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Research Article

Antioxidants, Total Phenolic and Flavonoid Content and Toxicity Assay of Ampelas (*Tetracera macrophylla* Wall.Ex Hook.F.& Thoms) From Kalimantan-Indonesia

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ABSTRACT

Background: High Reactive Oxygen Species (ROS) contribute to disease pathogenesis. Phenolic compounds and flavonoids are effective as antioxidants. Objective: This research aimed to measure the antioxidant activity, total phenolic and flavonoid content and leaf toxicity of *Tetracera macrophylla*. Methods: DPPH and FRAP were used to determine antioxidants, and the Folin–Ciocalteu method was used for total phenolic content, total flavonoid content with AlCl3 and toxicity with MTT assay against RAW 264.7 cells. Results: Methanol extract has antioxidant activity with IC50 = 81.582 µg/mL (DPPH) and 11840 mol/g (FRAP), total phenolic content of 353.781 mg GAE/g dry weight, and flavonoid content of 279.2 mg QE/g dry weight. The ethyl acetate and n-hexane extracts had weaker antioxidant activity than the methanol extracts. The IC50 toxicity assay methanol extract and ethyl acetate extract respectively showed 288.792 µg/mL and 541.472 µg/mL. Conclusion: The methanol extract of *Tetracera macrophylla* showed the highest yield, total phenolic content and total flavonoid content and had the highest antioxidant activity. Methanol extract has low toxicity to RAW 264.7 cells.
Key words: Antioxidants, Total phenolic, Total flavonoid, Toxicity, Tetracera macrophylla.

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INTRODUCTION

Balancing the level of free radicals with antioxidants in the body is a critical physiological function. A rise in the level of free radicals will trigger oxidative stress. Free radicals will injure cells or tissues, stimulating organ damage. Organ damage can subsequently induce chronic diseases such as diabetes, heart disease and gout. The administration of antioxidants will reduce the negative effects of free radicals on body tissues. Flavonoids and phenolic compounds are natural antioxidants contained in plants. Both are secondary metabolites with the ability to scavenge free radicals and inhibit lipid oxidation.

Indonesia has the second highest volume of natural resources in the world. One such resource with the potential to be developed is *Tetracera macrophylla*, which grows widely in Barito Utara, Kalimantan.[1] It belongs to the Dilleniaceae family and has synonyms in Indonesia of akar tembara and ampelas. In an ethnomedicinal method in Malaysia, a decocta of the stems of the plant was used to treat fatigue, while stembark was used to treat TB symptoms.[2] A decocta from the roots has also been used to treat diarrhoea and dysentery.[3] In West Nigeria, an infusion of fresh leaves was used to treat chronic diabetes. In previous research, *Tetracera macrophylla* with DPPH and ABTS methods has been shown to display the highest antioxidant activity.[4]

An ethyl acetate fraction of the ethanolic extract of *Tetracera macrophylla* leaves was found to contain 5, 7-dihydroxy-8-methoxy flavone (wogonin), betulinic acid, kaempferol, quercetin and norwogonin compounds.[4] Ethanol extract from the leaves of *Tetracera macrophylla* contains phenolic and flavonoid compounds. Based on traditional usage and its chemical content, this plant has the potential to be used as an antioxidant.[5] However, since no research has reported on the toxicity of this plant, it is important to determine its safety. The purpose of this study was therefore to determine the antioxidant potential of *Tetracera macrophylla* and analyse the total phenolic content (TPC) and total flavonoid content (TFC).

MATERIALS AND METHODS

Reagents

RAW 264.7 cells (ATCC), Fetal Bovine Serum (FBS) (Biosera), Penicillin-Streptomycin (Sigma, Jerman), 3-(4,5-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) (Bio Basic), Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Jerman), methanol, n-hexane, ethyl acetate, aquadest, aluminium chloride, ferric chloride hexahydrate, hydrochloride acid, dimethyl sulfoxide (DMSO), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma, Jerman), DPPH (Merck, Jerman), Quercetin, *Folin-Ciolcalteu*, Gallic acid (Sigma Aldrich, Jerman), TLC Silica gel 60 F 254 (Merck, Jerman).

Plant materials

Tetracera macrophylla leaf was the part of the plant used, which was obtained from the protected forest of Teweh Baru District, North Barito Regency, Central Kalimantan. This plant was determined at the National Research and Innovation Agency (BRIN) with collection number B-4/V/DI.05.07/11/2021.

Extraction

300 grams of *Tetracera macrophylla* leaves were extracted *via* the 3-times maceration method using

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n-hexane (3 litres), which was then stirred and allowed to stand for 24 hours. The solution was filtered and ethyl acetate and methanol were added to the residue, respectively. All extracts were evaporated with a rotary vacuum evaporator at 50°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°C before use.

Phytochemical screening

Phytochemical screening was carried out according to the Thin Layer Chromatography method using silica gel GF 254 as a stationary phase with a mobile phase for flavonoid: n-hexane-ethyl acetate-methanol (5:2:1) with $AlCl_3$ 10% spray reagent. A mobile phase was also used for phenolic: n-hexane-ethyl acetate (7:3) with $FeCl_3$ 5% spray reagent; and terpenoids/steroids: n-hexane-ethyl acetate (8:2) with spray reagent: Liebermann Burchard.[6]

Total Phenolic Content (TPC) assay

An amount of 20 µl of sample was added to 100 µl Folin–Ciocalteau (1:10) solution and shaken for 60 seconds in a 96-well microplate. This was then incubated for 4 minutes at room temperature. 80 µL of 7.5% Na_2CO_3 solution was then added, the solution was shaken and incubated for 2 hours in the dark. The absorbance of the sample solution was measured with a microplate reader at a maximum wavelength of 750 nm. The phenolic content was measured based on the regression equation of gallic acid.[7]

Total Flavonoid Content (TFC) assay

An amount of 20 µl of sample was added to 20 µL of aluminium chloride 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. The colour intensity of the solution was read for absorbance using a microplate reader at a wavelength of 415 nm. Total flavonoid levels were measured based on the regression equation of quercetin.[8]

Antioxidant assay with DPPH method

The DPPH free radical scavenging activities of the tested extracts were determined based on a protocol modified from Molyneux.[9] A total of 0,1 mM DPPH solution in absolute methanol was available. The sample concentration was made 1000 ppm in methanol. Concentration series of 40, 60, 80, 100 and 120 µg/mL were made from stock solution and the assay was sonicated for 2 minutes. For each concentration, 1 mL was pipetted, to which 3 mL of standard DPPH solution was added. It was then shaken until homogeneous and incubated for 30 minutes in a dark condition. The absorption was measured at a wavelength of 516 nm with a UV-Vis spectrophotometer. Quercetin was used as the positive control. 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:

% inhibition = (Absorbance sample/Absorbance control) x 100%

The value of IC_{50} 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[10,11] was modified slightly to estimate the antioxidant effect of the samples. The sample concentration was prepared at 1000 ug/mL in methanol, then diluted to 500 ug/mL. 30 µl of sample solution was pipetted and added to 270 µl of FRAP reagent. This was homogenised for ± 60 seconds and incubated for 30 minutes at 37°C in a dark condition. The absorbance was read at a wavelength of 595 nm. Tests were carried out in triplo. The blanks were prepared in the same way, with the same volume of sample replaced with methanol absolute. Plate blanks were made with 300 µl methanol. Quercetin was used as the positive control. Antioxidant activity was calculated based on the ferric iron equivalent antioxidant activity (FeEAC) with the following formula:

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

FeEAC: Equivalence between antioxidant activity (µmol/g) and ferric ion

A: Absorbance of samples that have been reduced by blank

GRAD: Gradient determined from the calibration curve on AFS

Av: Total volume for the test

Spv: Total sample volume in the test

D: Sample dilution factor

Cext: Sample stock concentration (g/L)

Toxicity assay on RAW 264.7 cells

RAW 264.7 cells were added to a 96-well microplate at a density of 5 x 10^3 cells/well and allowed to adhere for 24 hours at 37°C in a 5% CO_2 incubator. After 24 hours of incubation, the culture medium was replaced with fresh medium. The cells were then treated with various concentrations of the sample: 15.625 µg/mL, 31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 1000 µg/mL. Cells were incubated for 24 hours at 37°C in a 5% CO_2 incubator. After 24 hours, the medium was removed and the cells were washed with PBS. A total of 100 µL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 4 hours at 37°C in a CO_2 incubator. The formazan crystals that formed were then dissolved in 100 µL DMSO. Absorption was measured using a microplate reader at a wavelength of 570 nm.[12,13]

RESULTS

Phytochemical screening

The content of the secondary metabolites in the extract was further identified; this included flavonoids, polyphenol, triterpenoids and steroids. The three extracts of HE, EE, and ME contained phenolic and flavonoid compounds (Table 1, Figure 1).

DPPH assay

The DPPH assay was determined using quercetin as the standard (Figure 2). *Tetracera macrophylla* leaf n-hexane extract was found to be a weak antioxidant, while the ethyl and methanol extracts had strong antioxidant properties (Table 2).

FRAP assay

The FRAP assay was carried out using a microplate reader with a standard solution of AFS (ammonium ferrous sulfate) and positive control of quercetin. Figure 3 displays the AFS standard curve and

Table 1: Phytochemical screening of the extracts.

Phytochemical constituents	HE	EE	ME
Flavonoid	+	+	+
Polyphenol	+	+	+
Triterpenoids	+	-	-
Steroid	+	-	-

Note: Absent = -
Present = +

Table 3 shows the ability to reduce Fe^{3+} to Fe^{2+} from the *Tetracera macrophylla* extract. The slope of the linear regression obtained from the AFS standard, 0.0015, was used as the gradient to measure the antioxidant reduction capacity.

Table 2: Antioxidant activity of *T. Macrophylla* leaves by DPPH method.

Sample	Equation	R^2	IC_{50} (µg/mL)
n-Hexane Extract (HE)	Y= 0.1523x – 0.624	0.9799	**332.39**
Ethyl Acetate Extract (EE)	y = 0.3639x + 10.186	0.9907	109.409
Methanol Extract (ME)	y = 0.4189x + 15.825	0.9976	81.582
Quercetin	y = 5.7569x + 31.831	0.9929	3.15

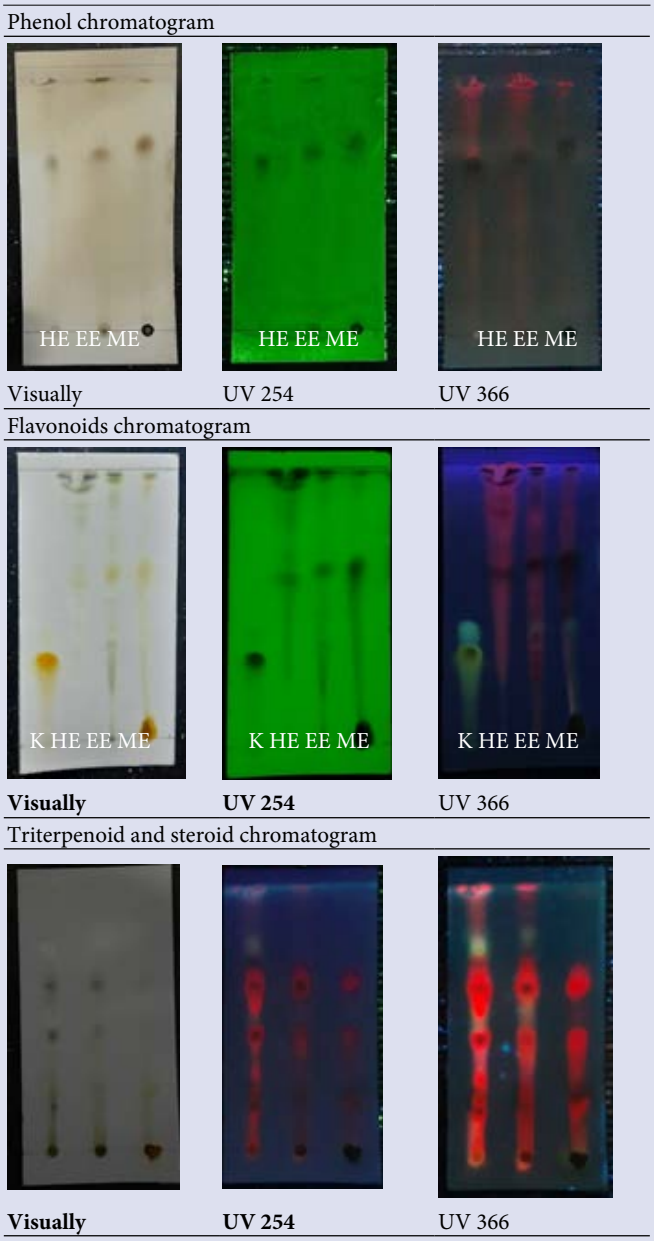

Figure 1: TLC results of n-hexane extract: ethyl acetate extract: methanol extract.

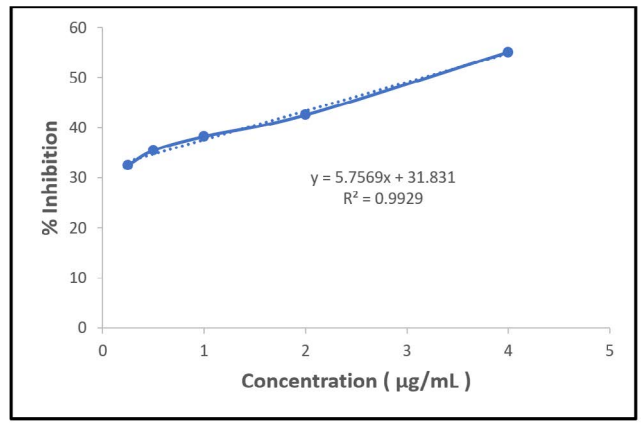

Figure 2: Curve of % inhibition and quercetin concentration.

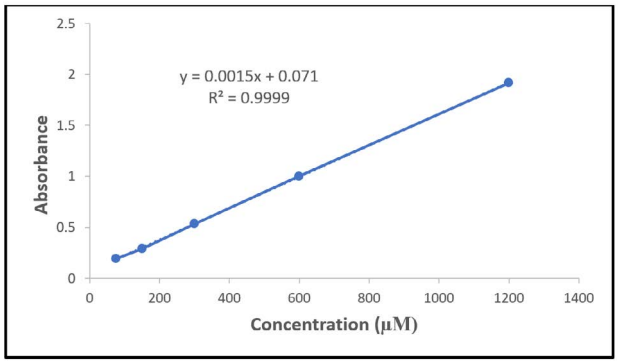

Figure 3: Calibration curve of AFS.

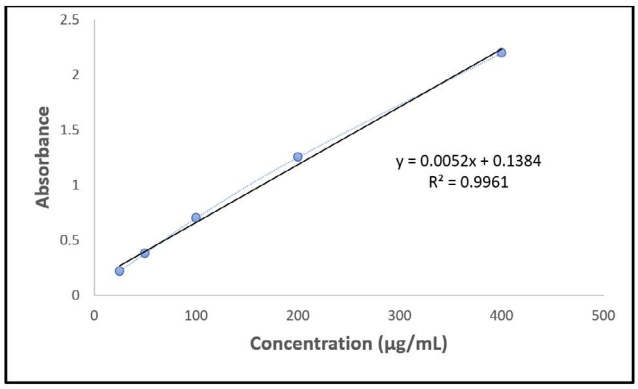

Figure 4: Calibration curve of gallic acid.

Table 3: Antioxidant activity of *T. Macrophylla* leaves by FRAP method.

Sample	FeEAC (mol/gram)
n-Hexane Extract (HE)	457.12 ± 0.019
Ethyl Acetate Extract (EE)	3823.33 ± 0.085
Methanol Extract (ME)	11840 ± 0.117
Quercetin	28200 ± 0.014

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 (Figure 4). TFC

was determined *via* a colorimetric method using $AlCl_3$ and quercetin as the standard (Figure 5). Table 4 contains the TPC and TFC results. The TPC and TFC for HE was not detected due to low absorbance.

Toxicity assay on RAW 264.7 cells

The toxicity assay was determined in triplo with six variations of concentration: 15.625; 31.25; 62.5; 125; 125; 250 and 1000 µg/ml. The sample IC_{50} value for RAW 264.7 cells can be seen in Table 5. The concentration curve for the extracts and inhibition of RAW 264.7 cell proliferation are shown in Figures 6 and 7. The formazan crystals can be dissolved with dimethyl sulfoxide (DMSO) to give a purple colour with characteristic absorption at 570 nm. The intensity of the purple colour is directly proportional to the cell number, thus indicating cell viability (Figure 8).

Table 4: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of *T. Macrophylla* extracts.

Sample	TPC (mg GAE/g Dry Weight) ± SD	TFC (mg QE/g Dry Weight) ± SD
Ethyl Acetate Extract (EE)	148.33 ± 17.474	104.98 ± 7.525
Methanol Extract (ME)	353.781 ± 5.796	279.2 ± 6.814

Table 5: IC_{50} value against RAW 264.7 cell.

y = bx ± c					
Sample	b	c	y	x/IC_{50}	IC_{50}
Ethyl Acetate Extract (EE)	51.528	-76.789	50	2.460585	288.792 µg/mL
Methanol Extract (ME)	37.583	-52.736	50	2.733576	541.472 µg/mL

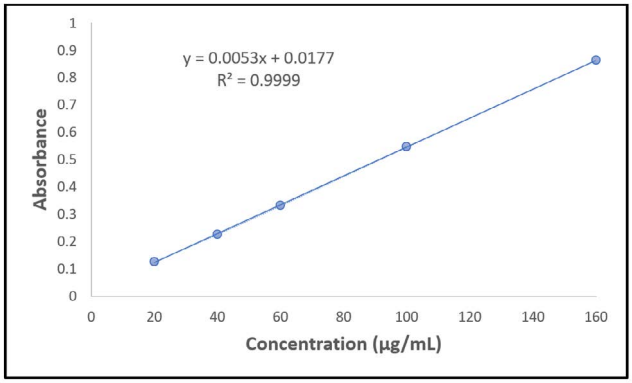

Figure 5: Calibration curve of quercetin .

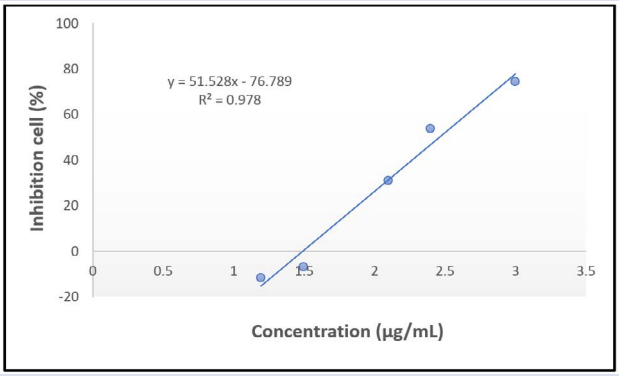

Figure 6: Curve of concentration of ethyl acetate extract and inhibition of RAW 264.7 cell proliferation.

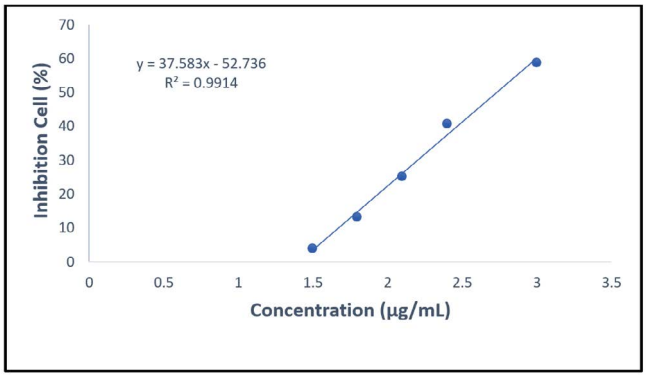

Figure 7: Curve of concentration of methanol extract and inhibition of RAW 264.7 cell proliferation.

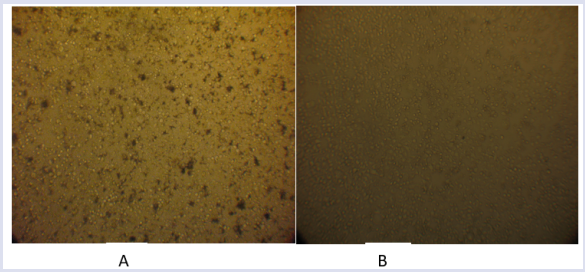

Figure 8: Appearance of raw cells after exposure to MTT.
A. EE at a concentration of 1000 µg/mL
B. ME at a concentration of 1000 µg/mL

DISCUSSION

Phytochemical screening showed that all of the extracts – HE, EE and ME – of *Tetracera macrophylla* contained flavonoid and polyphenol. Flavonoids and polyphenol can donate hydrogen and act as antioxidants. The determination of phenolic content in n-hexane extract did not produce absorbance data that met the standard curve. This was due to the presence of only minor amounts of phenolic compounds in the n-hexane extract so that they were not detected on the microplate reader. The high phenolic content resulted from a more polar solvent, namely methanol.

The determination of total flavonoid levels resulted in the formation of a stable complex between aluminium chloride and a keto group at the C-4 atom and a hydroxy group at the C-3 or C-5 atom in flavonols and flavones.[14] The formation of the complex was accompanied by a shift in the wavelength towards the visible, which was marked by a yellow solution. Potassium acetate was added to ionise the 3 and 4´-OH groups that were not complexed with the Al^{3+} and 7-hydroxyl groups so that they could maintain wavelengths in the visible region.[15]

The antioxidant assay with DPPH was faster and simpler. The DPPH molecule was more stable due to the delocalisation of the spare electrons along the entire molecule so that this molecule did not undergo dimerisation. In this process, the antioxidant compounds donated one electron to DPPH to produce a reduction in DPPH free radicals. The antioxidant power was expressed in IC_{50}, which was the concentration of the test compound that captured 50% of free radicals.[16] The antioxidant assay with DPPH showed that the antioxidant activity in methanol extract had the highest results, with IC_{50} 81.582 µg/mL, while hexane extract showed no antioxidant activity (the highest level of antioxidant activity was IC_{50} < 10µg/mL, the lower antioxidant activity was IC_{50} < 100 – 250 µg/mL, and no antioxidant activity was IC_{50} > 250 µg/mL).[17]

The principle of the FRAP method was electron transfer from antioxidant compounds to reduce the yellow Fe (III)-tripyrydyltriazine (TPTZ) complex to a blue Fe (II)-TPTZ complex. The more concentrated the blue colour produced, the more Fe2+ ions were formed, thus indicating a higher antioxidant potential.[18,19] Based on the table, methanol extract reduced ferrous ions more than hexane extract and ethyl acetate extract. This was presumably because the methanol extract of *Tetracera macrophylla* contained higher amounts of quercetin than the other two extracts. This was in line with the results of antioxidant activity under the DPPH method, where the methanol extract had an 11840 FeEAC mol/gram (FRAP method).

The MTT assay significantly helped to determine whether any compounds displayed cell toxicity or proliferative activity. Doses that produced a viability percentage of below 90% were categorised as toxic to cells.[20-22] The results of this study indicate that EE and ME have a lower ability to inhibit the proliferation of RAW 264.7 cells; therefore, ME and EE are not cytotoxic to RAW 264.7 cells. And HE toxicity test was not performed on RAW cells because it contained weak antioxidant compounds. The MTT assay was based on the reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by metabolically active cells. Viable cells contain NADPH-dependent oxidoreductase enzymes for use in reducing MTT to formazan. In terms of the MTT assay, its simplicity and effectiveness make it more suitable to assess the anti-inflammatory and anti-cancer activities of test samples at the preliminary level.

CONCLUSION

Differences in solvent polarity affect the levels of phenols, flavonoids and antioxidants. The methanol extract of *Tetracera macrophylla* showed the highest yield, total phenolic content and total flavonoid content and had the highest antioxidant activity. Methanol extract has low toxicity to RAW 264.7 cells.

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CONFLICTS OF INTEREST

No conflicts of interest.

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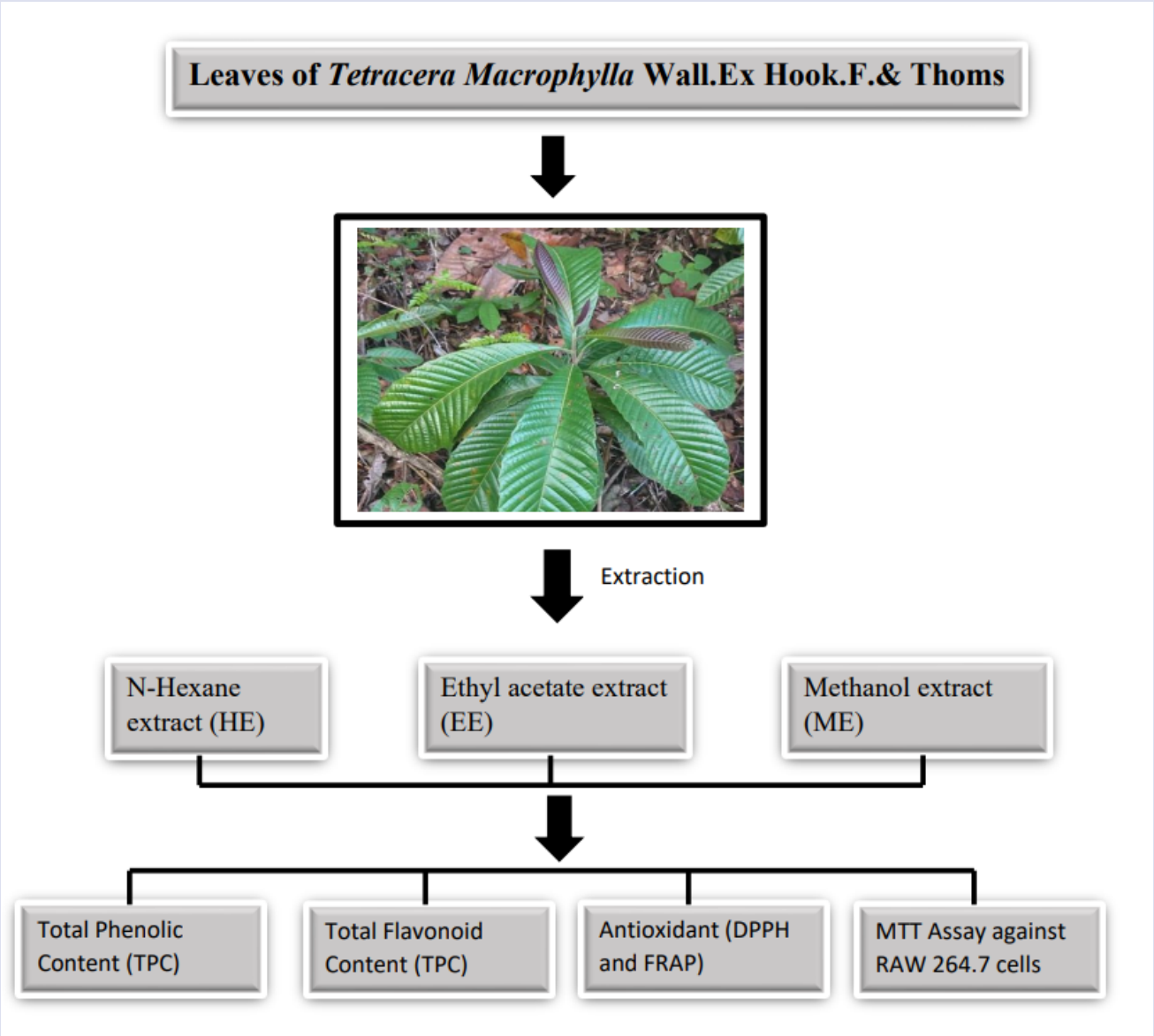
GRAPHICAL ABSTRACT



SUMMARY

ME showed highest antioxidant activity, TPC and TFC value (IC50 = 81.582 µg/mL (DPPH) and 11840 mol/g (FRAP), total phenolic content of 353.781 mg GAE/g dry weight, and flavonoid content of 279.2 mg QE/g dry weight).

IC50 toxicity assay methanol extract and ethyl acetate extract respectively showed 288.792 µg/mL and 541.472 µg/mL.

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