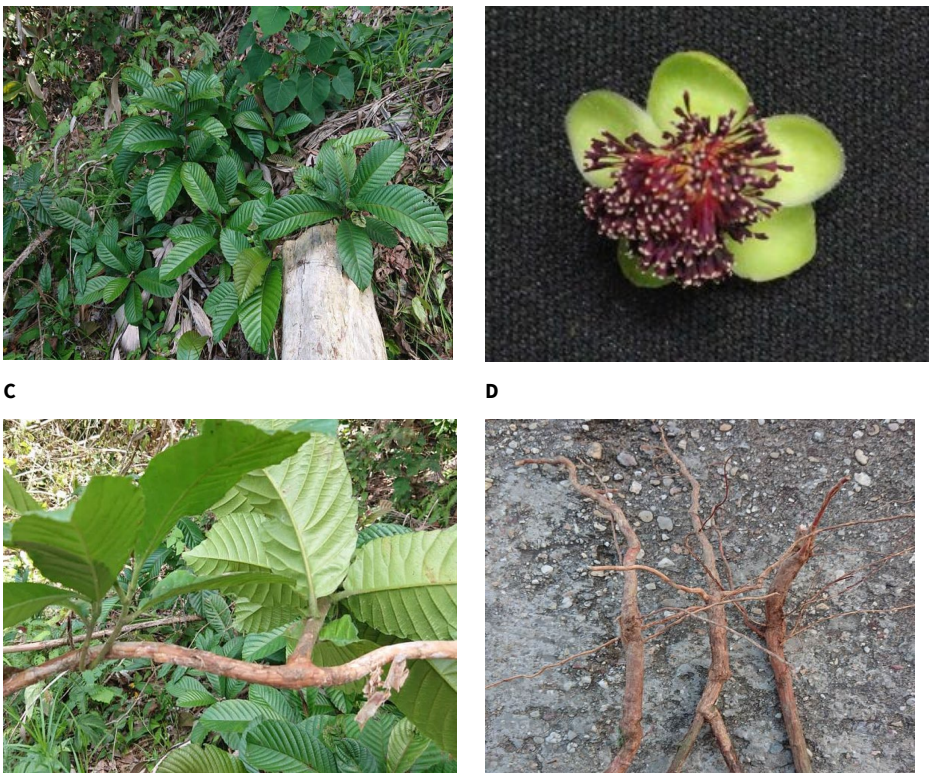

Figure 1. *Tetracera macrophylla* parts.
(A) Leaves; **(B)** Flowers; **(C)** Twig with leaves; **(D)** Roots.

MATERIAL AND METHODS

Materials

Methanol, hexane, ethyl acetate, Folin-Ciolcalteu, gallic acid, chloroform, and silica gel 60 F 254 TLC plates were obtained from Merck (Darmstadt, Germany). Ferric chloride hexahydrate ($FeCl_3.6H_2O$), hydrochloride acid (HCl), 2,2-diphenyl picrylhydrazyl (DPPH), quercetin, chloral hydrate, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Aldrich (St.Louis, MO, USA). Trinocular microscope (Nikon Eclipse E100, Japan), glass slides, coverslips from Marienfeld (Germany), twigs, roots, flowers, and leaves of *T. macrophylla* (source from Teweh Baru District, Central Kalimantan).

Collection and authentication of plant material

T. macrophylla was obtained from the protected forest of Teweh Baru District, North Barito Regency, Central Kalimantan (114°27'00" – 115°49'00" East Longitude and 0°58'30" North Latitude – 1°26'00" South Latitude). The species was authenticated by the plant taxonomist of the Biology Research Center, Indonesian National Research and Innovation Institute, Cibinong, Indonesia, with collection number B-644/V/D1.05.07/11/2021. The leaves and twigs of the plant collected were washed in running water, shade dried, powdered, passed through a 40-mesh sieve, and stored in an airtight container for further use.

Macroscopic, organoleptic, and microscopic

Morphological studies of the leaf and twigs were carried out through observation with the naked eye. Macroscopic and organoleptic characteristics such as size, shape, texture, color, surface, odor, and taste were examined using the terms outlined in Evans (2002). Microscopic examinations of transverse sections of fresh twig, lamina, midrib, and powder were performed. Leaves and twig powder were placed on slides and mounted in 2–3 drops of chloral hydrate or distilled water. Each slide was covered with a cover slip and examined under a microscope (Kumar et al., 2011). The different cell components were noted, and photographs (Camera Nikon D3400, Japan) were taken using a trinocular microscope (CST 10 Series, Nikon Eclipse e100, Japan) and at a magnification of 100×.

Extraction

Twigs of *T. macrophylla* (6 kg) were dried and powdered, extracted by gradual maceration using n-hexane, ethyl acetate, and methanol (Abubakar and Haque, 2020). Twig powder (1.8 kg) was soaked with n-hexane at a ratio of 1 g powder in 10 mL solvent. The maceration was carried out for 1 × 24 h at room temperature; after that, the macerated sample was filtered. The residue was re-macerated with the same solvent until a clear filtrate was obtained. The residue was then macerated with ethyl acetate and methanol solvent, respectively, in the same way as with n-hexane solvent. The solvent was evaporated with a rotary vacuum evaporator at 50°C (Eyela 05B-2100, China) and dried over a water bath to obtain n-hexane extract (HE), ethyl acetate extract (EE), and methanol extract (ME). The dry extract was stored at 2–8°C before use in phytochemical analysis with LC-MS/MS, total phenolic content (TPC), total flavonoid content (TFC), and antioxidant assays. The yield obtained for each extraction was HE 0.75%, EE 1.57%, and ME 10.94%.

Phytochemical screening

Phytochemical screening was performed by dissolving 20 g of twig powder with methanol. The phytoconstituents were analyzed by color reaction and precipitating reagents such as an alkaloid test (Mayer, Bouchardat, Wagner, and Dragendorff reagents), flavonoids (metal Mg + HCl 5 M), glycosides (Fehling solution), phenols ($FeCl_3$ test), saponins (shaken with warm water), steroids and terpenoids (Liebermann Burchard test), and tannins (gelatin + NaCl) (Evans, 2002; Harborne, 1998). The intensity of the color formed was observed.

Chromatographic profile

Phytochemical analysis of graded extracts of *T. macrophylla* twigs using the TLC method was undertaken. Silica gel 60 F 254 was used as the stationary phase. The plates were developed in a chamber using different solvent systems. The mobile phase for HE and EE was hexane-ethyl acetate (1:1), and the solvent system for ME was ethyl acetate-methanol (9:1). The plate was dried and sprayed with H_2SO4 10%. The formed spot was observed under UV 254 and visible light 366 nm (Harborne, 1998; Mac Fhionnlaoich et al., 2018).

The retention factor (Rf) value was determined using the formula [1].

$$Rf = \frac{Distance\ moved\ by\ the\ solute}{Distance\ moved\ by\ the\ solvent\ front} \quad [1]$$

Total phenolic content (TPC) assay

In a 96-well microplate, 100 µL of Folin-Ciocalteau solution (1:10) and 20 µL of a sample (HE, EE, and ME of *T. macrophylla* twig) were added and shaken for 60 s. The mixture was incubated at room temperature for

4 min. After adding 80 µL of 7.5% Na_2CO_3 solution, the mixture was stirred and incubated for 2 h in the dark room. The absorbance of the sample solution was calculated at a maximum wavelength of 750 nm using a microplate reader (iMark™, Bio-Rad, USA). Based on the regression equation for gallic acid (y = 0.0087x-0.1084 and R^2 = 0.9976), the phenolic content was calculated. (Fachriyah et al., 2020; García et al., 2015).

Total flavonoid content (TFC) assay

An amount of 20 µL of a sample (HE, EE, and ME of twig *T. macrophylla*) was added to 20 µL of 10% $AlCl_3$ solution, 20 µL of 1 M potassium acetate, and 180 µL of distilled water in a 96-well microplate. The mixture was shaken for 60 s and incubated for 30 min. The absorption was read at 415 nm using a microplate reader (iMarkTM, Bio-Rad, USA). The quercetin regression equation (y = 0.0053x + 0.0177 and R^2 = 0.9999) determined total flavonoid levels (Farasat et al., 2014; García et al., 2015).

Antioxidant assay with the DPPH method

Antioxidant assays with the DPPH method were determined based on a protocol modified by Molyneux (2004). A series of samples (40–120 µg/mL) was prepared. Into each sample was added 3 mL 0.4 mM DPPH solution. The volume was up to 5 mL in methanol, and the assay was vortexed (VM-300, Scientifica, Italy) to homogenize. The mixture was incubated for 30 min in the darkroom. The absorption was measured at a wavelength of 516 nm with a UV-Vis spectrophotometer (Shimadzu UV 1900, Japan). The active control employed was quercetin. The IC_{50} values were calculated based on the percentage of the inhibition of DPPH radicals from each concentration of the sample solution using the formula [2].

$$\% \text{ inhibition} = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100 \quad [2]$$

The value of IC_{50} was the concentration at which the sample reduced DPPH by 50% using a linear regression equation.

Antioxidant assay with the FRAP method

The FRAP method refers to the research of Prastiwi et al. (2020) and Wong et al. (2015) with modified slightly. The sample concentration was prepared at 1000 µg/mL in methanol, then diluted to 500 µg/mL. 270 µL of FRAP reagent was added to 30 µL of sample solution, homogenized for 60 s, and then incubated at 37°C in the darkroom for 30 min. The absorbance was measured at a wavelength of 595 nm with a microplate reader (iMark™, Bio-Rad, USA). 300 µL of methanol was used to create plate blanks. AFS was used as a reference compound in 1200 µM concentrations. From the AFS curve, the regression equation is obtained y = 0.0015x + 0.071 and R^2 = 0.9999. Antioxidant activity was calculated based on the ferric iron equivalent antioxidant capacity (FeEAC) with the following formula [3].

$$FeEAC = \frac{\triangle A}{GRAD} \times \frac{Av}{Spv} \times D \times \frac{1}{Cext} \times 10^5 \quad [3]$$

Where FeEAC: Equivalence between antioxidant activity (µmol/g) and ferric ion; $^{\triangle}$A: Absorbance of samples that have been reduced by blank; GRAD: Gradient determined from the calibration curve on ferrous ammonium sulfate (AFS); *Av*: Total volume for the test; *Spv*: Total sample volume in the test; D: Sample dilution factor; *Cext*: Sample stock concentration (g/L).

Identification of compounds by LC-MS/MS

Qualitative analysis of the compounds in the active extract was carried out using LC-MS/MS, referring to several references (Ahmad et al., 2018; Alhassan et al., 2019; Hasan et al., 2017). The measurements were obtained using a Waters Acquity UPLC I-Class equipped with XEVO G2-XS QTof mass spectrometry (Water Corp, Milford, MA, USA). The samples were separated using a column type ACQUITY UPLC® BEH C18 (1.7 µm × 2.1 mm × 50 mm) with injection volume 1 µL and full scan m/z 100 – 1200 (mode ESI). The mobile phase used solvent A (H_2O + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). The sample was prepared by dissolving 5 mg of solid and filtering through an 0.22 µm nylon filter. Mass fragmentation of compounds was detected using the instrument application's spectrum database of organic chemicals.

Statistical analysis

Tests were carried out in triples and presented as mean ± SD. One-way analysis of variance (ANOVA $p<0.05$) was conducted using SPSS version 23 to assess the significant difference between the mean values, followed by the Tukey test. The TPC and TFC data correlation with antioxidant capacity were analyzed using Spearman correlation by Minitab 20 version.

RESULTS

Macroscopic and organoleptic characterization

Leaf: *T. macrophylla* is a large and rough liana. The leaves have a length of 30 cm and a width of 12 cm. The young leaves are purple, while the old leaves have a dark green color, and the surface of the leaves has a rough texture. The macroscopic characteristics of *T. macrophylla* are shown in Tables 1 and 2.

Table 1. Organoleptic characters of *T. macrophylla.*

Organoleptic character	Leaf	Twig	Root	Flower
Surface	Rough	Scabrid, lignosus	Smooth	Snout with a smooth surface.
Color	Dark green (upper), light green (lower)	Dark brown,	Light brown	Maroon
Odor	No characteristic odor	No characteristic odor	No characteristic odor	No characteristic odor
Taste	No characteristic odor	Slightly bitter	Bitter	No characteristic odor

Table 2. Macroscopic characters leaf of *T. macrophylla.*

Phyllotaxy	Alternate
Type	Liana
Leaf	Length 12.5 - 8 cm, Width 10 - 12 cm
Shape	Elliptic-oblong
Apex	Acuminate
Margin	Entire to serrate
Venation	Reticulate
Base	Attenuate
Petiole	3.8 cm long

Flower: *T. macrophylla* is a complete flower with petals, sepals, and reproduction organs (male): stamen, anther, filament, and reproduction (female): carpel, stigma, style, pollen tube, ovary, and ovule. The length of the flower blooms is 2 cm, and the width is 2.5 cm; maroon color, flos terminalis, inflorescentia racemosa, corymbus ramosus type. There are ovarian with 2 or 3 carpellums that are oval in shape and have a snout with a smooth surface.

Twig: The twigs are branches from plant stems with a 0.3–0.5 cm diameter. The inside of the twig is fibrous and hard. The stem of *T. macrophylla* belongs to the lignosus, brown teres. The growth of the stem is scandens, and the stem is approximately 5 cm in diameter.

Root: The roots of *T. macrophylla* belong to the tap root, which has a primer root and lateral branches. The root is light brown.

Microscopic characterization of *T. macrophylla*

Leaf of *T. macrophylla:* Two parts of the leaf were observed, namely the lamina and midrib. The lamina of the adaxial epidermis lacks stomata. A tightly packed palisade network lies beneath the top epidermis, followed by spongy parenchyma. The abaxial epidermis has parasitic stomata. The single-layered, rectangular cells of the epidermis with a thin cuticle layer make up the midrib. Small rectangular cells and uniseriate trichomes with tapering ends are features of the abaxial epidermis. A total of 5–6 layers of parenchyma cortex follow 2–3 layers of collenchyma hypodermis (Fig. 2). Cortex also contains tightly packed parenchyma with raphide crystals, which are sharp (needle-shaped calcium oxalate crystals). Fig. 3 shows microscopic images of *T. macrophylla* leaf powder.

Twig of *T. macrophylla:* Fig. 4 show irregular starch grains, fibers, needle-shaped Ca oxalate crystals (raphide crystals), stone cells, trichomes, phloem, and xylem towards the center.

Phytochemical analysis

The results of the phytochemical analysis show that the crude extract contains flavonoids, phenols, saponins, tannins, glycosides, steroids, and terpenoids after phytochemical analysis (Table 3). The presence of metabolites in crude extracts significantly impacts their pharmacological effect.

Chromatographic evaluation of different extracts of twig *T. macrophylla*

The thin-layer chromatographic profile of the HE of *T. macrophylla* revealed the presence of 4 spots in the hexane:ethyl acetate (1:1) solvent system and ten spots on the EE. *T. macrophylla* extract in methanol (ME) underwent thin-layer chromatographic examination, which revealed the presence of two spots in the ethyl-methanol (9:1) solvent system (Table 4).

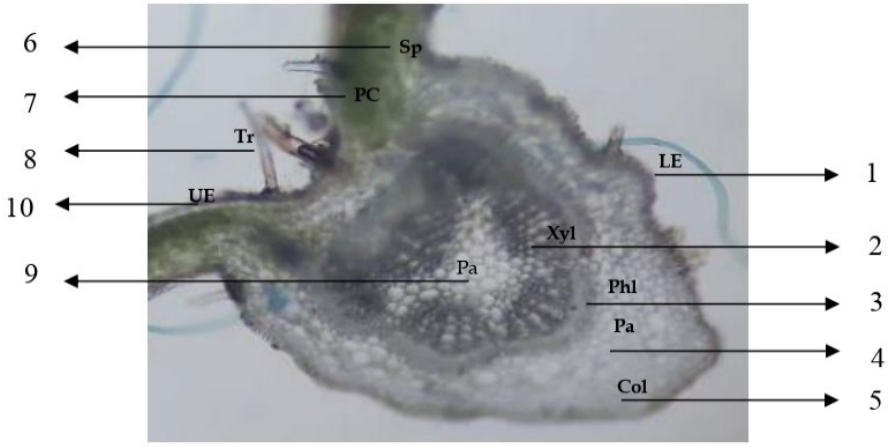

Figure 2. Transverse section of *T. macrophylla* midrib.

1. Lower epidermal (LE); 2. Xylem (Xyl); 3. Phloem (Phl); 4. Parenchyma (Pa); 5. Collenchyma (Col); 6. Sponge (Sp); 7. Palisade cells (PC); 8. Trichomes (Tr); 9. Parenchyma (Pa); 10. Upper epidermis (UE). Magnification 100×.

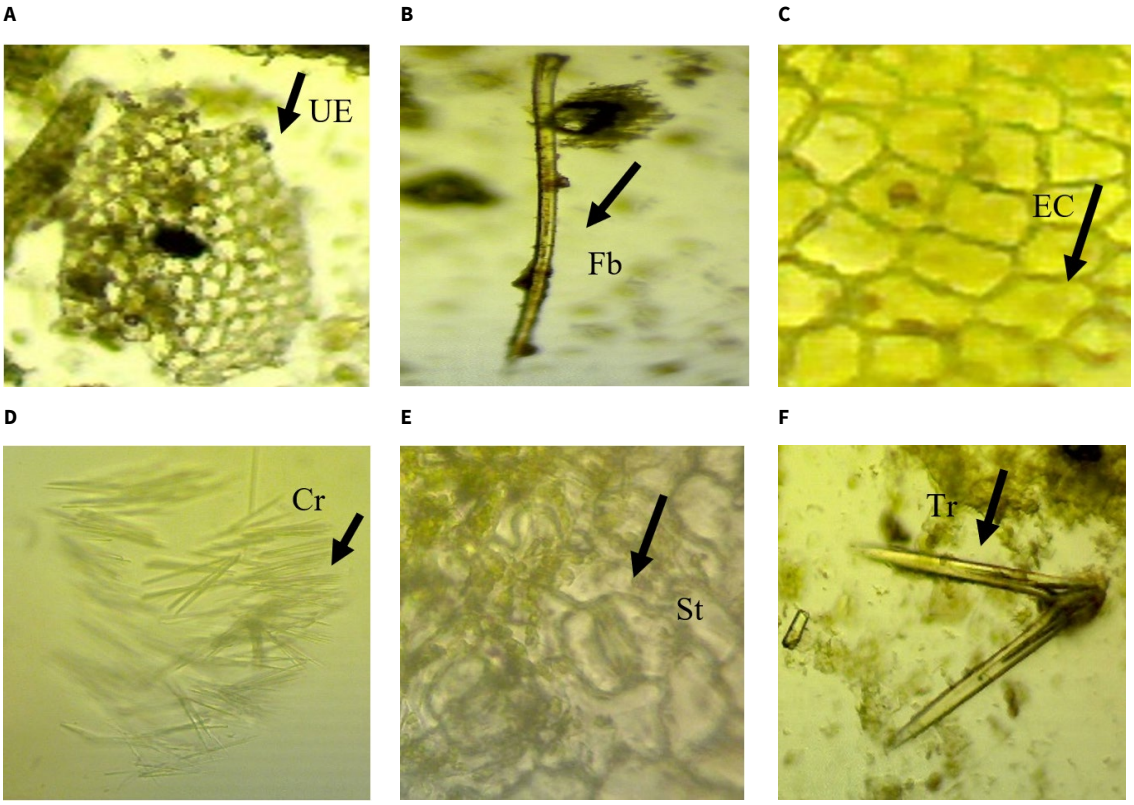

Figure 3. Microscopic of *T. macrophylla* leaf powder.

(A) Upper epidermis (UE); **(B)** Fiber (Fb); **(C)** Portion enlarged-upper epidermal layer (EC); **(D)** Crystalline Ca oxalate/Crystal raphide (Cr); **(E)** Trichome (Tr); **(F)** Paracytic Stomata (St). Magnification 100×.

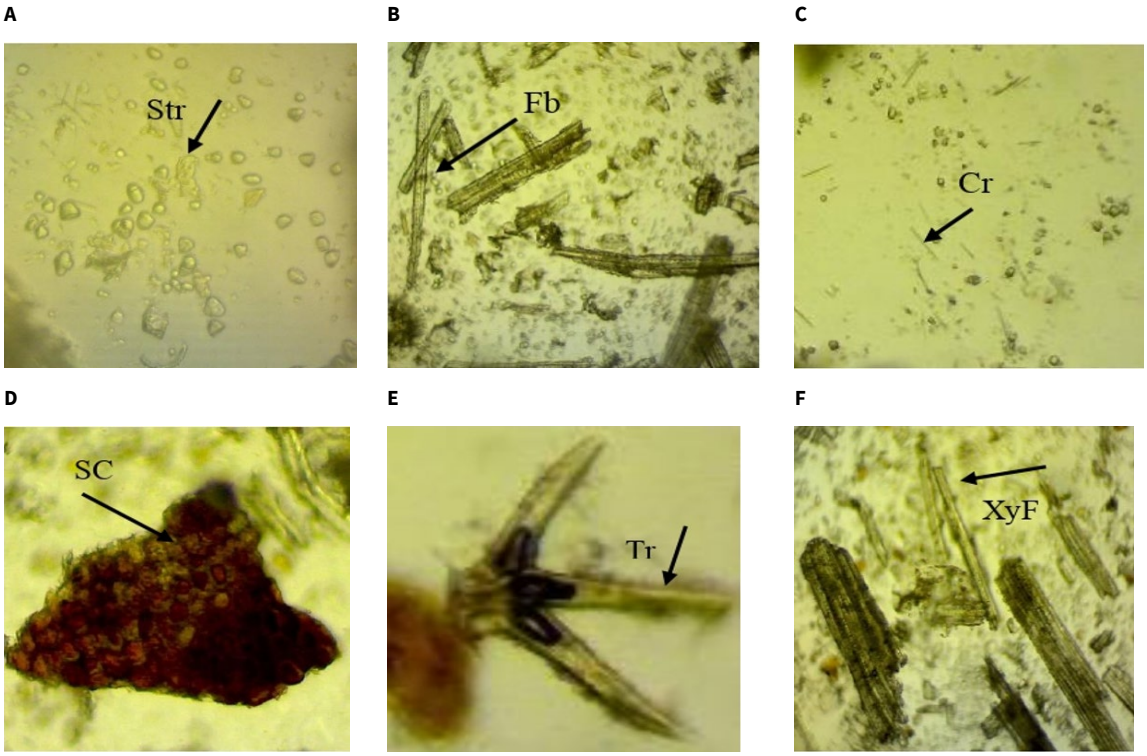

Figure 4. Microscopic of *T. macrophylla* twig powder.

(A) Starch (Str, magnification 400×); **(B)** Fiber (Fb); **(C)** Crystalline Ca oxalate (Cr); **(D)** Stone cells (SC); **(E)** Trichome (Tr); **(F)** Xylem Fiber (XyF). Magnification 100×.

Table 3. Phytochemical screening of material plant extracts of twig *T. macrophylla*.

Phytoconstituents	Reagents	Results
Alkaloids	Dragendorff	no detected
	Mayer	+ (white precipitate)
	Bouchardat	no detected
	Wagner	no detected
Flavonoids	Shinoda	++ (reddish orange)
Phenols	Ferric chloride	++ (dark blue)
Saponins	Foam	+ (stable foam)
Tannin	Gelatine 10%	+ (white precipitate)
Steroids and Terpenoids	Liebermann Burchard	+ (reddish brown)
Glycosides	Fehling	+ (reddish brown precipitate)

(+) Positive result; (++) Positive results with strong intensity.

Total phenolic content (TPC) and total flavonoid content (TFC)

TPC was determined using the Folin–Ciocalteu method at a wavelength of 750 nm with gallic acid as the standard with regression equation y = 0.0087x – 0.1084 and R^2 = 0.9976. This study showed that the ethyl acetate extracts (EE) had higher phenolic content than the hexane (HE) and methanol extracts (ME). The hexane, ethyl acetate, and methanol extracts had significantly different average phenol levels ($p<0.05$, n = 3, post hoc test/LSD).

TFC was determined with a colorimetric method using $AlCl_3$ and quercetin as the standard with regression equation y = 0.0053x + 0.0177 and R^2 = 0.9999. Based on the ANOVA results, p = 0.000 was obtained. EE had the highest TFC content of 337.367 mg QE/g, followed by ME and HE (305.942 and 6.786 mg QE/g, respectively). This result is supported by a

Table 4. Chromatographic evaluation of different extracts of twig *T. macrophylla.*

Extract	Mobile phase	Amount of spots	Visual	Spraying reagent H_2SO_4 10%	Rf (UV 366 nm)
Hexane	Hex:EtOAc (1:1)	4	1. Yellowish	1. Blue	0.48
			2. Yellowish	2. Pink	0.53
			3. Green	3. Light blue	0.61
			4. Yellow	4. Reddish	0.69
Ethyl acetate	Hex:EtOAc (1:1)	10	1. Green	1. Pink	0.11
			2. -	2. Yellowish	0.14
			3. -	3. Green	0.21
			4. -	4. Pink	0.26
			5. Yellow	5. Green	0.38
			6. Yellow	6. Green	0.42
			7. -	7. Pink	0.48
			8. -	8. Yellowish	0.59
			9. Yellow	9. Yellowish	0.65
			10. Yellow	10. Yellowish	0.68
Methanol	EtOAc:MeOH (9:1)	2	1. Light blue	1. Blue	0.15
			2. Light blue	2. Blue	0.21

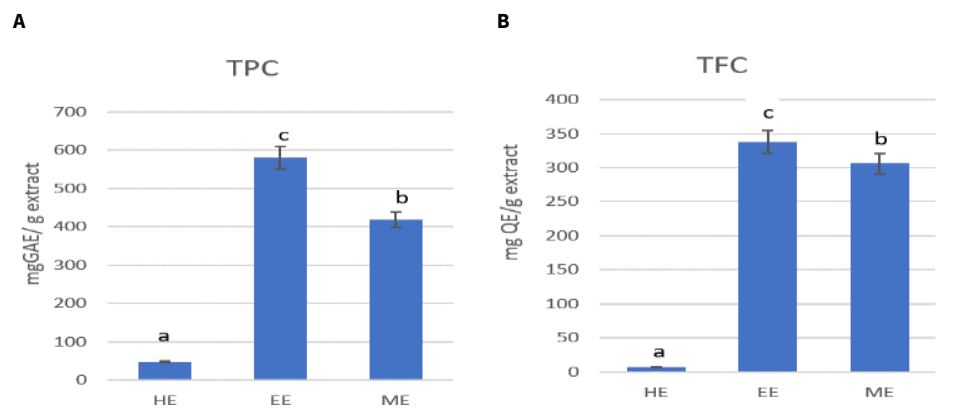

Figure 5. (A) Total phenolic content (TPC); **(B)** Total flavonoid content (TFC) of *T. macrophylla* twig.

Data represent mean ± SD (n = 3). Different letters indicate statistically significant differences ($p<0.05$, Tukey post hoc test, LSD). HE: Hexane extract; EE: Ethyl acetate extract; ME: Methanol extract.

study by Roheem et al. (2020), which states that ethanol extracts of *T. macrophylla* leaves have high phenols and flavonoids. The solvents of different polarities will affect plant extractability. TPC and TFC values are shown in Fig. 5.

Antioxidant assay with DPPH and FRAP method

The DPPH assay was determined using quercetin as the standard. The hexane extract of *T. macrophylla* twigs showed a weak potential in reducing DPPH with IC_{50} = 168.006 ± 2.520 µg/mL. In contrast, the ethyl acetate and methanol extract had substantial antioxidant effects with IC_{50} = 13.85 ± 0.065 and 15.56 ± 0.01 µg/mL, respectively (Fig. 6). Quercetin as the standard has a regression equation y = 5.7569x + 31.831, R^2 = 0.9929 with IC_{50} = 3.156 ± 0.002 µg/mL. IC_{50} was used to determine the antioxidant capacity of the sample compared to the standard. Based on the classification by Blois (1958), the antioxidant power in reducing free radicals was categorized as robust activity (IC_{50} < 50 µg/mL), potent antioxidant (50-100 µg/mL), medium antioxidant (101-150 µg/mL), and weak (>150 µg/mL).

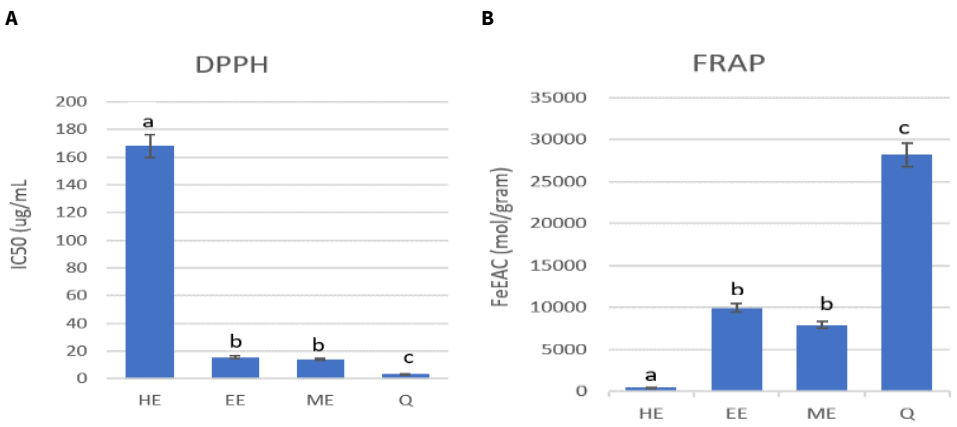

Figure 6. Antioxidant activity of *T. macrophylla* twig.

(A) DPPH; and **(B)** FRAP methods.

Data represent mean ± SD (n = 3). Different letters indicate statistically significant differences ($p<0.05$, Tukey post hoc test, LSD). HE: Hexane extract; EE: Ethyl acetate extract; ME: Methanol extract; Q: Quercetin.

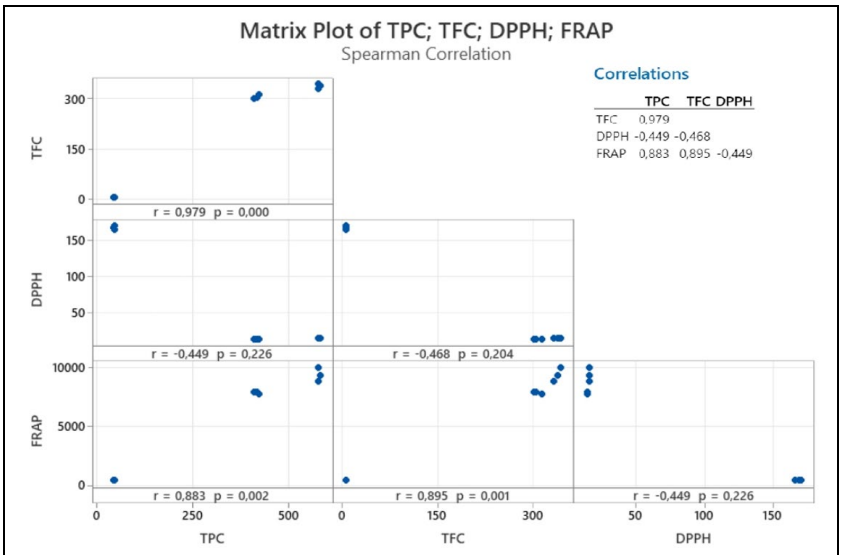

Figure 7. Spearman correlation coefficient analysis between total phenolic content (TPC) and total flavonoid content (TFC) against antioxidant activity (DPPH and FRAP).

P<0.01 indicates the variable significance.

The FRAP assay was performed using an AFS standard solution and quercetin as a positive control (Fig. 6). From the AFS curve, the regression equation is obtained y= 0.0015x + 0.071, and R^2 = 0.9999 was used as the gradient to measure the antioxidant reduction capacity. Ethyl acetate and methanol extract can reduce ferric ions (FRAP) by 9955 ± 0.022 mol/g and 7926.67 ± 0.034, respectively. It could be associated with the high content of phenols and flavonoids in both extracts.

Correlation analysis between TPC and TFC with antioxidant capacity

The influence of phenolic and flavonoid compounds on the antioxidant activities of *T. macrophylla* extracts were investigated. Analysis of linear correlation coefficient was performed using Spearman's correlation between TPC and TFC and their antioxidant capacity (Fig. 7). A robust category correlation between TPC and antioxidant capacity was found in the FRAP method with r = 0.883, $p<0.01$. These results indicate that the antioxidant capacity of each extract is influenced by the presence of phenolic compounds by 88.3%, whereas other compounds influence only by 11.7%. The correlation coefficient of TPC on antioxidant capacity using the DPPH methods was categorized as moderate (r = - 0.449).

On the other hand, a robust correlation was also given by TFC with antioxidant capacity in the FRAP method with r = 0.895, $p<0.01$. Meanwhile, the correlation between TFC and DPPH methods was categorized as moderate (r = -0.468).

Table 5. Identification of the active extract by LC-MS/MS of twig *T. macrophylla.*

No.	Extract	Observed m/z	Observed RT	Component name	Formula
1	Ethyl acetate extract (EE)	267.10	4.54	6-hydroxy-2-(2-phenylethyl) chromone	$C_{17}H_{14}O_3$
		291.08	2.40	d-catechin	$C_{15}H_{14}O_6$
		317.06	5.25	Isorhamnetin	$C_{16}H_{12}O_7$
		443.09	3.27	Epicatechin gallate	$C_{22}H_{18}O_{10}$
2	Methanol extract (ME)	443.09	3.27	Epicatechin gallate	$C_{22}H_{18}O_{10}$

Identification of active extract by LC-MS/MS

The presence of phenolic compounds contributes to inhibiting free radicals. Extracts from *T. macrophylla* have been found to have potent antioxidants *in vitro*. Spearman correlation analysis showed a strong correlation between the presence of phenolic compounds on the antioxidant capacity. Therefore, the phenolic compounds in the extracts of *T. macrophylla* were analyzed using the LC-MS/MS.

The identification of active extracts of ethyl acetate (EE) revealed the compounds 6-hydroxy-2-(2-phenyl ethyl) chromone (267.10 m/z), catechin (291.08 m/z), epicatechin gallate (443.09 m/z), and isorhamnetin (317.06 m/z). The presence of phenolic compounds in the form of Epicatechin gallate (443.09 m/z) was identified in methanol extracts (Fig. 8). All of these compounds in the twigs of *T. macrophylla* have not been previously reported. Table 5. shows a group of phenolic compounds identified based on LC-MS/MS analysis.

DISCUSSION

Macro-microscopic observation of plants *T. macrophylla* is part of the pharmacognostic evaluation. This is very important for anatomical authentication and early identification (Hanani et al., 2017). Macro-microscopic characteristics are specific parameters of the standardization of raw materials. The number of requests for raw materials for traditional medicines has encouraged the research of specific fragments to establish the authenticity of the plant. Macro-microscopic analysis can also identify adulteration and improper treatment of crude pharmaceuticals (Ragesh et al., 2016).

Phytochemical screening showed that the crude extract contained flavonoids, glycosides, phenols, saponins, steroids, terpenoids, and tannins. The results in Table 3 show that the crude extract shows a dark blue color (addition of $FeCl_3$) and reddish-orange color (Shinoda test) with high color intensity. The high color intensity indicates the presence of high levels of phenolic and flavonoid compounds. TLC results following qualitative analysis of different extracts from *T. macrophylla* twigs. The TLC results of the ethyl acetate extract showed many yellow spots after being sprayed with H_2SO_4. The chromatographic profiles of hexane, ethyl acetate, and methanol extracts showed different phytoconstituents with different Rf values. The Rf value is significant in understanding the degree of polarity of a compound (Hanani et al., 2017; Ragesh et al., 2016).

TPC was determined using the Folin–Ciocalteu method at a wavelength of 750 nm with gallic acid as the standard. The standar curve equation with regression value $y = 0.0087x - 0.1084$ and $R^2 = 0.9976$. The TPC assay involves a reaction with phenolic compounds in an alkaline state and decomposes protons into phenolic ions. The phosphotungstate-phosphomolybdate heteropoly acid is reduced by phenolic ions, indicated by a change in the solution to a blue color (Chen et al., 2015). Meanwhile, TFC was determined with a colorimetric method using $AlCl_3$ and quercetin as the standard with regression equation $y = 0.0053x + 0.0177$ and $R^2 = 0.9999$. TFC assay, $AlCl_3$ binds with the keto group on the C-4 atom and the hydroxyl group on the adjacent C-3 or C-5 atom of the flavone and flavonol groups (Chang et al., 2002). The addition of $AlCl_3$ produces a shift in the wavelength towards the visible marked with a yellow solution and stabilized with sodium acetate. The phenolic and flavonoid are the significant compounds of twig *T. macrophylla* with TPC 580.69 ± 2.63 mgGAE/g extract and TFC 337.367 ± 5.67 mgQE/g extract. Similarly, studies by Roheem et al. (2020) showed that crude extract and ethyl acetate fraction of *T. macrophyla* leaf provided high phenolic content compared to hexane and methanol fraction. Generally, phenolic and flavonoids are the major compounds of the genus *Tetracera*. Ethanol extract of *T. indica* yielded kaempferol, quercetin, wogonin, and norwogonin (Alhassan et al., 2019; Hasan et al., 2017). Meanwhile, ethanol extract of *T. scandens* yielded kaempferol, quercetin, and other known flavonoids (Thanh et al., 2015). 7-O-methylkaempferol, quercetin 3-sulphate, procyanidin were isolated from the stems and leaves of *T. breyniana* (Lima et al., 2014). These compounds are included in the phenolic and flavonoid groups.

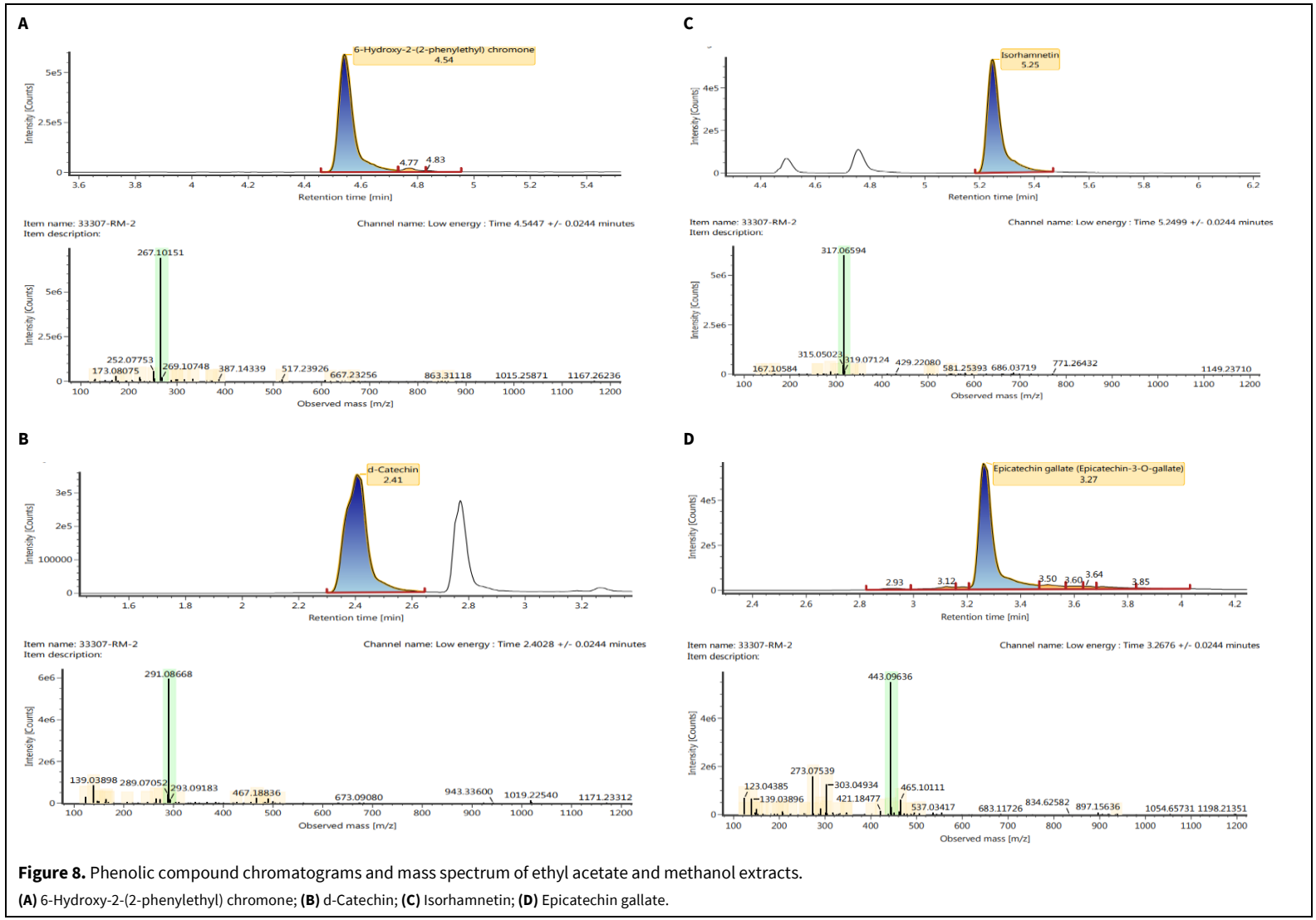

Figure 8. Phenolic compound chromatograms and mass spectrum of ethyl acetate and methanol extracts.

(A) 6-Hydroxy-2-(2-phenylethyl) chromone; **(B)** d-Catechin; **(C)** Isorhamnetin; **(D)** Epicatechin gallate.

The DPPH assay was determined using quercetin as the standard. The methanol extract (ME) showed the highest activity with IC_{50} 13.85 ± 0.065 µg/mL, followed by EE (15.56 ± 0.01 µg/mL), while HE had the lowest activity (168.01 ± 2.52 µg/mL). This research is relevant to data obtained by Roheem et al. (2020), which states that the potential of *T. macrophylla* leaves to reduce DPPH radicals in ethanol extract and ethyl acetate fraction maybe because it contains a relatively high phenolic content. The phenolic compounds have significant antioxidant capacity. Its ability is a reducing agent, hydrogen donor, and singlet oxygen quencher due to its redox characteristics. Due to the delocalization of the spare electrons throughout the entire molecule, the DPPH molecule was more stable and did not dimerize. Antioxidant substances assisted DPPH by donating one electron, decreasing DPPH free radicals (Alam et al., 2013; Chang et al., 2002). The concentration of the test substance at which 50% of free radicals are captured is the IC_{50} and was used to measure the antioxidant capacity.

The FRAP method's fundamental tenet was the reduction of the yellow Fe (III)-tripyridyltriazine (TPTZ) complex to a blue Fe (II)-TPTZ complex through electron transfer from antioxidant chemicals (Munteanu and Apetrei, 2021). Increased blue color concentration generated more Fe^{2+} ions, indicating a better antioxidant capability (Chang et al., 2002; Xiao et al., 2020). In this study, DPPH and FRAP assays were used to observe the radical scavenging activity of compounds. The results from these two methods indicated that the ethyl acetate and methanol extracts from the *T. macrophylla* twigs provided potential antioxidant activity.

Analysis of the linear correlation coefficient was performed using Spearman's correlation between TPC and TFC and their antioxidant capacity (Fig. 7). The antioxidant activity of each extract correlated with the phenolic content (r = 0.883) and flavonoids (r = 0.895) while other compounds affect the rest. This research showed that phenolic compounds and flavonoids are predicted to contribute to their activity as antioxidants.

The LC-MS/MS analysis results describe the differences in the compound content of the n-hexane, ethyl acetate, and methanol extracts from twigs of *T. macrophylla*. The difference in the content of these compounds is described by the chromatogram peaks of compounds with different molecular weights. The results of the LC-MS/MS examination of the ethyl acetate and methanol extracts of *T. macrophylla* indicate catechin compounds, epicatechin gallate, and isorhamnetin, which are well known for their antioxidant effects (He et al., 2018; Pietta, 2000). Catechins are natural polyphenolic compounds –flavan-3-ols (or flavonols) – belonging to the flavonoid groups. The chemical structure of catechins and their diastereoisomers is the same and includes phenolic, which has a hydroxyl group and the ability to stabilize free radicals. Catechins reduce free radicals by donating one electron to the phenolic OH group, while the aromatic group is kept stable by the resonance of the resulting peroxyl radicals. A radical form of the antioxidant is created after interaction with the initial reactive species and is stabilized by charge delocalization caused by the interaction of the phenolic hydroxyl groups with the π-electrons of the benzene ring (Bernatoniene and Kopustinskiene, 2018; He et al., 2018).

CONCLUSION

The pharmacognostic evaluation of *T. macrophylla* revealed the presence of characteristic morphological features of the plant. A microscopic study of the leaf showed the presence of uniseriate trichomes and paracytic stomata on the abaxial epidermis. Powder analysis of twigs showed the presence of irregular starch grains, raphide crystals, and stone cells. It can help check for adulteration and the improper management of crude medication. Ethyl acetate extracts exhibited the highest phenolic and flavonoid contents among all extracts and subsequently exhibited the highest antioxidant. TPC dan TFC in twigs of *T. macrophylla* were robustly correlated with the antioxidant capacity by the FRAP method. In contrast, the DPPH methods moderately correlate to TPC and TFC. This research revealed that *T. macrophylla* prospects for being developed as a natural antioxidant.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors are grateful to The Ministry of Research, Technology, and Higher Education, The Government of Indonesia Republic for Research Foundation via Doctoral Dissertation Research Grant (PDD) No. NKB-885/UN2.RST/HKP.05.00/2022. The authors acknowledge the facilities, scientific, and technical support from Advanced Characterization Laboratories Serpong, National Research and Innovation Institute through E- Layanan Sains, Badan Riset dan Inovasi Nasional (BRIN).

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AUTHOR CONTRIBUTION:

Contribution	Ladeska V	Elya B	Hanafi M	Kusmardi
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x	x	x	x
Experimental studies	x			
Data acquisition	x	x	x	x
Data analysis	x			
Statistical analysis	x			
Manuscript preparation	x	x	x	x
Manuscript editing	x			
Manuscript review	x	x	x	x

Citation Format: Ladeska V, Elya B, Hanafi M, Kusmardi (2023) Pharmacognostic evaluation and antioxidant capacity of *Tetracera macrophylla* Hook. F. & Thoms twigs. J Pharm Pharmacogn Res 11(3): 523–536. https://doi.org/10.56499/jppres23.1613_11.3.523