Vera Ladeska-MACROS-MICROSCOPIC, PHYTOCHEMICAL, AND ANTIOXIDANT STUDY OF TWIG Tetracera macrophylla Hook. F. & Thoms

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Submission date: 30-Dec-2022 09:35AM (UTC+0700)
Submission ID: 1987384797
File name: RJC_TURNITIN_-_Ladeska_Vera.pdf (963.02K)
Word count: 4393
Character count: 23582

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ABSTRACT

Tetracera macrophylla Hook. f. & Thoms is used for traditional medicine, the twig empirically to treat dysentery, diarrhea, diabetes, and tuberculosis symptoms. The plant contains phenolic and flavonoid compounds that have the potential as antioxidants. The study aimed to identify macros-microscopic, phytochemical, total phenolic, and flavonoid content analysis, to find possible sources for future novel antioxidants of twig *Tetracera macrophylla*. Morphological and microscopic identification using chloral hydrate and aqua dest, observed under a light microscope, DPPH and FRAP methods were used to determine antioxidants, and the Folin–Ciocalteu method was used for total phenolic content, total flavonoid content with AlCl3. The microscopic analysis of leaf powder showed the presence of raphide crystals, uniseriate trichomes, and parasitic stomata meanwhile twig powder showed the presence of raphide crystals, xylem fiber, vessel, and tracheids. Preliminary phytochemical analysis of a crude extract of *Tetracera macrophylla* revealed the presence of phytoconstituents like phenols, flavonoids, saponin, glycosides, steroids, and terpenoids. Methanol extract has the highest antioxidant activity with IC50 = 13.86 μg/mL (DPPH) and 7926.67 ± 0.034 mol/g (FRAP), total phenolic content of 418.23 ± 5.757 mg GAE/g extract, and flavonoid content of 305.94 ± 6.360 mg QE/g extract. The ethyl acetate and methanol extract of twig *Tetracera macrophylla* exhibited good antioxidant activity. The primary phenolic compounds in active extracts were discovered using LC-MS/MS data, and they included 6-hydroxy-2-(2-phenylethyl) chromone, d-catechin, isorhamnetin, and epicatechin gallate.

Keywords: Antioxidants, Macro-microscopic, Phytochemical, *Tetracera macrophylla*

INTRODUCTION

Medicinal plants have secretory structures that contribute to metabolite production.[1] Chemical compounds such as phenolic, flavonoids, steroids, and terpenoids can be identified qualitatively with Thin Layer Chromatography (TLC). To find out the specific fragments of the *T.macrophylla* plant, macroscopic and microscopic observations were carried out. However, there has been no evaluation of the phytochemical, morpho-anatomical, and microscopic

of *T.macrophylla* which will provide some valuable markers for identifying raw drugs. This study will help in authenticating the genuine plant material and also help in detecting adulterants.

Tetracera macrophylla Hook. f. & Thoms belongs to the family Dilleniaceae and is a woody climber that widely spread across many Asian countries and some parts of Africa.[2]

The decoction from the stems of *T. macrophylla* is efficacious for treating throat problems.[3,4] The roots and leaves are mixed to treat itching, and the decoction of the roots to treat diarrhea and dysentery.[5,6,7] *T. macrophylla* is rich in phenol and flavonoid content so it has good radical scavenging activity.[8]

Antioxidants are compounds that can reduce free radical attacks. Many studies have shown that antioxidant compounds can reduce the risk of chronic diseases such as diabetes, hypertension, cancer, and coronary heart disease.[9,10]

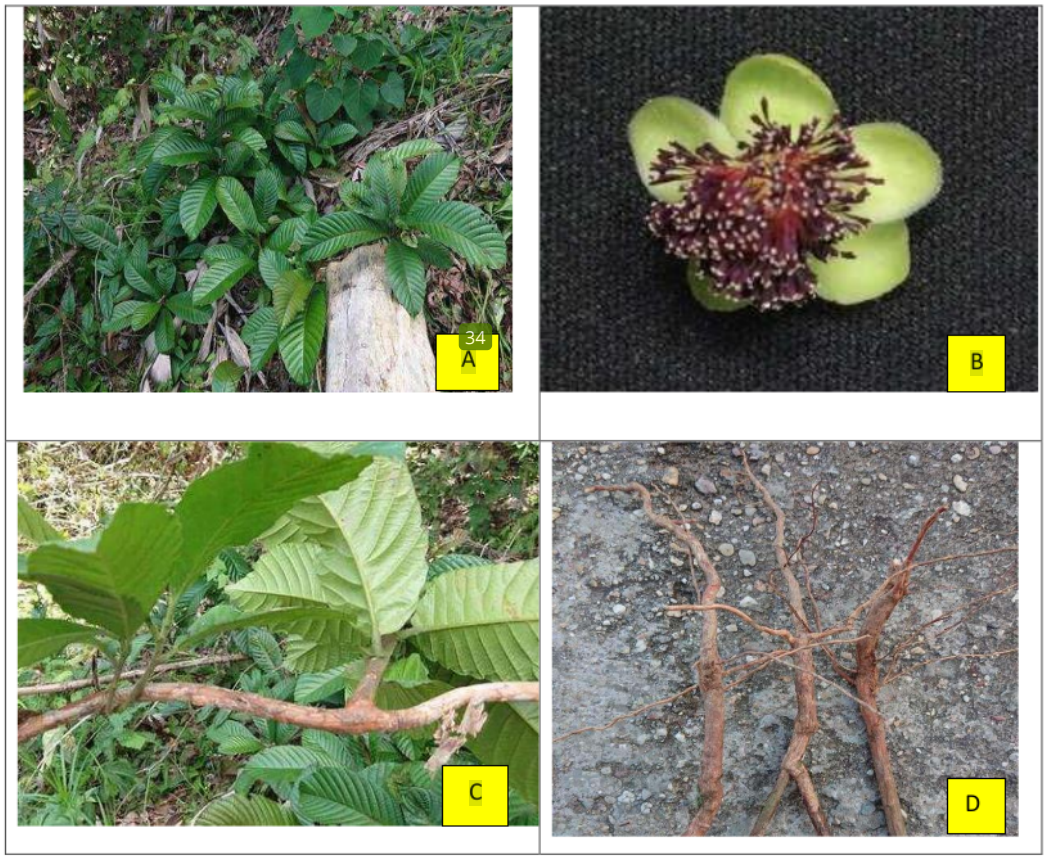

Fig 1. A. Leaves, B. Flowers, C. Twig with leaves, D. Roots of *Tetracera macrophylla*

EXPERIMENTAL

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 authenticated by the plant taxonomist of the Biology Research Center, Indonesian National Research and Innovation Institute, Cibinong, Indonesia with collection number B-4/V/DI.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 study

Morphological studies leaf and twigs were carried out by observing with the naked eye. The macroscopic and organoleptic characteristics like size, shape, texture, color, surface, odor, and taste of leaf and twig were observed. Microscopic of transverse sections of fresh twig, lamina, midrib, and powder was performed. A small number of leaves and twig powder were placed on slides and mounted in 2-3 drops of chloral hydrate or aqua dest, each slide was covered with a cover slip and then examined under a microscope. Different cell components were noted and photographs were taken using a microscope.[10,29]

Extraction

Twigs of *T. macrophylla* were extracted by graded maceration using n-hexane, ethyl acetate, and methanol. The solvent was evaporated with a rotary vacuum evaporator at 50^{0}C 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^{0}C before being used for phytochemical screening, Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant assay.

Phytochemical Analysis

Phytochemical screening was carried out by dissolving 20 g of twig powder with methanol. Phytoconstituents were analyzed by color reactions and precipitating reagents such as alkaloid test (Mayer, Bouchardat, and Dragendorff reagents), flavonoids (metal Mg + HCl 5 M), glycosides (Fehling solution), phenols (FeCl3 test), saponins (shaken with warm water), steroids and

terpenoids (Liebermann Burchard test) and tannins (gelatin + NaCl).[11,12]The intensity of the color formed is observed.

Chromatographic profile

Phytochemical analysis of graded extracts of *T. macrophylla* twigs using thin layer chromatography (TLC) method. Silica gel 60 F 254 is used as the stationary phase. The plates were developed in a chamber using different solvent systems. The mobile phase for HE and EE is hexane-ethyl acetate (1:1), and the solvent system for ME is ethyl acetate-methanol (9:1). The plate was dried and sprayed with H2SO4 10%. The formed spot was observed under UV 254 and visible light 366 nm.[12,14,30]

The retention factor (Rf) value was determined by the formula:

$$Rf = \frac{Distance\ moved\ by\ the\ solute}{Distance\ moved\ by\ the\ solvent\ front}$$

Total Phenolic Content (TPC) Assay

In a 96-well microplate, were added 100 μl of the Folin-Ciocalteau (1:10) solution and 20 μl of the sample (n-hexane, ethyl acetate, and methanol extract of *T. macrophylla* twig) and shaken for 60 seconds. Then, this was incubated at room temperature for 4 minutes. Following the addition of 80 μL of 7.5 % Na2CO3 solution, the mixture was agitated and incubated for 2 hours in the dark. Using a microplate reader, the sample solution's absorbance was calculated at a maximum wavelength of 750 nm. Based on the regression equation for gallic acid, the phenolic content was calculated.[15,28]

Total Flavonoid Content (TFC) Assay

An amount of 20 μl of a sample (n-hexane, ethyl acetate, and methanol extract of twig *T. macrophylla*) was added to 20 μL of 10% AlCl3 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 seconds and incubated for 30 minutes. Using a microplate reader, an absorbance measurement of the solution color intensity was made at a wavelength of 415 nm. Based on the quercetin regression equation, total flavonoid levels were determined.[15,16]

Antioxidant Assay with DPPH Method

The DPPH free radical scavenging activities of the tested extracts were determined based on a protocol modified from Molyneux.[17,19] The sample concentration was made at 1000 μg/mL in methanol. From the stock solution, sample concentration series of 40, 60, 80, 100, and 120 μg/mL were made, and the assay was sonicated for 2 minutes. 3 mL of standard DPPH solution were pipetted into 1 mL sample for each concentration. It was then homogenized and incubated for 30 minutes in the dark. The absorption was measured at a wavelength of 516 nm with a UV-Vis spectrophotometer. The active control employed was quercetin. IC_{50} values were calculated based on the presentation of the inhibition of DPPH radicals from each concentration of the sample solution with the formula: [17]

$$\% \text{ inhibition} = \frac{Absorbance\ sample - Absorbance\ control}{Absorbance\ control}\ x\ 100$$

The value of IC50 was the concentration at which the sample reduced DPPH by 50% using the linear regression equation y = a + bx.

Antioxidant Assay with FRAP Method

The FRAP radical method was modified slightly to estimate the antioxidant effect of the samples. The sample concentration was prepared at 1000 μg/mL in methanol, then diluted to 500 μg/mL. 270 μl of FRAP reagent were pipetted with 30 μl of sample solution added. This was homogenized for about 60 seconds and then incubated at 37°C in the dark for 30 minutes. A wavelength of 595 nm was used to measure the absorbance. Triplo was used for the tests. 300 μl of methanol was used to create plate blanks. The active control employed was quercetin. Antioxidant activity was calculated based on the ferric iron equivalent antioxidant activity (FeEAC) with the following formula.[17,18]

$$FeEAC = \frac{\triangle A}{GRAD} x \frac{Av}{Spv} x\ D\ x\ \frac{1}{Cext}\ x\ 10^5$$

Identification of compounds by LC-MS/MS

Qualitative analysis of the compounds contained in the active extract was carried out using a Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). Measurement

conditions is a Waters Acquity UPLC I-Class equipped with XEVO G2-XS QTof mass spectrometer. Separation of samples using column type ACQUITY UPLC® BEH C18 (1.7 μm x 2.1 mm x 50 mm) with injection volume 1 μl and full scan m/z 100 – 1200 (mode ESI). Mobile phase used solvent A (H2O + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). Utilizing the instrument application's spectrum database of organic chemicals, mass fragmentation of compounds was detected.

Statistically Analysis

Tests are carried out in triples and presented as mean ± SD. One-way analysis of variance (ANOVA $p<0.05$) was used by SPSS 23 version to assess the significant difference between mean values followed by Tukey test.

RESULTS AND DISCUSSION

Macroscopic and organoleptic characterization

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

Flower – *T. macrophylla* is a complete flower that has petals, sepals, reproduction (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 that have 2 or 3 carpellums that are oval in shape and have a snout with a smooth surface.

Twig – The stem of *T. macrophylla* belongs to the lignosus, brown color, the growth of the stem is climbing, and the diameter of the stem is approximately 1 cm.

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

Table 1. Organoleptic characters of *Tetracera macrophylla*

Organoleptic characters				
	Leaf	Twig	Root	Flower
Surface	Rough	Scabrid, lignosus	Smooth	Snout with a smooth surface.
Colour	Dark green (upper), Light green (lower)	Dark brown,	Light brown	Maroon
Odour	No characteristic odour	No characteristic odour	No characteristic odour	No characteristic odour
Taste	No characteristic odour	Slightly bitter	Bitter	No characteristic odour

Table. 2 Macroscopic characters Leaf of *Tetracera macrophylla*

Macroscopic Observation	
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.

Microscopic characterization of T. macrophylla

Leaf of *Tetracera macrophylla*. There are 2 parts of the leaf observed, namely the lamina and midrib. The lamina of the adaxial epidermis lacks stomata. A tightly packed palisade network lies underneath 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. 5-6 layers of parenchyma cortex follow 2-3 layers of collenchyma hypodermis (Fig 3). Cortex also contains tightly packed parenchyma with raphide

crystals, which are sharp (needle-shaped calcium oxalate crystals). Figure 2 shows microscopic images of *T. macrophylla* leaf powder.

Twig *of Tetracera macrophylla.* A microscopic cross-section of the fresh twig of *T. macrophylla* can be seen in Figure 4. There are irregular starch grains, fibers, needle-shaped Ca oxalate crystals (raphide crystals), stone cells, multicellular trichomes, phloem, and xylem towards the center.

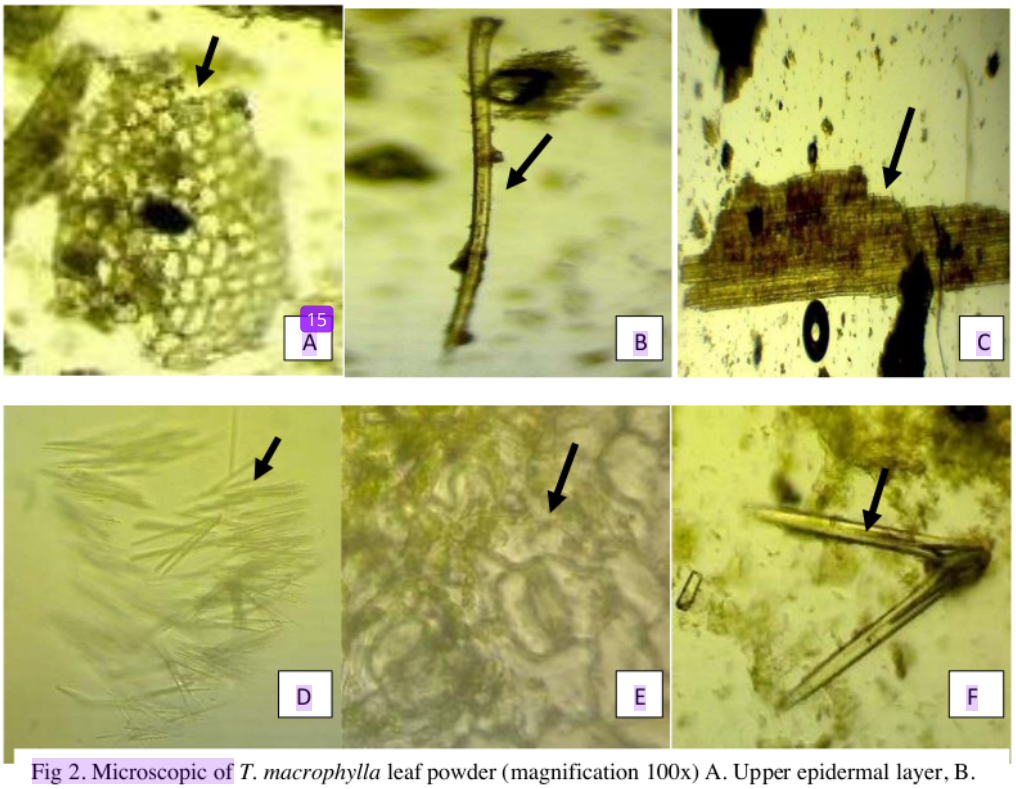

Fig 2. Microscopic of *T. macrophylla* leaf powder (magnification 100x) A. Upper epidermal layer, B. Fiber , C. Longitudinal section of midrib D. Crystalline Ca oxalate (crystal raphide), E. Paracytic Stomata, F. Trichome

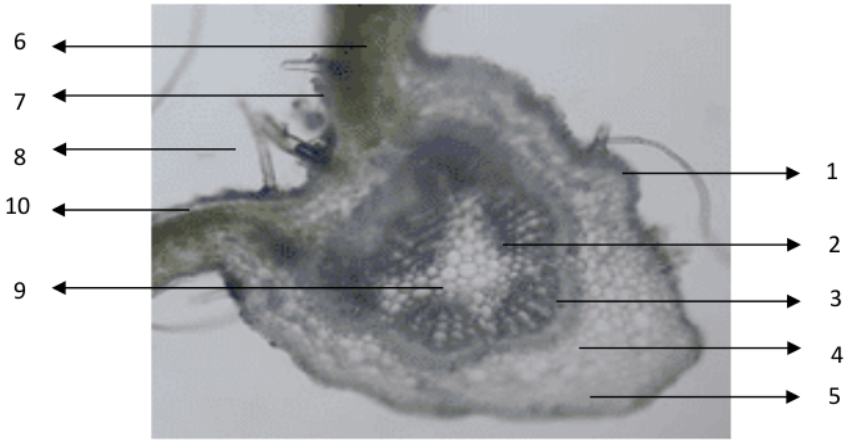

Fig 3. Transverse section of the midrib. 1. Lower epidermal 2. Xylem 3. Phloem 4. Parenchyma 5. Collenchyma 6. Sponge 7. Palisade cells 8. Uniseriate Trichomes 9. Parenchyma 10. Upper epidermis

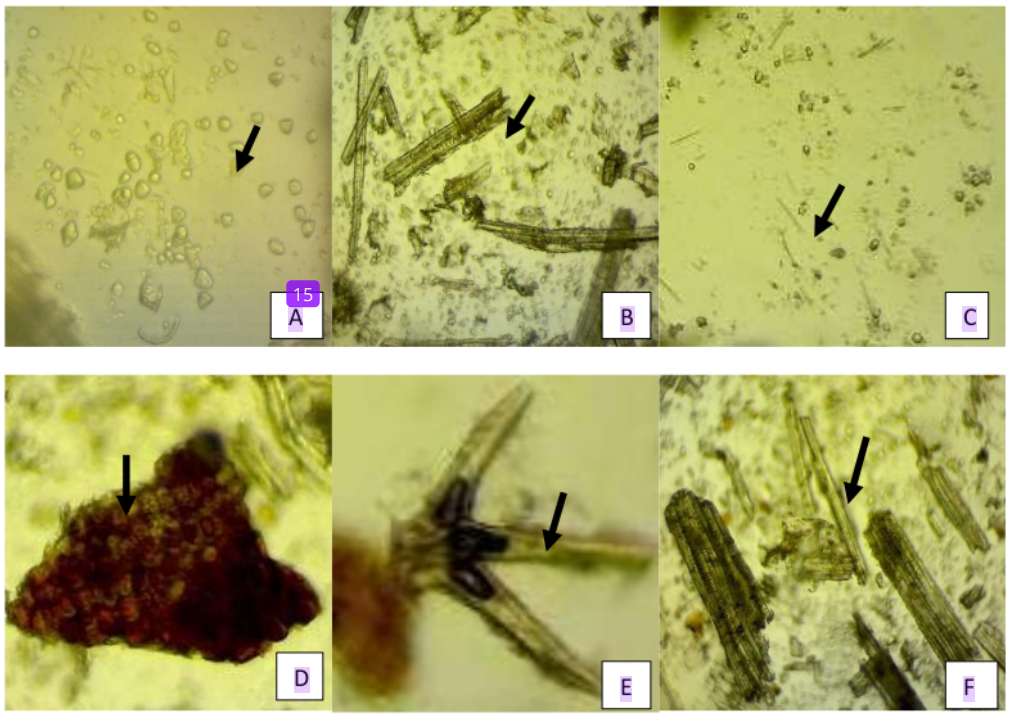

Fig 4. Microscopic of *T. macrophylla* twig powder (magnification 100x). A. Starch (magnification 400x), B. Fiber C. Crystalline Ca oxalate D. Stone cells E. Trichome, F. Xylem fiber

Phytochemical Analysis

The extract tested positive for flavonoids, phenols, saponins, tannins, glycosides, steroids, and terpenoids after undergoing phytochemical analysis (Table 3). The presence of metabolites in crude medicine has a significant impact on its pharmacological effect.

Table 3. Phytochemical screening of material plant extracts of Twig *Tetracera macrophylla*

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

Note: (+) = Positive result

Chromatographic evaluation of different extracts of twig Tetracera macrophylla

The thin layer chromatographic profile of the hexane extract (HE) of *T. macrophylla* revealed the presence of 4 spots in the hexane: ethyl (1:1) solvent system and revealed the presence of 10 spots on the ethyl acetate extract (EE). *T. macrophylla* extract in methanol underwent thin layer chromatographic examination, which revealed the presence of 2 spots in the ethyl-methanol (9:1) solvent system (Table 4).

Table 4. Chromatographic evaluation of different extracts of Twig *Tetracera macrophylla*

Extract	Mobile phase	Amount of spots	Visual	Spraying reagent H2SO4 10%	Rf (UV 366 nm)
n-Hexane	Hex: Ethyl (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: Ethyl (1:1)	10	1. Green	1. Pink	0,11
			2. -	2. Yellowish	0,14
			3. -	3. Green	0,21
			4. -	4. Pink	0,26
			Yellow	5. Green	0,38
			6. Yellow	6. Green	0,42
			7. -	7. Pink	0,48

				8. - 9. Yellow 10 Yellow	8. Yellowish 9. Reddish 10. Gray	0,59 0,65 0,68
Methanol	Ethyl: (9:1)	MeOH	2	1. Light blue 2. Light blue	1. - 2. -	0,15 0,21

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 – 01084. TFC was determined via a colorimetric method using $AlCl_3$ and quercetin as the standard with regression equation y = 0,0053x + 0,0177. Value of TPC and TFC as shown in Figure 5.

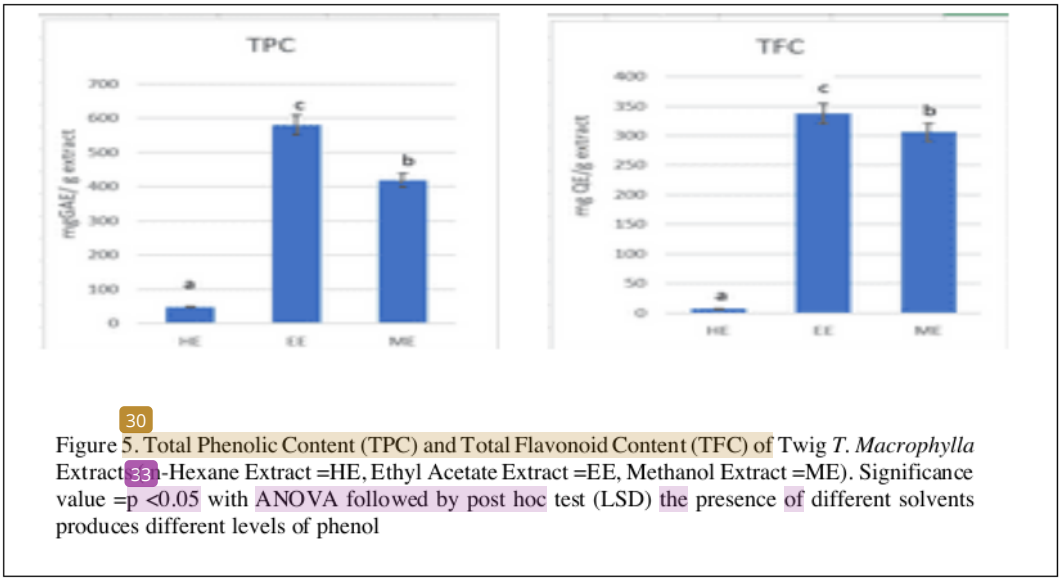

Figure 5. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of Twig *T. Macrophylla* Extract (-Hexane Extract =HE, Ethyl Acetate Extract =EE, Methanol Extract =ME). Significance value =p <0.05 with ANOVA followed by post hoc test (LSD) the presence of different solvents produces different levels of phenol

Antioxidant Assay with DPPH and FRAP Method

The DPPH assay was determined using quercetin as the standard. The n-hexane extract of *T. macrophylla* twigs was shown to be weak antioxidant, whereas the ethyl acetate and methanol extracts had substantial antioxidant effects (Figure 6).

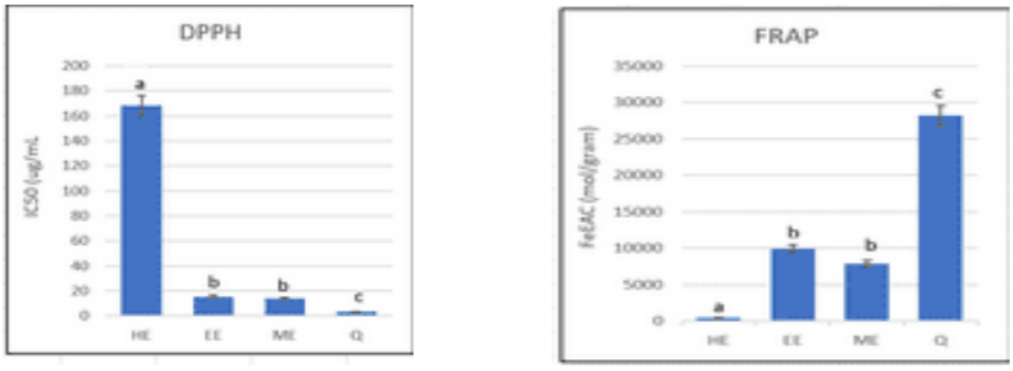

Fig 6. Antioxidant Activity of *T. Macrophylla* Twig by DPPH and FRAP Method (N-Hexane Extract =HE, Ethyl Acetate Extract =EE, Methanol Extract =ME, Q = Quercetin). The potential antioxidant activities gave significantly different effects ($p<0.05$, post hoc test, LSD) among each extract of different solvent)

Using a microplate reader, the FRAP assay was performed using an AFS (ammonium ferrous sulfate) standard solution and quercetin as a positive control. The slope of the linear regression obtained from the AFS standard, 0.0015, was used as the gradient to measure the antioxidant reduction capacity.

Identification of active extract by LC-MS/MS

The presence of phenolic compounds contributes to inhibiting free radicals. Identification of active extracts of ethyl acetate and methanol by LC-MS/MS revealed the compounds 6-hydroxy-2-(2-phenylethyl) chromone, catechin (291.08 m/z), epicatechin gallate (443.09 m/z), isorhamnetin (317.06 m/z). z) on ethyl acetate extract and methanol extract of *T. macrophylla* twigs (Figure 7). The finding of this phenolic compound in the twigs of *T. macrophylla* has not been previously reported.

Table 5. Results 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	267.10	4.54	6-hydroxy-2-(2-phenylethyl) chromone	C17H14O3
2	Ethyl acetate extract	291.08	2.40	d-catechin	C15H14O6

3	Ethyl acetate extract	317.06	5.25	Isorhamnetin	C16H12O7
4	Methanol extract	443.09	3.27	Epicatechin gallate	C22H18O10

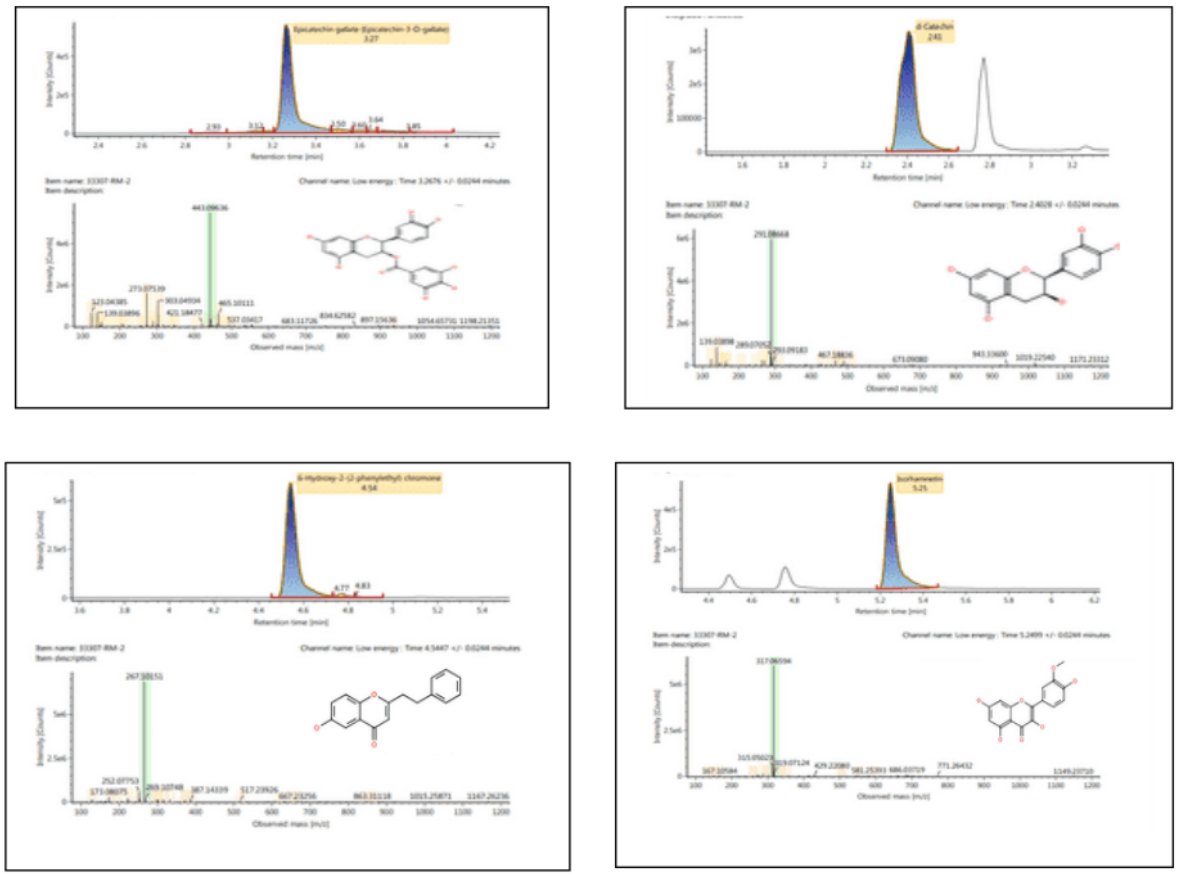

Fig 7. Phenolic compounds chromatograms and mass spectrum of ethyl acetate and methanol extract of twig *Tetracera macrophylla*

Discussion

Macro-microscopic evaluation of the plant *T. macrophylla* is important for anatomical authentication and early identification of the plant.[20] The number of requests for raw materials for traditional medicines has encouraged the research of specific fragments as the identity of the authenticity of the plant. In order to identify adulteration and improper treatment of crude

pharmaceuticals, macro-microscopic analysis can be useful.[20,21] Numerous significant classes of phytoconstituents, including flavonoids, glycosides, phenols, saponins, steroids, terpenoids, and tannins, were detected during the phytochemical screening and may have an impact on the pharmacological activities of the plants.[20]

To determine the initial chemical compound content of plants, chemo profiling, and marker compound analyses were all done.[20] Numerous significant phytoconstituents, including flavonoids, glycosides, phenols, saponins, steroids, terpenoids, and tannins, were detected during the phytochemical screening and may have an impact on the pharmacology of plants.[20] Phenolic and flavon compounds in *T. macrophylla* have a major role in antioxidant activity. Chromatographic profiles of n-hexane extract, ethyl acetate, and methanol extract showed different amounts of phytoconstituents present in plants with different Rf values. The value of Rf is important in understanding the degree of polarity.[20,21]

TPC assay using the Folin-Ciocalteu method which reacts with phenolic compounds in an alkaline state and decomposes protons into phenolic ions. The phosphotungstate-phosphomolybdate heteropoly acid is reduced by phenolic ion, which is indicated by a change in the solution to a blue color.[22] In TFC, $AlCl_3$ 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.[23]

Because of their ability as reducing agents, hydrogen donors, and singlet oxygen quenchers due to their redox characteristics, phenolics compounds have significant antioxidant activity. Due to the spare electrons being delocalized throughout the entire molecule, the DPPH molecule was more stable and did not dimerization. Antioxidant substances assisted DPPH in this process by donating one electron, resulting in a decrease in DPPH free radicals.[23,24] The concentration of the test substance at which 50% of free radicals were captured was known as the IC50, which was used to measure the antioxidant power.

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 the use of electron transfer from antioxidant chemicals. More Fe^{2+} ions were generated with increased blue color concentration, indicating a better antioxidant capability.[23,25] In this study, DPPH and FRAP assays were used to observe the radical scavenging activity of compounds. The results obtained from these two methods indicated that the ethyl acetate and methanol extracts from the twigs of *T. macrophylla* provided potential antioxidant activity.

The results of the LC-MS/MS analysis can describe the differences in the compound content of the extracts of n-hexane, ethyl acetate, and methanol 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* present catechin compounds, epicatechin gallate, and isorhamnetin which are well known for their antioxidant effects (Table 5).[25,26,32] Catechins are natural polyphenolic compounds—flavan-3-ols (or flavanols), belonging to the flavonoid groups. The chemical structure of catechins and their diastereoisomers is the same, and this 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, and 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 brought on by the interaction of the phenolic hydroxyl groups with the π-electrons of the benzene ring.[26,27]

CONCLUSION

The crucial first step in locating the source of the crude medicine was to identify the macro and microscopic features of this plant. The phytoconstituents and physicochemical parameters that were established can be useful in spotting adulteration and improper management of crude medication. The results of this study prove that *T. macrophylla* extracts in ethyl acetate and methanol showed strong antioxidant activity.

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