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Antioxidant Activity-Guided Isolation of Phenolic Compounds from Leaves of *Vitex pinnata* (Lamiaceae)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study is to isolate phenolic compounds from the ethyl acetate fraction of the leaves of *V. pinnata Linn*, which have antioxidant properties, guided by a TLC-Bioautography (Thin-layer chromatography) with a reagent of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The TLC-antioxidant bioautography technique has several advantages. It does not require a long time in testing, the sample used is small, and it can potentially obtain promising antioxidant-positive compounds.The isolation process led to obtaining two phenolic compounds. The UV-Vis, FTIR, and NMR spectrum analysis showed that the compounds were (**1**) p-hydroxybenzoate and (**2**) luteolin. Further antioxidant evaluation by DPPH assay showed a potential radical scavenging DPPH of luteolin (2) with an IC_{50} of 1.56±0.18 µg/mL.

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1. INTRODUCTION

Phenolic compounds are secondary metabolites widely found in plants, with more than 50.000 molecules identified so far [1]. Phenolic compounds have one or more hydroxyl groups (-OH) attaching to the aromatic ring, such as benzoic acid, cinnamic acid, flavonoids, tannins, and stilbenes [2-3]. Phenolic compounds have many benefits for human health, such as antioxidant, anti-diabetic, antifilarial, anticancer, cardio-protective, anti-inflammatory, and antiviral of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [4–10]. For example, chlorogenic acid is an anti-influenza virus; quercetin is antioxidant and anti-inflammatory in cardiovascular disease; antiviral, naringin is an anti-malignant, anti-proliferative, anticarcinogenic, antiviral, and inhibitor of hepatitis C infection; resveratrol to treat heart failure; and catechins to treat insulin insufficiency and chronic inflammation or fibrosis [11–14].

As the antioxidant function, Phenolic transfers an electron from hydroxyl groups, resulting in stable Phenol• due to resonance in the aromatic ring [15]. The presence of a methoxy group ($-OCH_3$) and a hydroxyl group (-OH) in phenolic compounds can reduce bond dissociation enthalpy leading to the release of hydrogen atoms [16]. Phenolic also plays a role in single electron transfer described by the energy gap between the Highest Occupied Molecular Orbital (HOMO) and Lowest Empty Molecular Orbital (LUMO). The smaller the energy gap, the greater the radical scavenging activity that can occur due to the electron delocalization system in the aromatic ring of phenolic compounds [17-18].

Vitex is a plant species belonging to the Lamiaceae family, with 270 species of trees and shrubs spread in tropical and subtropical regions [19]. There are 161 compounds reported from this species, such as iridoids, diterpenoids, ecdysteroids, flavonoids, and phenolic compounds [20]. Phenolic compounds commonly found in this genus are vanillic acid, ferulic acid, p-coumaric acid, 3,4-dihydroxybenzoate acid, casticin, caffeic acid, gallic acid, chlorogenic acid, isochlorogenic acid, vitexin [21–25]. *Vitex pinnata Linn* is a plant species from the *Vitex* genus with a wide range of phenolic compounds, such as methyl p-hydroxybenzoate, vicioside, apigenin, retusin, and kaemferol trimethyl ether [26–28].

This study aimed to find a phenolic compound with good antioxidant activity. Furthermore, the TLC-bioautography with DPPH was used as guidance to reach the pure antioxidant-phenolic compound. The TLC-bioautography method is a simple, inexpensive, and fast screening method in providing initial information about the biological activity of the examined plants [29]. Generally, secondary metabolites exist in small amounts in plants. Therefore, the utilization of guidance of TLC-bioautography using DPPH reagents might be an effective method to find the potential antioxidant compounds.

2. MATERIALS AND METHODS

2.1 Materials

The leaves of *V. pinnata* Linn were air-dried and grinded to produce the leave powder. The materials used in this study were silica gel GF254 (Merck KgaA, Germany), silica gel 60 (70-230 mesh; Merck KgaA, Germany), Sephadex LH-20 (Merck, Germany), powder 2,2-diphenyl- 1-picrylhydrazyl (DPPH) (Himmedia-India).

2.2 Methods

2.2.1 Extraction

A total of 1.6 kg of dried leaf powder of *V. pinnata* were soaked with methanol (MeOH) for three times at room temperature during 72 hours. The solvent-material ratio was maintained at 2:1 (w/w). The filtrate was concentrated with a rotary evaporator to obtain a crude extract. The crude extracts were partitioned with n-hexane (H), dichloromethane (DCM), and ethyl acetate (EtOAc), respectively. The % yield is calculated with the formula below:

$$\%\ \text{Yield} = \frac{\text{Extract weight (g)}}{\text{Air dried leaves (g)}} \times 100$$

2.2.2 TLC-bioautography assay

Potential antioxidant compounds from the *V. pinnata* Linn leaves can be determined through TLC-bioautography using the DPPH reagent procedure [30] with slight modification. Samples on TLC plates that had been eluted were air-dried at room temperature for 1 hour. Then, the TLC plates were sprayed with 0.02 mM DPPH reagent (w/v in methanol-acetone). The tested plates were allowed to react with the DPPH reagent for 30 minutes. The presence of a yellow spot on a purple background indicated the antioxidant activity of the samples [31].

2.2.3 Purification and characterization

Ethyl acetate extract (50 gr), which showed better antioxidant potential, was purified by Vacuum Liquid Chromatography (VLC) technique with silica gel GF_{254} as a stationary phase and H-EtOAc-MeOH as the mobile phase. The positive antioxidant fraction was separated by column chromatography with silica gel (70-230 mesh) as the stationary phase and H-EtOAc-MeOH as eluent. The isolated compounds were characterized by UV-Vis, FTIR, and NMR spectroscopy. The DPPH assay determined the antioxidant activity of the isolated compound.

2.2.4 DPPH radical scavenging activity test

The antioxidant activity was carried out by DPPH assay [32] with modifications. A total of 1 mL of 0.02 mM DPPH solution was added to a test tube containing 3 mL of the tested sample dissolved with MeOH-Acetone (9.5:0.5) in several concentrations (0.5, 1, 2, 3, 4 mg/L). The mixture was incubated for 30 minutes at room temperature, and the absorbance was measured at λ 517 nm. Ascorbic acid was used as a positive control. All analysis was conducted in triplicate. The formula calculates the following formula calculated inhibition percentage:

% Inhibition= (Absorbance control-Absorbance sample / Absorbance control) ×100

3. RESULTS AND DISCUSSION

3.1 Extraction

In this study, the *V. pinnata* Linn leaves was extracted by a maceration method using MeOH solvent at room temperature to prevent the degradation of the thermo-labile compounds [33], since heated-assisted extraction can cause a decrease in the levels of phenolic compounds and increase radical activity in the material [34]. In addition, MeOH was used due to its universal properties that can extract polar, semi-polar, and non-polar compounds out of the plant cell matrix. MeOH is also a suitable solvent for extracting phenolic compounds [35-36].

Further fractionation of the crude methanolic extract produced several fractions with different yield percentages (Table 1). The results showed the yield variation of the fractions in the order of ethyl acetate > methanol > dichloromethane > hexane. The ethyl acetate fraction had the highest yield (Fig. 1). It indicated that *V. pinnata Linn* leaves have a wide range of semi-polar compounds, such as polyphenolic compounds of flavonoids [37-38]. High contents of phenolic content in the ethyl acetate fractions were also reported by Shafie et al. [39] working with *Vitex pinnata* leaves (33.1 ± 0.1 mg QE/g), de Brum et al. [40] working with *Vitex megapotamica* leaves (522.4 ± 1.12 mg GAE/g), Gothai et al. [41] with *Moringa oleifera* leaves (65.81 ± 0.01 mg GAE/g), Lasboi et al. [42] with *Muntingia calabura* L leaves (74.90 mg GAE/g), Okselni et al. [43] with *Elaeocarpus mastersii* Kings roots, leaves, and stem bark (362.88 ± 1.89 mg GAE/g, 380.99 ± 2.14 mg GAE/g, and 341.89 ± 3.97 mg GAE/g), and studies Tinco-Jayo et al. [44] with *Jatropha macrantha* Müll Arg. L leaves and stems (359 ± 5.21 mg GAE/g and 306 ± 1.93 mg GAE/g).

3.2 Purification and Characterization

TLC with DPPH reagent was applied for all extracts of *V. pinnata L* (Fig. 2). The results showed that the ethyl acetate extract has clear spots among the other extracts. A potential compound with a yellow spot was detected at a retention factor (Rf) of 0.4. Therefore, the ethyl acetate fraction was selected to be purified by several column chromatography types. This purification process led to obtaining compounds 1 and 2.

Table 1. Yield % yield of hexane, dichloromethane, ethyl acetate, and methanol fractions of *Vitex pinnata* Linn

Solvent	Fraction weight (g)	% Yield (w/w)
Hexane	87.48	5.47
Dichloromethane	72.69	4.54
Etyl Acetate	147.16	9.20
Methanol	130.15	8.13

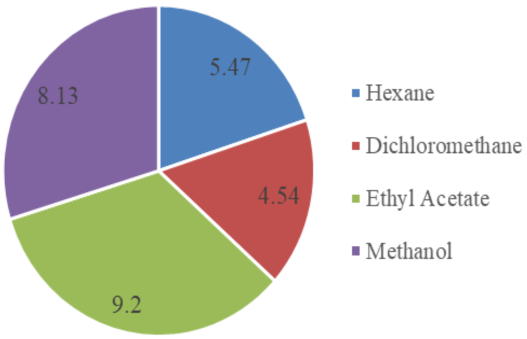


Fig. 1. Fractions distribution of *Vitex pinnata* Linn

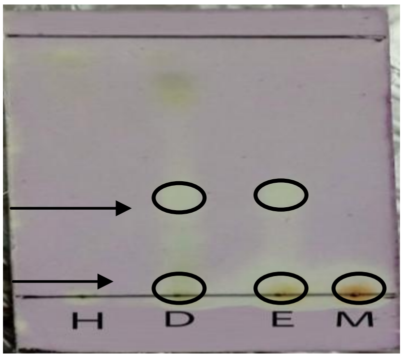


Fig. 2. TLC-DPPH with the eluent of DCM-EtOAc (5:5) for *V. pinnata L* fractions

The purity of the isolated compounds was evaluated by TLC using several eluent ratios. A constant single spot on the TLC plate in various eluent ratios indicated the high purity of the isolated compound with the Rf value of 0.28, 0.42, and 0.56 for compound **1** and 0.13, 0.31, and 0.47 for compound **2**. While a yellow spot on the TLC with DPPH reagent was only detected from compound **2**. It indicated that compound 1 was not active as a DPPH radical scavenging.

The structure of compound 1 was determined by UV, IR, and NMR data. Based on the UV spectrum, compound 1 gives an absorption band at a maximum wavelength of λ_{max} 253 nm, indicating there is benzoate acid with para hydroxyl substituent [45]. Supported by IR spectral data showed represents the acid compound that indicated absorption at wave number (vmax 3448 cm^{-1}) from the (–OH) stretching in alcohols, a strong peak from carbonyl group (C=O) at wave number (vmax 1651 cm^{-1}), the broader peaks from (-OH) stretching vibration from acids that were overlapping with (C-H) stretching at (vmax 2818 cm^{-1}- 2542 cm^{-1}) [46].

The ^{1}H NMR spectrum from compound 1 (Table 2) indicated the presence of phenolic protons by a chemical shift at δ 4.9-7.5 ppm [47]. The presence of protons in the signaling δ 7.872 ppm indicates their position is less protected from the magnetic field when compared to the protons that appear in the signaling 6.887 ppm. The presence of an electron donor group (-OH) in a benzene ring causes resonance with high electronegativity in ortho and para proton substituents [48]. The increasing electronegativity in the area closest to the electron donor group causes the proton to be more protected so that it has a slight chemical shift value (δ) [49]. The appearance of two signaling protons at 7.87 ppm and 6.89 ppm, which are paired together, indicates that the protons are in the same chemical environment. Based on the analysis of the coupling constant (J) according to field et a [50], it is known that there is a proton at the H-4 position paired with a doublet of doublets multiplicity with H-3 at the ortho position with a J value of 6 Hz and paired at the meta position with H-6 which is indicated by a value of J at 2.5 Hz.

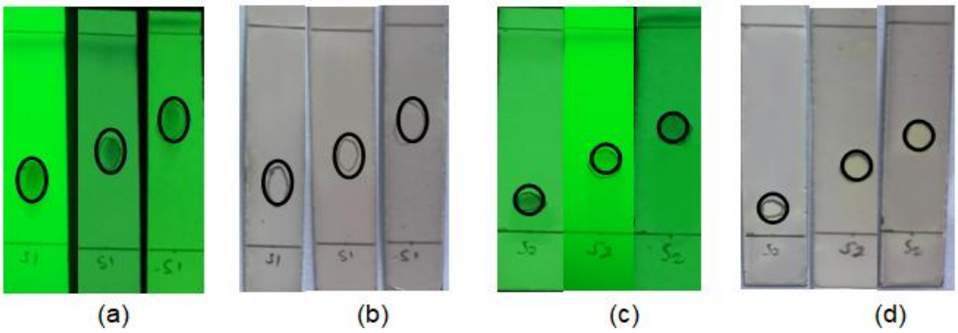


Fig. 3. TLC bioautography two pure compound DCM: EtOAC with eluent (6:4, 4:6, 3:7) (a) compound 1 UV_{254} nm, (b) with DPPH reagent, (c) compound 2 UV_{254} nm dan (d) with DPPH reagent

The presence of a carbonyl group (C=O) in the structure of compound **1** at ^{13}C NMR (Table 2) was indicated by a chemical shift at C-1 (δ 166.79 ppm) [51]. Chemical shifts δ 131.91 ppm and δ 115.14 ppm from DEPT 135 data indicate the presence of four methine carbons (C-H) with overlapping spectra due to the same chemical environment. The structure of compound **1** was determined by comparing the spectral data with the literature [52] and assigned as p-hydroxybenzoic acid has been isolated from bark (Fig. 4a). This compound has been reported to have been isolated from the leaves of *V. pubescens Vahl* by Mastura et al. [53] *V. agnus-castus* [54], bark and leaves of *V. negundo Linn* [55].

Srtructure determining of compound 2 was identified using UV, IR, and NMR data. Based on the UV spectrum, it is known that flavonoid is indicated by absorption at a maximum wavelength λ_{max} 247 nm (aromatic chromophore), and λ_{max} 345 nm (carbonyl chromophore that are conjugated with the aromatic ring). The predicted structure from UV data was supported by IR data that showed the presence of (-OH) stretching with very broad and unstructured band at 3411-3501cm^{-1}, 2688-2627cm^{-1} (C-H), 1646cm^{-1} (C=O) [56]. A chemical shift at δ 182.232 ppm at ^{13}C NMR (Table 2) indicated the presence of a carbonyl group (C=O). The appearance of six peaks in the DEPT 135 spectrum indicates the presence of six methine carbons (CH) at chemical shifts δ 93.847 ppm, δ 98.848 ppm, δ 103.379 ppm, δ 113.265 ppm, δ 115.780 ppm, and δ 119.322 ppm, as well as nine quaternary carbon (C). This finding was confirmed by ^{1}H NMR spectral data (Table 2), which showed six protons signaling at a chemical shift > δ 6 ppm representing aromatic protons.

The presence of three aromatic protons signaling in the spin ABX system, at proton 7.48 ppm (H-6') with doublet-doublet multiplicity matched with a proton at the meta position of proton 7.46 ppm (H-2') with coupling constant (*J*) 2.5 Hz and the ortho of the proton 6.98 ppm with a *J* value of 8.5 Hz. This statement is also supported by the interpretation of COSY data, which shows the formation of a diagonal field at 7.48 ppm, 7.46 ppm, and 6.98 ppm signaling [47].

The results from elucidating structure of compound 2, it was assigned as a luteolin compound supported by HMBC analysis (Fig. 4b). The HMBC analysis showed the correlation between the proton at δ 6.55 ppm (H-3) and several carbons, including C-9, C-1', C-2, and C-4, which confirmed the presence of aliphatic carbon at C-3. The leaves of *V. pinnata L* have reported four isolated compounds by Ata et al. [26]: pinnatoside, viscoside, apigenin, and luteolin. Luteolin has also been reported to be isolated from aerial parts of *Ixeris sonchifolia* [57], flowers of *Dendranthema morifolium* Ramat Tzvel [58], leaves of *Eclipta alba* [59], *Syzygium myrtifolium* leaf [60], *Sterculia foetida* Linn leaf [61], *Codonopsis clematidea* leaf [62], *Gymnanthemum amygdalinum* flower [63].

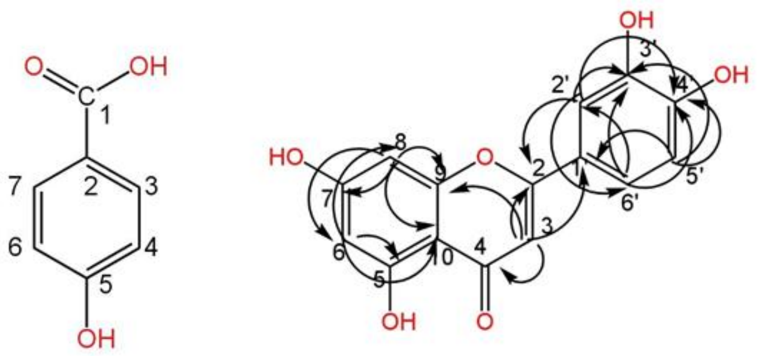
3.3 DPPH Radical Scavenging Activity

Antioxidant properties testing with DPPH showed that the isolated luteolin compound had an intense DPPH radical scavenging activity with an IC_{50} value of 1.56 ± 0.18 μg/mL. Furthermore, at the concentration of 3 μg/mL, luteolin showed a higher radical inhibition percentage than ascorbic acid as a positive control, with a value of 80.29% for luteolin and 56.34% for ascorbic acid. This finding was in line with previous studies through several antioxidant assays. For instance, its value was IC_{50} 18.3 ± 0.2 μg/mL [64], EC_{50} 49.36 ± 0.22 μM [64], and IC50 2.099 ± 0.0587 [65] for DPPH assay, EC_{50} 15.69 ± 0.23 μM [66] and IC_{50} 0.59 ± 0.0208 μg/mL [65] for ABTS assay and 37.58 ± 0.51 μM [66] for FRAP assay. Moreover, luteolin was reported to have excellent 3T3-L1 adipocyte differentiation inhibitory activity through reduced ROS generation [66]. It confirmed that the isolated flavonoid compound of luteolin is a promising antioxidant compound.

The antioxidant activity of a flavonoid is caused by the number and position of hydroxy substituents in ring B. Luteolin is higher in antioxidants than naringenin and pinocembrin because it has more hydroxy substituents in ring B [67]. The hydroxy substituent acts as a donating electron which will weaken the bond dissociation energy (BDE) [68]. The smaller the BDE value, the greater the potential for hydrogen atom transfer (HAT), which means the radical scavenging activity becomes greater. The effect of the hydroxy group on the ortho position (C-3') will weaken the electron density from 4'-OH so that it experiences HAT [69]. In addition, the presence of a 3-OH substituent in the C ring, the double bond between C2-C3 will have a conjugation effect on the 4-oxo bond (C=O), which allows electron delocalization to occur, thereby increasing radical scavenging activity [70].

Table 2. NMR chemical shift of isolated compound

No	Compound 1		Compound 2		
	δC in ppm	**δH in ppm (multiplicity, J)**	**δC in ppm**	**δH in ppm (multiplicity, J)**	**HMBC**
1	166.79	-	-	-	
2	121.84	-	164,01	-	
3	131.91	7.87 (dd, J = 2.5 and 6 Hz)	103.38	6.56 (brs)	C-9, C-1', C-2, C-4
4	115.14	6.89 (dd, J = 2.5 and 6 Hz)	182.23	-	
5	161.78	-	162.55		
6	115.14	6.89 (dd, J = 2.5 and 6 Hz)	98.85	6.22 (brd, $J_{H(6\rightarrow8)}$ = 2.0 Hz)	C-5, C-10, C-8
7	131.91	7.87 (dd, J = 2.5 and 6 Hz)	164.29		
8			93.85	6.50 (brd, $J_{H(8\rightarrow6)}$ = 2.5 Hz)	C-6, C-10, C-9, C-7
9			157.95	-	
10			104.55	-	
1'			122.95	-	
2'			113.27	7.46 (d, J = 2.5 Hz)	C-2, C-3', C-4', C-6'
3'			145.62		
4'			149.20	-	
5'			115.78	6.98 (d, J = 8.5 Hz)	C-1', C-3', C-4'
6'			119.32	7.48 (dd, $J_{H(6'\rightarrow2', 6'\rightarrow5')}$= 2.0 Hz and 8.0 Hz)	C-2', C-3', C-4',



(a) (b)

Fig. 4. Prediction structure for p-hydroxybenzoate (a); and luteolin with key HMBC (H →C) (b)

On the other hand, compound **1** (p-hydroxybenzoic acid), although a derivative of phenolic compounds, did not show antioxidant activity when bioautography was performed with TLC. It is assumed due to the absence of transfer of hydrogen atoms by p-hydroxybenzoic acid to form DPPH-H [71]. The influence of the number of hydroxyl groups and the position of the hydroxyl substituent (-OH) in benzoic acid affects the release of hydrogen atoms. The ortho position of the monohydroxy substituent shows potent antioxidant activity, but for the dihydroxy, the meta position is preferred [72]. Compound **1** has one hydroxy substituent (-OH), which is in the para position to the carboxylic group (-COOH), which causes the hydrogen atom release process to take longer. As a result, the DPPH radical scavenging that occurs is not significant. The DPPH radical scavenging activity of p-hydroxybenzoic is also evidenced by the research of Farhoosh et al. [73] who reported no antioxidant activity of p-hydroxybenzoic compounds against DPPH radicals. Therefore, the carboxylic acid groups affect the antioxidant activity of phenolic acids according to their electron-donating ability in the following order: -CH_2COOH > -CH = CHCOOH > -COOH [71].

4. CONCLUSION

The results of the isolation of compounds guided by TLC-bioautography with DPPH reagents obtained *p*-hydroxybenzoate (**1),** and luteolin (**2**), where compound **2** showed a 50% DPPH radical scavenging activity of 1.56±0.18 µg/mL with a powerful antioxidant agent category. Thus, it can be concluded that the isolation of compounds in the ethyl acetate extract of the leaves of *Vitex pinnata Linn* with this technique succeeded in isolating compounds that have the potential as antioxidants. The TLC-antioxidant bioautography technique has several advantages. It does not require a long time in testing, the sample used is small, and it can potentially obtain promising antioxidant-positive compounds.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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