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Pharmacognostic Evaluation and Antioxidant Activities of *Tetracera indica* (Christm. and Panz.) Merr

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ABSTRACT

The community uses *Tetracera indica* (Christm. and Panz.) Merr extensively as a traditional medicine. This plant was the main source of raw materials for herbal medicinal products, so identification was needed to ensure its authenticity through pharmacognosy evaluation. The present study evaluated macros- and microscopic characteristics, performed phytochemical analysis, and performed total phenolic (TPC) and flavonoid content (TFC) analysis. The organoleptic and macroscopic studies were carried out through sensory organs. The microscopic characteristics of leaf powder and twigs identification using chloral hydrate and distilled water. Numerous chemical reagents, TLC methods and LC-MS/MS analysis were employed in the phytochemical study. Research employed DPPH and FRAP tests to assess the antioxidant activity. Furthermore, quercetin was used as a marker of antioxidant activity. The morphological marker of the leaf *T. indica* showed the surface of the leaves is rough bristles, shape elliptic-oblong, margin serratus and phyllotaxy alternate. The stem is lignosus, and the rough stem surface is slightly peeling. The lower leaf surface had paracytic stomata and uniseriate trichomes with tapering ends. Raphide crystals and cork cells were detected in the powder analysis of the twigs. The ethyl acetate extract's twig had the highest TPC and TFC values. Ethyl acetate extract from *Tetracera indica* twigs has potent antioxidant activity with IC_{50} = 99.84±0.011 µg/ml (DPPH) and 4296.67±0.024 mol/g (FRAP). The study showed that *Tetracera indica* contains possible active components that could be developed as novel antioxidants. The result of this study might provide early verification and identification to obtain quality medicinal raw materials.

1. Introduction

The world's greatest consumer of medicinal herbs is Indonesia (Widjaja 2014). *Tetracera indica* (Christm. and Panz.) Merr, often known locally as "mempelas," is a member of the Dilleniaceae family and is one of those that has been used historically to cure a variety of ailments. Asian forests from China, Malaysia, Indonesia, Thailand, Vietnam, and Guyana are home to this plant. It is a large, woody climber from the rainforest. It has white-colored flowers with small pink calyxes, and the leaves have a medium shape and are simple. Various *T. indica* plant sections have different pharmacological effects, including treating fever, sickness, nasal issues, skin rashes, itching, and diarrhea. Pharmacological properties of this plant include anti-hyperuricemia, anti-diabetic, and dilation of hypertension (Ahmed *et al.* 2012; Hasan *et al.* 2017). Betulinic acid in the twigs provides biological activities such as inhibiting the human immunodeficiency virus (HIV), antibacterial, antimalarial, anthelmintic, and free radical scavenger (Abdullah *et al.* 2014). Decoction of the

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leaves in hypercholesterolemia mice can reduce cholesterol levels (Samitra and Rozi 2017). *T. indica* leaf extract has been shown to have antidiabetic effects in both *in vitro* and *in vivo* studies. This plant is utilized as a raw material in Malaysia for the herbal remedy Plantisol®, which traditional healers frequently recommend for the treatment of diabetes (Ahmed *et al.* 2012). In summary, *T. indica* presents a promising subject for more study due to its diverse pharmacological properties, beginning with a pharmacognosy assessment.

Pharmacognosy evaluation is a method of measuring specific and non-specific parameters to identify a natural medicine and determine the quality and purity of the raw material. This is needed because of variations in drug biochemistry, the influence of drug treatment and storage, as well as adulteration and substitution. Pharmacognosy evaluation parameters are numerous, and these are the initial stages, which can be classified as organoleptic, morphological, and microscopic analysis. Specific fragments in plants can be used as an identity to prevent the counterfeiting of a plant (Lima *et al.* 2014; Hanani *et al.* 2017). Phytochemical analysis and quantitative measurements about the main active constituent present in the crude drugs are also crucial in pharmacognosy evaluation.

Furthermore, many plants have different vernacular names in each country, causing identification problems and leading to counterfeiting. On the other hand, plants belonging to the same genus sometimes have similar morphology. This resemblance could progress to the stage of falsification if not anticipated. Profile chromatography and established phytoconstituents can be used to screen for adulteration and poor handling of crude pharmaceuticals (Lima *et al.* 2014; Hanani *et al.* 2017). Currently, no data is available regarding the pharmacognosy evaluation of *T. indica*. This is important to do because, ethnomedicinally, the use of this plant is quite extensive. *T. indica* is the main raw material used to prevent diabetes. Therefore, pharmacognostic studies are important to ensure the plant's identity, establishing standardization parameters that will help prevent counterfeiting.

Previous research has shown *T. indica* contains flavonoid compounds such as quercetin, kaempferol, wogonin, apigenin, rhamnetin, azaleatin, 5,7-dihydroxyflavone-O-8-sulfate, and techtochrysin, (Hasan *et al.* 2017; Alhassan *et al.* 2019). Isolation of the ethyl acetate extract of *T. indica* stems yielded 5,7-dihydroxyl-8-methoxyflavone and betulinic acid (Abdullah *et al.* 2014; Ogunlakin and Sonibare 2022). The hexane extract from the bark and leaves of *T. indica* contains β-sitosterol, betulinic acid, betulin, and lupeol (Muharni *et al.* 2018, 2019). The number of phenolic and flavonoid derivative compounds in *T. indica* has become a target for developing antioxidant compounds. An active antioxidant compound produced from ethyl acetate showed potent antioxidant activity (IC_{50} 8.25 µg/ml) in the previous study (Muharni *et al.* 2018), surpassing the level of standard ascorbic acid (IC_{50} 11.3 µg/ml). A pharmacognostic study has not yet been conducted on this plant. Therefore, this study aims to evaluate various pharmacognostic properties, including the macro-microscopic and phytochemical characterization of *T. indica*. This will yield some important markers for recognizing crude pharmaceuticals and looking into their antioxidant capabilities.

2. Materials and Methods

2.1. Materials

Analytical standard compounds were employed in the investigation. Merck (Darmstadt, Germany) provided the chloroform, methanol, *n*-hexane, ethyl acetate, aluminum trichloride, ferric chloride hexahydrate, hydrochloride acid, 2,2-diphenyl picrylhydrazyl (DPPH) and silica gel GF 254 TLC plates. Sigma Aldrich (Germany) provided the 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ). Sigma Aldrich (St. Louis, MO, USA) provided the quercetin, Folin-Ciolcalteu, gallic acid, chloral hydrate, and sodium carbonate. Basic equipment and tools for the investigation included a compound microscope, cover slips, watch glass, glass slides and other common glass items.

2.2. Collection and Determination of Plant Material

T. indica was acquired from Bogor Agricultural University in West Java, under the Tropical Biopharmaca Study of LPPM (Latitude: -6.556731, Longitude: 106.725945, Latitude DMS: 6° 33'24.23''S, Longitude DMS: 106° 43'33.4" E). It was verified by the Indonesian National Research and Innovation Institute's Biology Research Center plant taxonomist in Cibinong, Indonesia, using collection number

B-689/V/DI.05.07/11/2021. The leaves and twigs of the plant were removed, washed under running water, shade-dried, ground into a powder, and stored in an airtight container for later use.

2.3. Methods

2.3.1. Macroscopic, Organoleptic, and Microscopic Study

Taste, smell, color, texture, and surface are all perceived organoleptically. These morphological traits that are visible to the unaided eye include phyllotaxy, size, shape, texture, petiole presence or absence, and the leaf's apex, edge, base, and lamina. The microscopic properties of the powdered leaves and twigs of *T. indica* were examined. The lamina and midrib were cut into transverse pieces by hand, and a tiny quantity of powdered leaves and twigs was tested along with drops of distilled water or chloral hydrate on a glass slide (Kumar *et al.* 2011). A trinocular microscope (Olympus CX23, China) was used to analyze various cell components.

2.3.2. Extraction

The leaves (6 kg) and twigs (6 kg) of *T. indica* were dried, ground into a powder, and extracted by gradual maceration using *n*-hexane, ethyl acetate, and methanol as solvents (Abubakar and Haque 2020). The sample was filtered after being soaked for one 24-hour period with *n*-hexane (1 g powder in 10 ml solvent). Using the same solvent, the residue was again macerated until a clear filtrate was produced. The residue was subsequently macerated in the same manner as with *n*-hexane solvent using ethyl acetate and methanol solvents, respectively. A vacuum rotary evaporator (Eyela, China) and a water bath temperature of 40°C were used to concentrate the filtrate. *N*-hexane (TH), ethyl acetate (TE), and methanol (TM) extracts were made from *T. indica* twigs. Meanwhile, *n*-hexane extract (LH), ethyl acetate extract (LE), and methanol extract (LM) were obtained from *T. indica* leaves.

2.3.3. Phytochemical Analysis

Methanol was used to dissolve every 20 g of leaf and twig powder to carry out phytochemical screening. Alkaloid screening uses precipitating reagents such as Mayer, Bouchardat, Wagner and Dragendorff. Flavonoid compounds using Mg metal with 5M HCl reagent, carbohydrates with Fehling's reagent, phenol with 3% $FeCl_3$ reagent, saponin test shaken with warm water until foam appears, tannins with 1% gelatin + 10% NaCl reagent, and amino acid analysis with reagent ninhydrin was used to analyze phytoconstituents (Junaid *et al.* 2020; Nortjie *et al.* 2022).

2.3.4. Chromatographic Profile

Thin Layer Chromatography (TLC) was used to grade *T. indica* leaf and twig extracts in phytochemical assays. As the stationary phase, aluminium TLC plates (GF 254) were employed. For TH, LH, TE, and LE, a solvent combination of *n*-hexane and ethyl acetate (1:1) was utilized as a mobile phase. For TM and LM, solvent mixture of ethyl acetate and methanol (1:1) was used. The plate was dried and sprayed with 10 % H_2SO_4 (in water, v/v). The spots were seen at wavelengths of 254 and 366 nm using UV lamps (CAMAG, Germany) (Pyka 2014; Abubakar and Haque 2020). The formula below is used to calculate the retention factor (Rf) value (Mwankuna *et al.* 2023):

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

2.3.5. Total Phenolic Content (TPC) Assay

The microplate was filled with 20 µL of the sample and 100 µl of the Folin-Ciocalteau (1:10) solution. After shaking the mixture for 60 seconds, it was allowed to sit at room temperature for 4 minutes. Following the addition of 80 µL of 7.5% Na_2CO_3 solution, the mixture was mixed and allowed to incubate for two hours in the dark. Using a microplate reader (iMark, Bio-Rad, USA), the absorbance of the sample solution was measured at a maximum wavelength of 750 nm. The regression equation for gallic acid was utilized to determined the phenolic content (Bobo-García *et al.* 2015; Fachriyah *et al.* 2020).

2.3.6. Total Flavonoid Content (TFC) Assay

In a microplate with 96 wells, 20 µL of a sample was added to 10% $AlCl_3$, 1 M potassium acetate (20 µL each) and 180 µL of distilled water. After shaking the mixture for 60 seconds, it was incubated for 30 minutes. At a wavelength of 415 nm, the absorbance of the solution color intensity was measured using a microplate reader (iMark, Bio-Rad, USA). Total flavonoid levels were calculated using the quercetin regression method (Farasat *et al.* 2014; Bobo-García *et al.* 2015).

2.3.7. Antioxidant Assay with DPPH Method

The methodology for antioxidant tests was adapted from Molyneux P (2004). A sample solution of 1,000 µg/ml in methanol was prepared. The sample solution was diluted to concentrations of 40, 60, 80, 100, and 120 µg/ml, it was vortexed for 30 seconds (VM-300, Scientifica, Italy). For every concentration, 1 ml of the standard DPPH solution was pipetted into 3 ml of the sample. It was homogenized and then let to sit in the dark for 30 minutes. The absorbance at a wavelength of 516 nm was measured with a UV-Vis spectrophotometer (Shimadzu UV 1900, Japan). Quercetin was used as a comparison. To get the IC_{50} values, the following formula was utilized.

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

The IC_{50} value was defined as the concentration at which the sample reduced DPPH by 50%. The linear regression equation was used to calculate this, y = a + bx (y=50, a = intercept, b = regression coefficient). The more effective an antioxidant is at scavenging free radicals, the lower its IC_{50} value.

2.3.8. Antioxidant Assay with FRAP Method

Slight modifications were made to the Wong *et al.* (2015) methodology to perform the FRAP approach. The sample was diluted to 500 µg/ml from 1,000 µg/ml in methanol. After pipetting 270 µL of FRAP reagent, 30 µL of the sample solution was added. The mixture was vortex (VM-300, Scientifica, Italy) and then let to sit in the dark for 30 minutes at 37°C. A wavelength of 595 nm was used to measure absorption. The positive control was quercetin, while the blank was methanol. Antioxidant activity was measured using the following formula, which was based on ferrous iron equivalent antioxidant activity (FeEAC) (Wong *et al.* 2015; Prastiwi *et al.* 2020).

$$\text{FeEAC} = \frac{\Delta A}{\text{GRAD}} \times \frac{\text{Av}}{\text{Spv}} \times D \times \frac{1}{\text{Cext}} \times 10^5$$

FeEAC : Ferric ion and antioxidant activity (µmol/g) equivalency
ΔA : Sample absorbance that has been reduced by a blank
GRAD : Gradient found on AFS based on the calibration curve
Av : Overall volume for the test
Spv : Overall volume of the test sample
D : Factor of dilution for samples
Cext : Concentration of sample stock (g/L)

2.3.9. Identification of Compounds by LC-MS/MS

A Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) apparatus was utilized to carry out a qualitative analysis of the constituents present in the active extract. A Waters Acquity UPLC I-Class with an XEVO G2-XS QTof mass spectrometer is used for measurements. Sample separation with column type ACQUITY UPLC® BEH C18 (1.7 µm × 2.1 mm × 50 mm), full scan m/z 100–200 (mode ESI), and injection volume 1 µL. Solvents A (water plus 0.1% formic acid) and B (acetonitrile plus 0.1% formic acid) were utilized in the mobile phase. An organic chemical spectrum database was used to identify the mass fragmentation of molecules (Harmita *et al.* 2019).

2.3.10. Statistically Analysis

Triple testing was used, and the results were shown as mean ± SD. After a one-way analysis of variance (ANOVA $p<0.05$) using SPSS version 26, the Tukey test was run to see if there was a significant difference in the mean values.

3. Results

3.1. Macroscopic and Organoleptic Characterization

T. indica is a small shrub that can grow up to 2 m in height or a small liana that can grow up to 6 m in length. It can creep along the ground or clamber over other plants, branching widely. Pictures of plant components of *T. indica* are presented in Figure 1, and the organoleptic properties of *T. indica* are shown in Table 1.

3.1.1. Leaf

The leaves have rough bristles on their surface. Older leaves are dark green, whereas young leaves are frequently purple, sparingly pubescent on the midrib, glossy and glabrous on the intervenium. Table 2 displays the macroscopic character leaf of *T. indica*.

3.1.2. Flower

2.5–3 cm across, pedicel 8–15 mm, strigose, with hairs patent toward the apex and dense hairs directly below the flower, either without or with 1-3 lanceolate bracteoles. The sepals are green or have a faint red tint on both sides, and are glabrous. Except for a few 0.5 mm long, appressed, and stiff hairs on the back, the carpels are glabrous. The petals are white, the stamens are red, white at the base, and 6–8 mm long.

3.1.3. Twigs

The stem of *T. indica* is classified as lignosus. It is an old bald stem with greyish- brown bark and a rough, slightly peeling surface.

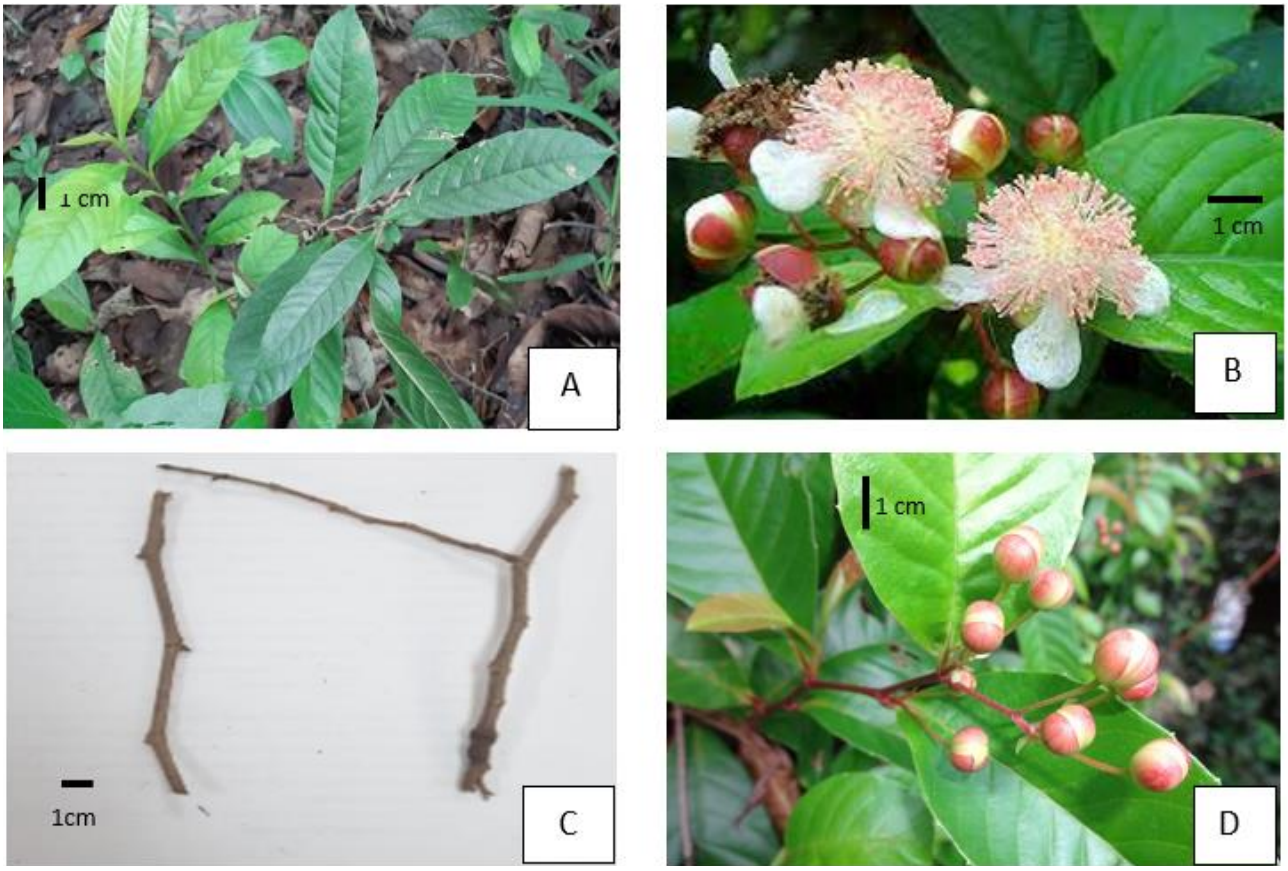


Figure 1. Parts of *Tetracera indica*. (A) leaves, (B) flowers, (C) twigs, (D) fruits

Table 1. Organoleptic characters of *T. indica*

	Organoleptic characters			
	Leaf	Twig	Root	Flower
Surface	Rough bristles	Scabrid, lignosus	Smooth	Snout with a smooth surface
Color	Dark green (upper), Light green (lower)	Dark brown,	Light brown	Maroon
Odor	No specific odor	No specific odor	No specific odor	No specific odor
Taste	No specific taste	a little bit bitter	Bitter	No specific taste

Table. 2 Macroscopic features of *T. indica* leaf

Macroscopic Observation	
Phyllotaxy	Alternate
Type	Small liana
Leaf	Length 9-11 cm, width 3.5-6 cm
Shape	Elliptic oblong
Apex	Obtuse to acute
Margin	Entire to serrate or less dentate
Venation	Reticulate
Base	Acute
Petiole	4-15cm long, sparsely pubescent above, strigose beneath

3.1.4. Fruits

Fruits with capsules formed in each flower are nearly spherical, with a diameter of approximately 10 mm, glabrous, glossy, red, or reddish brown, with a beak ranging in length from 2-6 mm.

3.2. Microscopic Characterization of *T. indica*

T. indica powder is microscopically characterized using only leaves and twigs. Based on previous studies, this plant's two sections were picked because they were most frequently reported to have pharmacological activity. Compared to roots and flowers specific fragments are more commonly found on leaves and twigs. Characterization of macroscopically discovered roots and flowers.

3.2.1. Leaf of *T. indica*

There are two sections of the leaf visible: the midrib and lamina. Abaxial epidermis has a large number of paracytic stomata. The midrib is made up of single-layered, rectangular epidermis cells covered in a thin layer of cuticle. The abaxial epidermis is characterized by small rectangular cells and uniseriate trichomes with tapered ends. Figure 2 shows that 2-3 layers of collenchyma hypodermis are followed by 5–6 layers of parenchyma cortex. Additionally, the cortex has densely packed parenchyma containing sharp raphide crystals (calcium oxalate crystals in the form of needles). Paracytic stomata, trichomes, mesophyll fragments and crystalline Ca oxalate (crystal raphide) were found in *T. indica* leaf powder (Figure 3).

3.2.2. Twig of *T. indica*

Figure 4 shows a microscopic view of *T. indica* twig powder. There are cork cells, uniseriate trichomes, needle-shaped Ca oxalate (raphide crystals), and epidermal layers.

3.3. Phytochemical Analysis

Phytochemical analysis of the extract revealed positive results for flavonoids, phenols, saponins, tannins, glycosides, steroids, and terpenoids

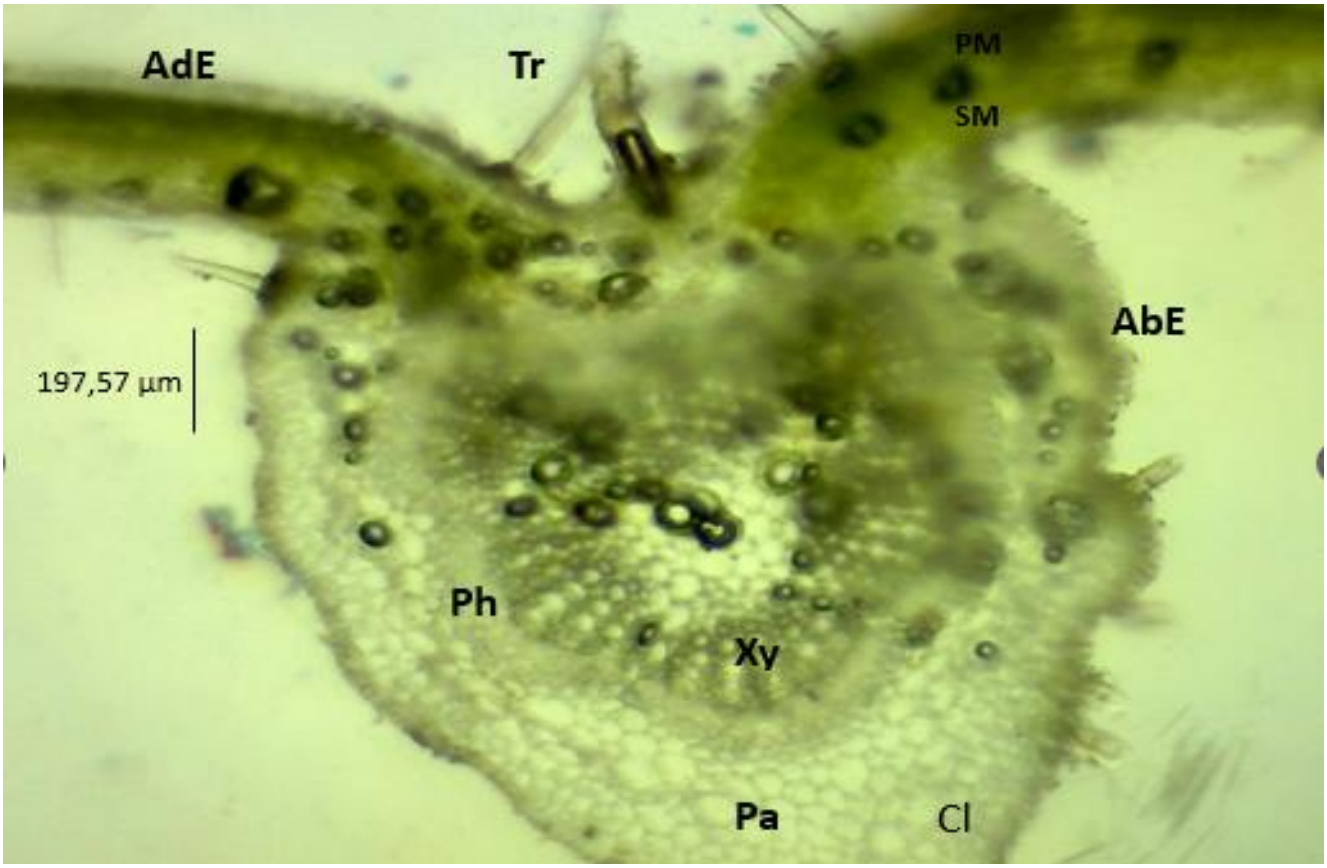


Figure 2. Transverse section of *T. indica* midrib. AbE–Abaxial Epidermal, AdE-Adaxial Epidermal, PM-Palisade mesophyll, SM-Spongy Mesophyll, Tr- Trichomes, Pa-Parenchyma, Cl-Collenchyma, Ph-Phloem, Xy-Xylem. Magnification 100x

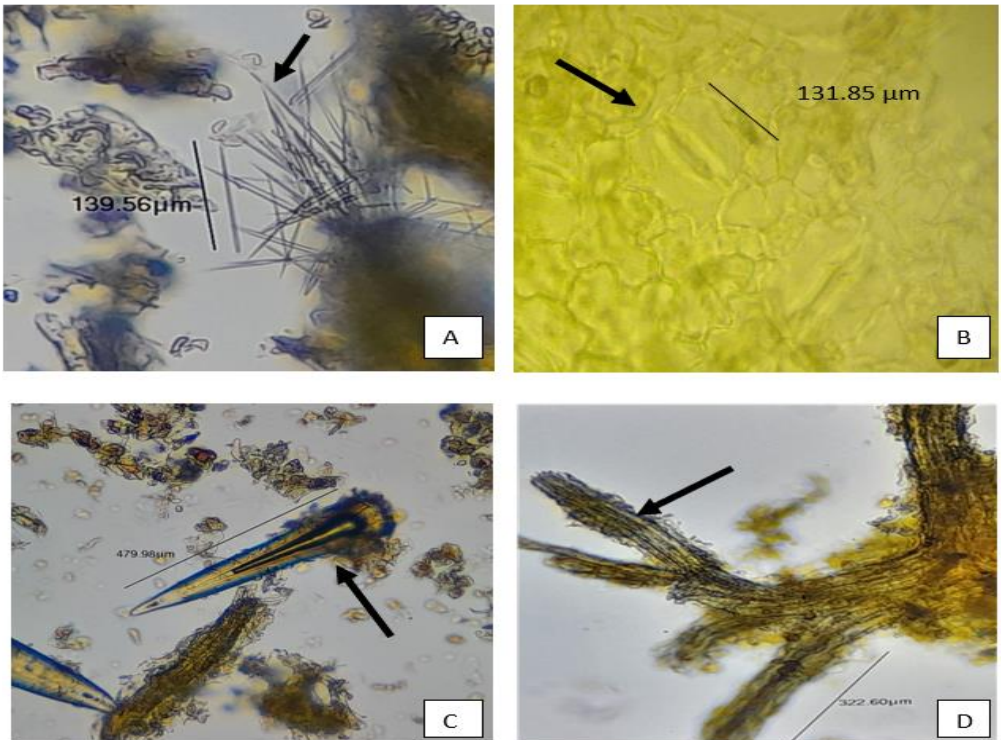


Figure 3. Microscopic views of *T. indica* leaf powder (magnification 100x). (A) Crystalline Ca oxalate (crystal raphide), (B) paracytic stomata, (C) trichome, (D) mesophyll Fragment

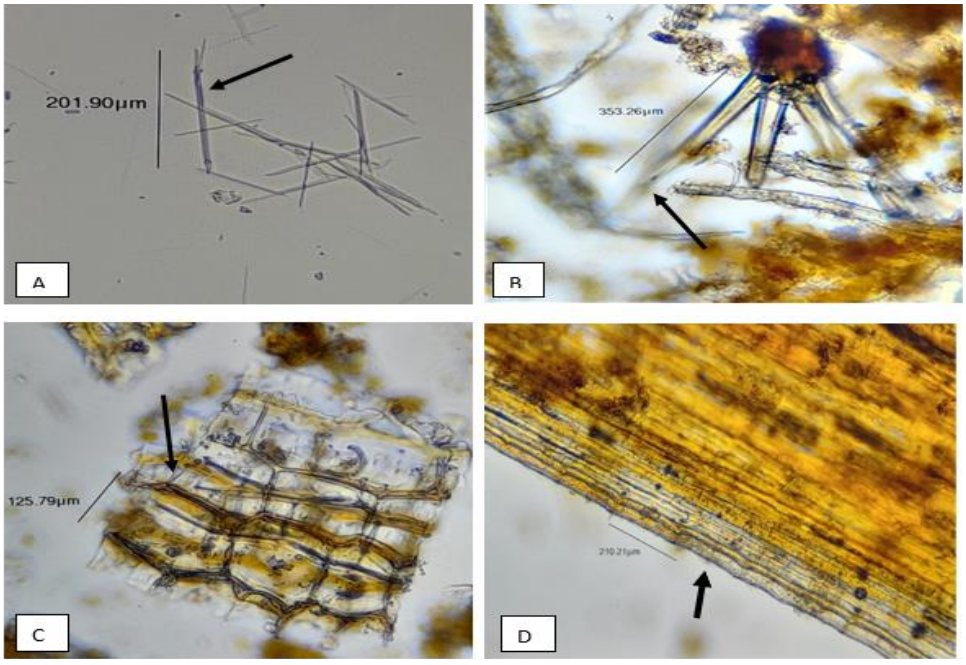


Figure 4. Microscopic of *T. indica* twig powder (magnification 100x). (A) Crystalline Ca oxalate, (B) trichome, (C) cork cell, (D) epidermal layer

(Table 3). Metabolites have a major impact on the pharmacological activity of crude extract. The absence of alkaloid compounds was likely caused by the false negative reactions. It may occur as a result of the substance being harmed by hydrolysis or enzymatic reactions during the extraction process. The free amino acids in the sample can be seen to form a purple-colored complex in the ninhydrin test. The sample may not include free amino acids in this assay, but rather amino acids attached to other peptide bonds, which could explain negative response results.

3.4. Chromatographic Evaluation of Different Extracts of *T. indica*

The presence of two and three spots, respectively, in the hexane: ethyl acetate (1:1) solvent system was indicated by the thin layer chromatographic profile of the hexane extract of twigs (TH) and leaves (LH). The twigs (TE) and leaves (LE) ethyl acetate extracts showed the presence of five and two spots, respectively. In the meantime, in the ethyl acetate-methanol (1:1) solvent solution, the methanol extract of twig (TM) and leaves (LM) both yield four spots. H_2SO_4 10% was the spray reagent that was employed. Visual observations were made at 254 nm and 366 nm at UV wavelengths (Table 4).

3.5. The Total Content of Phenol (TPC) and Flavonoids (TFC)

The Folin–Ciocalteu reagent and gallic acid as the standard were used to determine TPC at 750 nm using the regression equation y = 0.0087x–0.1084, which has a correlation coefficient of R^2 = 0.9976.

Table 3. Phytochemical screening of *T. indica* leaf and twig extracts

Phytoconstituents	Reagents	Leaves	Twig
Alkaloids	Dragendorff	nd	nd
	Mayer	nd	nd
	Bouchardat	nd	nd
	Wagner	nd	nd
Flavonoids	Shinoda	+ (reddish)	+ (red)
Phenols	Ferric chloride	+ (dark blue)	+ (dark blue)
Saponins	Foam	+ (foam 1.5 cm)	+ (foam 1cm)
Tannin	Gelatine 10%	+ (white precipitate)	+ (white precipitate)
Steroids and terpenoids	Liebermann Burchard	- (dark blue)	+ (purple)
Carbohydrate	α-Naphthol + H_2SO_4	+ (reddish brown)	+ (purple)
Amino acid	Ninhydrin	- (light green)	- (brownis yellow)

(+): positive result, (nd): not detected, (-): negative result

Table 4. Chromatographic analysis of several twig and leaf extracts from *T. indica*

Sample	Mobile phase	Number of spots	Spraying reagent H_2SO_4 10%	Rf 366 nm
TH		2	1. Green	0.8
			2. Green	0.78
LH		3	1. Green	0.8
			2. Yellow	0.72
			3. Yellowish green	0.33
TE	*n*-hexana: EtOAc(1:1)	5	1. Green	0.8
			2. Green	0.79
			3. Purple	0.75
			4. Yellow	0.71
			5. Yellowish	0.52
LE		2	1. Yellow	0.8
			2. Yellow	0.71
TM		4	1. Yellowish green	0.89
			2. Yellowish green	0.81
			3. Yellow	0.76
			4. Yellow	0.70
LM	EtOAc:MeOH (1:1)	4	1. Yellowish green	0.88
			2. Yellowish green	0.82
			3. Yellow	0.70
			4. Yellow	0.21

The graph (Figure 5) shows that in the ethyl acetate extract of *T. indica* (TE), the TPC value with the highest value was 340.652 mg GAE/gram ± 6.303. The antioxidant properties are influenced by the high phenol concentration. This was further supported by the LC-MS/MS qualitative analysis, which revealed that most of the chemicals in the *T. indica* twig ethyl acetate extract were phenol derivatives. Using $AlCl_3$ and quercetin as the standard, a colorimetric approach was used to calculate TFC, with the regression equation being y= 0.0053x + 0.0177 and R^2 = 0.9999. It can be explained that $AlCl_3$ with the OH group on the C-3 or C-5 atom of flavonols and flavones and the keto group on the C-4 atom form a stable combination. Additionally, it can form complexes containing OH at the location in the flavonoid B ring. The complex compound causes the wavelength to shift toward the visible. This is indicated by the appearance of a yellow solution. To keep the wavelength within the visible range, potassium acetate is added to ionize the 3 and 4-OH groups, which do not form a compound with Al3+ and 7-OH groups. The graph indicates that the ethyl acetate extract of *T. indica* (TE) twigs had the highest TFC value, 427,967 ± 37,037 mg QE/gram extract. The values of TPC and TFC are shown in Figure 5.

3.6. Antioxidant Assay with DPPH and FRAP Method

Quercetin was used as the standard to determine the DPPH assay. The *T. indica* twigs' ethyl acetate and methanol extracts showed potent antioxidants. However, the *n*-hexane extract was only shown to have weak antioxidant capabilities (Figure 6). AFS (ammonium ferrous sulfate) standard solution and quercetin as a positive control were used in the FRAP test, which was conducted using a microplate reader. The antioxidant reduction capacity was determined using the linear regression slope of 0.0015, derived from the AFS standard as the gradient.

3.7. Identification of Active Extract by LC-MS/MS

Phenolic chemicals help to prevent free radicals from forming. Many phenolic compounds were found in the ethyl acetate and methanol extracts

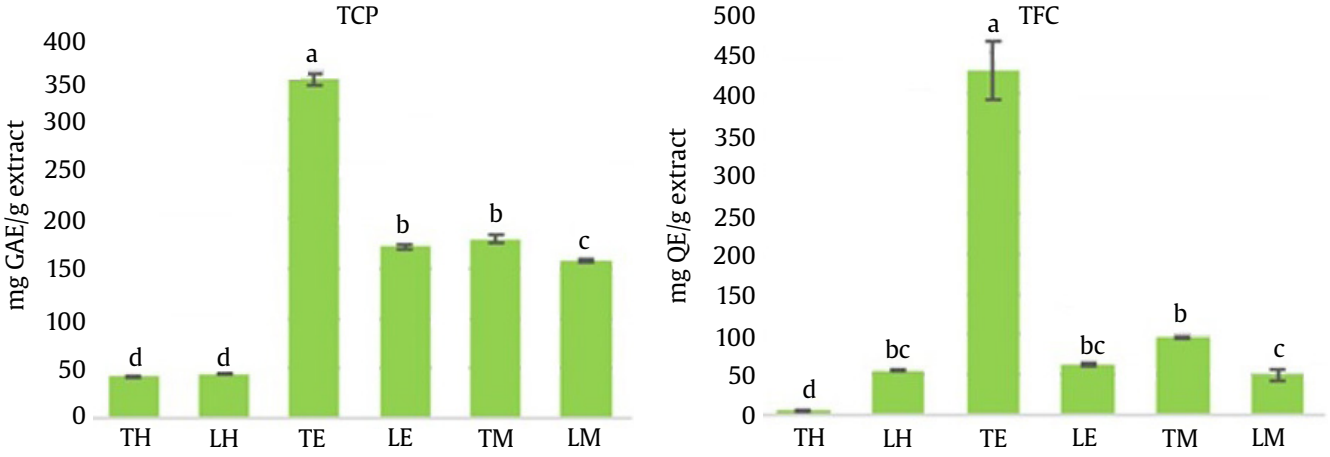


Figure 5. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of T.indica extracts n-hexane extract of twigs (TH), ethyl acetate extract of twigs (TE), methanol extract of twigs (TM), n-hexane extract of leaf (LH), ethyl acetate extract of leaf (LE), methanol extract of leaf (LM). The data are presented mean ± SD (n = 3), one-way ANOVA, and the Tukey test (P value <0.05). Significant differences are indicate by different letters

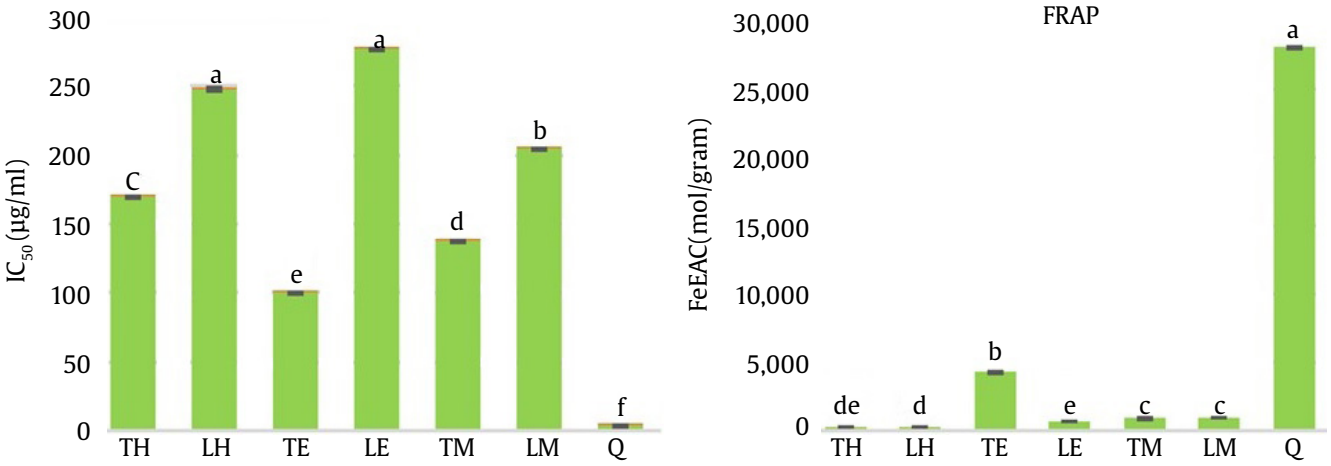


Figure 6. Antioxidant activity of *T. indica* twigs and leaves by DPPH and FRAP method, *n*-hexane extract of twigs = TH, ethyl acetate extract of twigs =TE, methanol extract of twigs = TM, *n*-hexane extract of leaf = LH, ethyl acetate extract of leaf = LE, methanol extract of leaf = LM, Q = Quercetin. The data are presented mean ± SD (n = 3), one-way ANOVA, and the Tukey test (P value <0.05). Significant differences are indicate by different letters

Table 5. Phenolic compounds of *T. indica* identified by LC-MS/MS

Sample	Observed m/z	Observed RT	Compound	Formula
TE/TM	301.07	4.59	3-hydroxy-7-methoxy baicalein	$C_{16}H_{12}O_6$
TE/TM/LM/LE	269.08	4.63	7-hydroxy-3-(4'-hydroxybenzylidene)-chroman-4-one	$C_{16}H_{12}O_4$
TE/TM/LM	285.07	5.63	5,7-dihydroxy-3-(4'-hydroxybenzyl) chromone	$C_{16}H_{12}O_5$
LE/LM	285.07	5.68	Oroxylin A	$C_{16}H_{12}O_5$
LE/LM	285.11	5.53	p-hydroxy phenyletanol coumarate	$C_{17}H_{16}O_4$
LM	431.13	3.60	Ononin Ononin	$C_{22}H_{22}O_9$
LM	303.05	4.20	Quercetin	$C_{15}H_{10}O_7$

TE and TM (Ethyl acetate & methanol extract of twig *T. indica*), LE and LM (Ethyl acetate and methanol extract of leaves *T. indica*), RT (Retention Time), m/z = Mass unit (Mass number/charge number)

when were identified by LC-MS/MS (Table 5). The presence of these phenolic compounds help produce antioxidant effect by suppressing radical activity in phenolics through electrons transfer and hydrogen atom transfer from the OH group. The mass spectrum and chromatograms of the phenolic chemicals found in *T. indica* twigs and leaves are shown in Figure 7.

4. Discussion

Various vernacular names for the same plant may exist in different parts of the nation. It makes it difficult to identify plants (Hanani *et al.* 2017; Muanyishay *et al.* 2018). The current investigation into the macro-microscopic characteristics and the phytochemical makeup of *T. indica* yields important data for verification and identification. The quality of herbal remedies largely depends on the origin and caliber of their primary materials. As a result, the standardization of herbal medicinal plants with therapeutic potential through pharmacognostic evaluation has received increased attention in recent years. Pharmacognosy characterization, which includes macros-microscopic and phytochemical assessment, is intended for raw drug material identification, adulteration detection, and quality control compilation (Ragesh *et al.* 2016; Wulansari *et al.* 2020). *Tetracera indica* grows in different country regions and has different vernacular names. This causes severe problems in identifying plants and can cause counterfeiting (Zhang *et al.* 2012; Ragesh *et al.* 2016). So far, no data is available regarding this plant's macros-microscopic.

The morphological characters of *T. indica* observed can be used to distinguish it from closely related species like *Tetracera akara*. Organoleptically, these two species have different leaf morphology. *T. indica* has prominent leaf veins and a symmetrical elliptical shape, while T.akara does not. Microscopically, both species have raphide crystals, unicellular trichomes, and paracytic stomata. The cross-section of the lamina

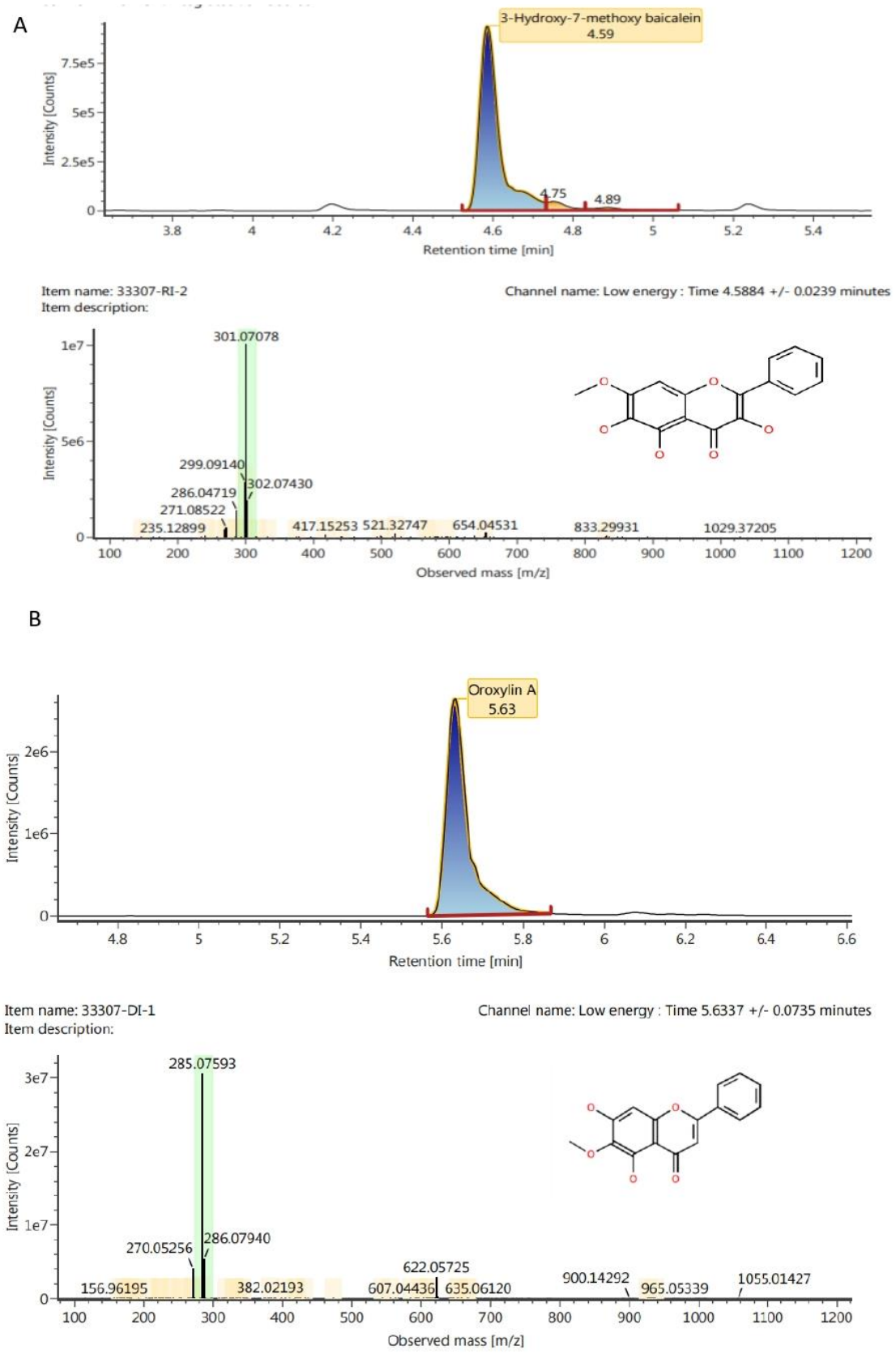
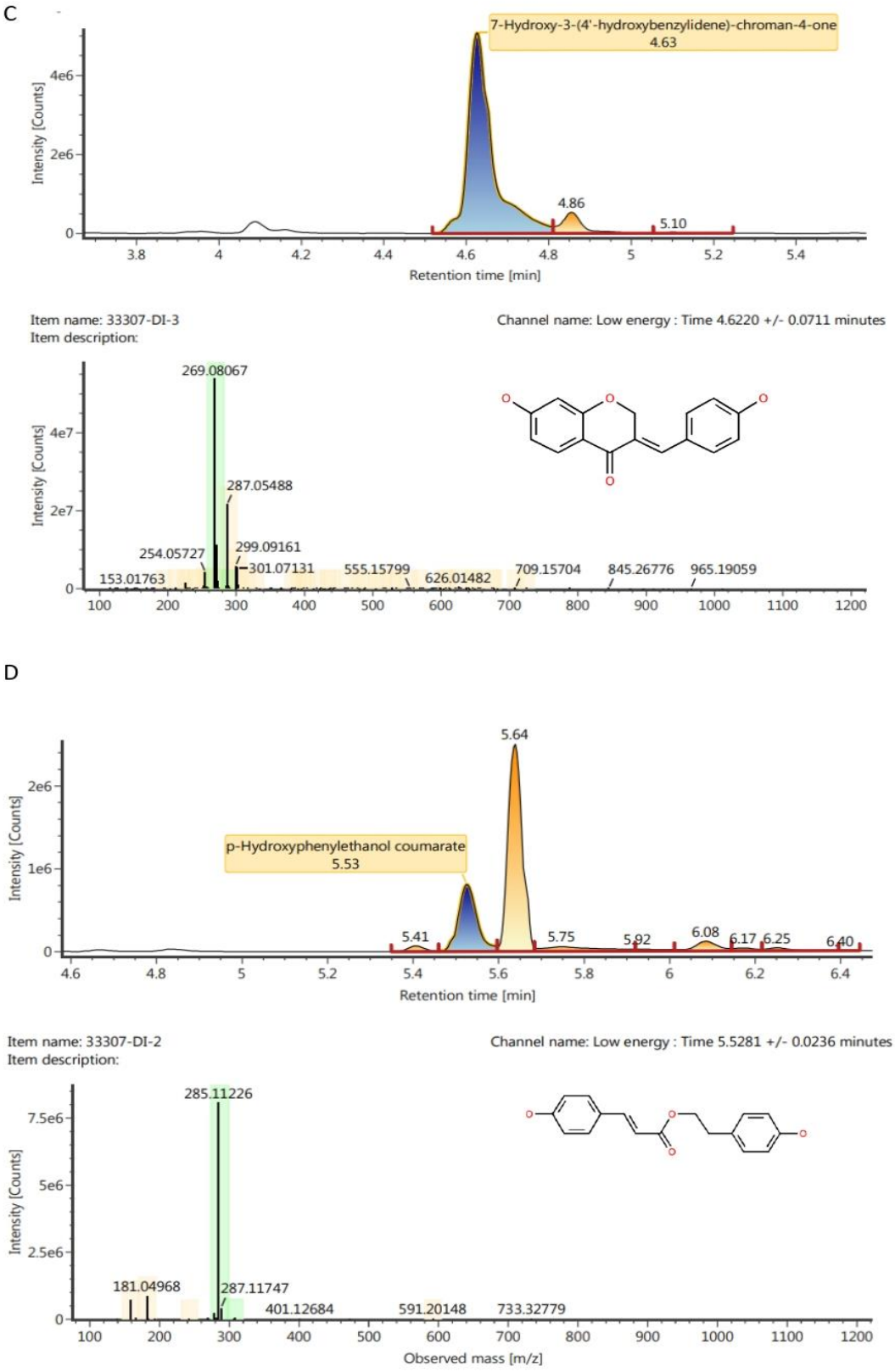


Figure 7. Phenolic compound chromatograms and mass spectrum of twig and leaves *T. indica* (A) 3-hydroxy-7-methoxy baicalein, (B) Oroxylin A, (C) 7-hydroxy-3-(4'-hydroxybenzylidene)-chroman-4-one, (D) p-hydroxy phenyletanol coumarate, (E) 5,7-dihydroxy-3-(4'-hydroxybenzyl) chromone, (F) Ononin, (G) Quercetin



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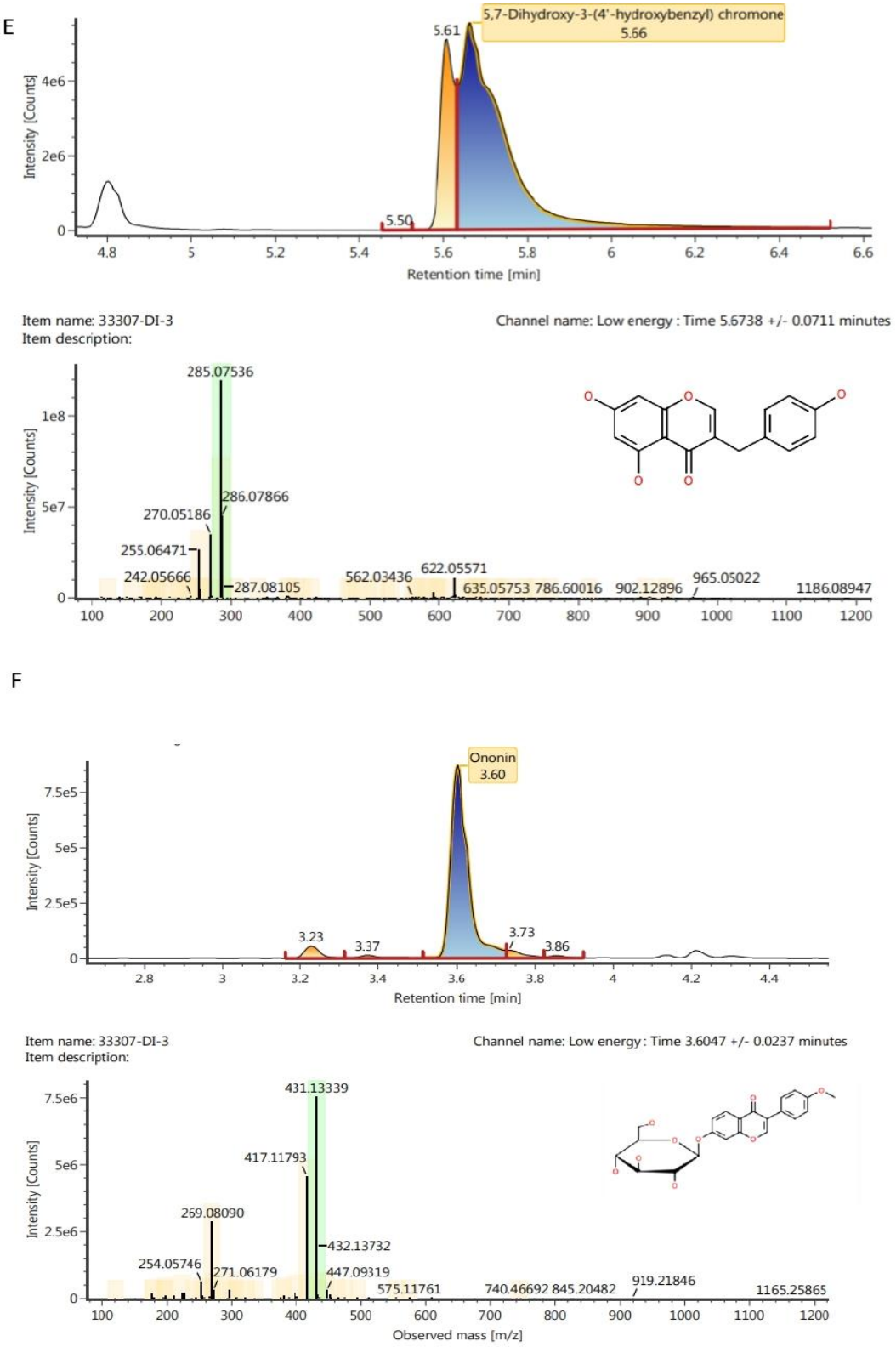

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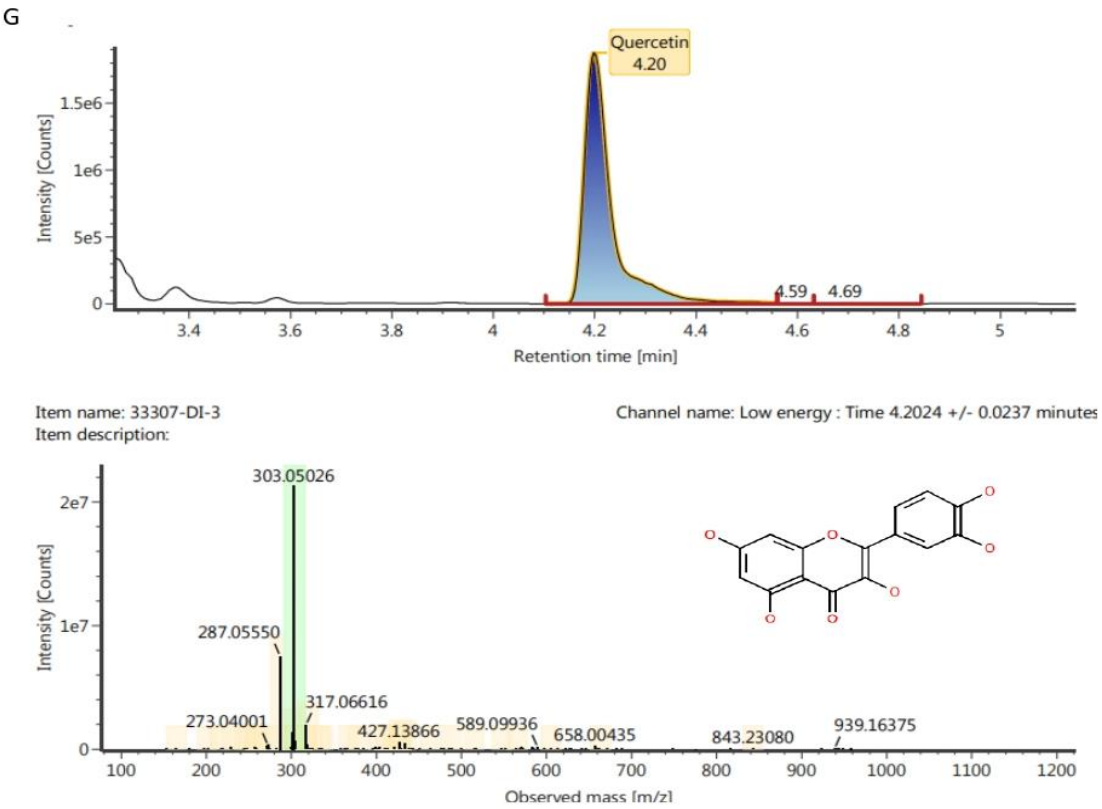

Figure 7. Continued

presents a single-layered epidermis. Below the adaxial epidermis, compactly arranged palisade tissue is present, followed by spongy parenchyma. The abaxial epidermis possesses paracytic stomata. A powder examination of the twigs revealed the presence of Ca oxalate crystals with needle-like shapes and cork cells.

Numerous significant classes of phytoconstituents, including flavonoids, carbohydrates, phenols, saponins, steroids, terpenoids, amino acids, and tannins, were detected during the phytochemical screening and may have an impact on the pharmacological activities of the plants. The previous studies succeeded in isolating five phenolic compounds where the kaempferol compound can inhibit alpha-glucosidase, which is robust (Alhassan *et al.* 2019), and wogonin, norwogonin has an insulin-like effect (Hasan *et al.* 2017). Meanwhile, wogonin can act as a potent xanthin oxidase inhibitor (Abdullah *et al.* 2014). The same result was also obtained from this study, based on analysis from the phytochemical screening, chromatographic profile, and LC-MS/MS test of *T. indica* leaves and twigs, which gave an overview of the dominant phenolic compounds and flavonoids. It is also consistent with the results of TPC and TFC. Phenolic and flavonoid compounds in *T. indica* have a significant role in antioxidant activity.

Plants with different Rf (Retention factor) values were shown to have different phytoconstituents amounts based on chromatographic profiles of *n*-hexane, ethyl acetate, and methanol extract. The value of Rf is crucial for comprehending the degree of polarity (Ragesh *et al.* 2016; Hanani *et al.* 2017). The kind of solvent that will be utilized to separate the chemicals can be predicted from the chromatographic profile. Different compounds will be attracted to solvents with varying polarity. Phenolic and flavonoid chemicals have been found in the ethyl acetate fraction of *T. indica* stems, according to several prior investigations (Hasan *et al.* 2017). The fruit grading section's phytochemical test revealed the existence of phenolic chemicals in the methanol solvent (Sukmawati *et al.*2020). Four terpenoids compounds, namely β-sitosterol, lupeol, betulin, and betulinic acid, as well as six flavonoid compounds, namely 5,7-dihydroxy-8-methoxylavone, 5,7,8-tryhydroxyflavone, isocutellarein methyl ether, kaempferol, quercetin, and techtochrysin, have been identified through chemical analysis of *T. indica* leaves (Ahmed *et al.* 2012).

The Folin-Ciocalteu method is used in the TPC assay, which reacts with phenolic compounds in an alkaline state, breaking protons down into phenolic ions. The phenolic ion reduces the phosphotungstate-phosphomolybdate heteropoly acid, as shown by a blue color shift in the solution (Chen *et al.* 2015). Sodium carbonate improves the reduction of phenol, resulting in more stable colors. Aluminum trichloride binds to the OH group on the neighboring C-3 or C-5 atom of flavone and flavonol groups as well as the keto group on the C-4 atom in TFC (Sjahid *et al.* 2020).

The redox characteristics of phenolic compounds, such as their ability as hydrogen donors, reducing agents, and singlet oxygen quenchers, give rise to their potent antioxidant activity. According to Zeb (2020), the hydroxyl group on the benzene ring is responsible for the antioxidant properties of the phenolic compound. The DPPH molecule did not dimerize because the spare electrons were delocalized throughout the entire molecule, making it more stable.The antioxidants reduced DPPH free radicals by helping DPPH by contributing one electron (Alam *et al.* 2013; Chang *et al.* 2002). The IC_{50} value was used to calculate the antioxidant power (Flieger *et al.* 2021). The ethyl acetate extract of twigs yielded IC_{50} values of 99.84 µg/ml (strong category). Weak activity was shown by the hexane extract. The leaf extract was ineffective but the methanol extract of *T. indica* twigs showed moderate activity. These findings support earlier research that suggests the ethyl acetate extract may have antioxidant properties (Muharni *et al.* 2018).

The reduction of the yellow Fe (III)-tripyridyltriazine (TPTZ) complex to a blue Fe (II)-TPTZ complex by means of electron transfer from antioxidant chemicals was the basic principle of the FRAP method. An improved antioxidant capacity was indicated by the generation of more Fe^{2+} ions with enhanced blue color concentration (Chang *et al.* 2002; Xiao *et al.* 2020). The DPPH and FRAP tests were utilized in this investigation to monitor the compounds' capacity to scavenge radicals. The outcomes of these two techniques suggested that the ethyl acetate from *T. indica* twigs may have some antioxidant potential.

The variations in the chemical composition of the *n*-hexane extracts, ethyl acetate, and methanol from *T. indica* twigs and leaves can be explained by the LC-MS/MS analysis results (Table 5). The chromatogram peaks of compounds with varying molecular weights describe the differences in content of these compounds. The phenolic components found in the ethyl acetate and methanol extracts of *T. indica*, as determined by LC-MS/MS investigation, are widely recognized for their antioxidant properties (Pietta 2000; He *et al.* 2018). Previous research indicates that no prior reports have been made for 3-hydroxy-7-methoxy baicalein, 7-hydroxy-3-(4'-hydroxybenzylidene)-chroman-4-one, and p-hydroxy phenyletanol coumarate. Quercetin, ononin, and oroxylin A are naturally occurring polyphenolic substances. Because phenolic has a hydroxyl group and can stabilize free radicals, it shares the same chemical structure as quercetin and its diastereoisomers. Three theories have been proposed to explain why phenolic compounds exhibit antioxidant properties, and they are known as the Bors criteria. First, the catechol group (Bors 1) of the B-ring is engaged, raising the stability of the resulting antioxidant radical. The second component that promotes electron delocalization is the presence of a 2,3 double bond and a 4-oxo group on the C-ring (Bors 2). The presence of 4-oxo and OH groups at positions 3 and 5 is the third, which enable hydrogen bonding to enable electron delocalization (Bors 3). In Figure 8, the three Bors criteria are briefly explained. The phenolic substances lower free radicals by giving one electron to the phenolic OH group. The ensuing peroxyl radicals' resonance stabilizes the aromatic group (Bors *et al.* 1990; Platzer *et al.* 2021). An antioxidant becomes a radical once it interacts with the initial reactive species. Charge delocalization arises from the interaction between the π-electrons in the benzene ring and the hydroxyl groups in phenol, stabilizing the molecule. Figure 9 presents the resonance stabilization of the phenol radical (Bernatoniene and Kopustinskiene 2018; Zeb 2020; Gunathilake *et al.* 2022).

However, this pharmacognostic study is the first to be useful for verification and identification to obtain quality medicinal raw materials. This study provides specific markers, such as raphide crystals and paracytic stomata. This research also paves the way for the search for new antioxidants from natural ingredients.

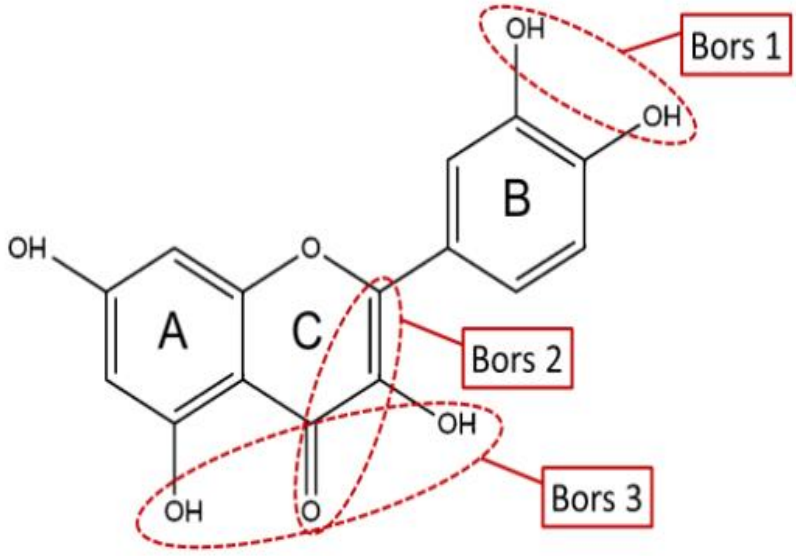

Figure 8. Structure activity relationship based on Bors criteria. Bors 1-catechol group on the B-ring; Bors 2-2,3 double bond and 4-oxo group on the C-ring; Bors 3-OH groups at position 3 and 5 OH group on the A- and C- rings and 4-oxo group on the C-ring (Platzer *et al.* 2021)

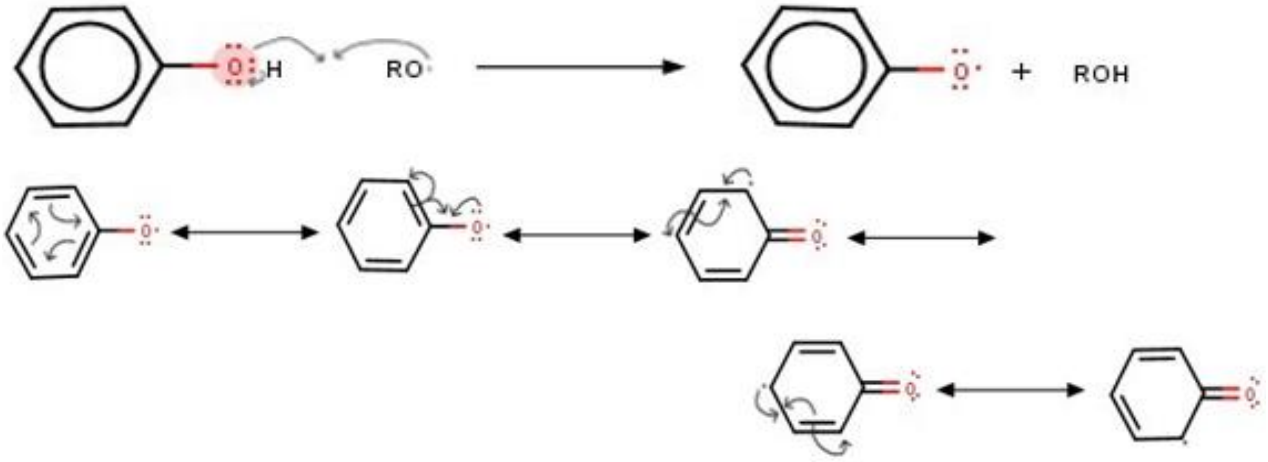

Figure 9. The phenol radical's resonance stabilization (Gunathilake *et al.* 2022)

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