

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

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 $1.56 \pm 0.18 \mu\text{g/mL}$.

Keywords: Antioxidant; Structure Elucidation; TLC-Bioautography; Vitex pinnata Linn.

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 (-OCH₃) 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. MATERIAL AND METHODS

2.1 Material

The leaves of *V. pinnata* Linn were air-dried and grinded to produce the leaf 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) (Himedia-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 GF₂₅₄ 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.

2.3 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:

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

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 (Figure 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).

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 |
| Ethyl Acetate | 147.16 | 9.20 |
| Methanol | 130.15 | 8.13 |

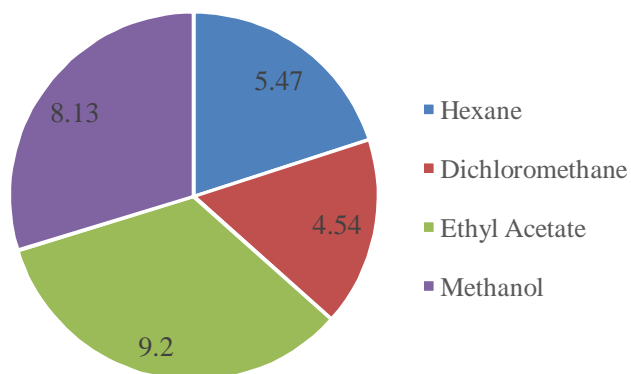


Figure 1. Fractions distribution of *Vitex pinnata* Linn

3.2. Purification and Characterization

TLC with DPPH reagent was applied for all extracts of *V. pinnata* L (**Figure 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**.

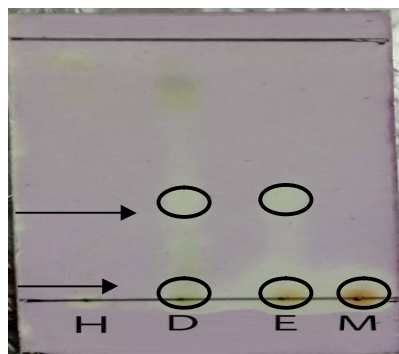


Figure 2. TLC-DPPH with the eluent of DCM-EtOAc (5:5) for *V. pinnata* L fractions

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.

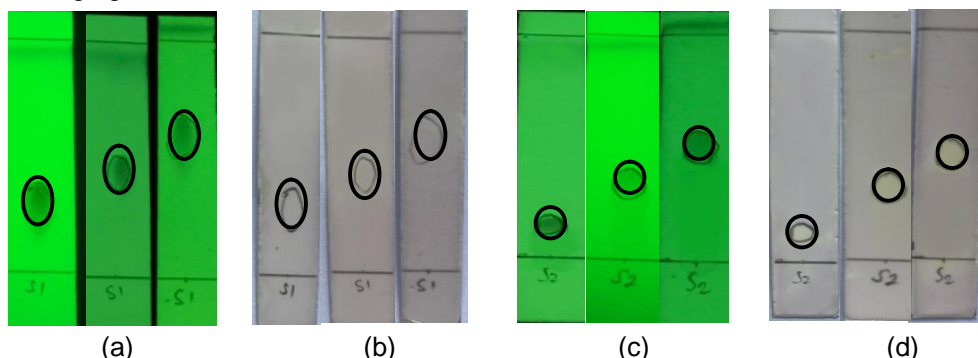


Figure 3. TLC bioautography two pure compound DCM: EtOAc with eluent (6:4, 4:6, 3:7) (a) compound **1** UV₂₅₄ nm, (b) with DPPH reagent, (c) compound **2** UV₂₅₄ nm dan (d) with DPPH reagent.

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 (ν_{\max} 3448 cm^{-1}) from the (-OH) stretching in alcohols, a strong peak from carbonyl group (C=O) at wave number (ν_{\max} 1651 cm^{-1}), the broader peaks from (-OH) stretching vibration from acids that were overlapping with (C-H) stretching at (ν_{\max} 2818 cm^{-1} - 2542 cm^{-1}) [46].

The ^1H 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.

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 (**Figure 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].

Structure 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 $3411\text{-}3501\text{cm}^{-1}$, $2688\text{-}2627\text{cm}^{-1}$ (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 ^1H NMR spectral data (**Table 2**), which showed six protons signaling at a chemical shift $> \delta$ 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 (**Figure 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].

Table 2. NMR chemical shift of isolated compound

| No | Compound 1 | | Compound 2 | | HMBC |
|----|-------------------------|---|-------------------------|--|-----------------------|
| | δC in ppm | δH in ppm (multiplicity, J) | δC in ppm | δH in ppm (multiplicity, J) | |
| 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, $J_{\text{H}(6\rightarrow 8)} = 2.0$ 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, $J_{\text{H}(8\rightarrow 6)} = 2.5$ 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, $J_{\text{H}(6'\rightarrow 2')}, 6'\rightarrow 5') = 2.0$ Hz and 8.0 Hz) | C-2', C-3', C-4' |

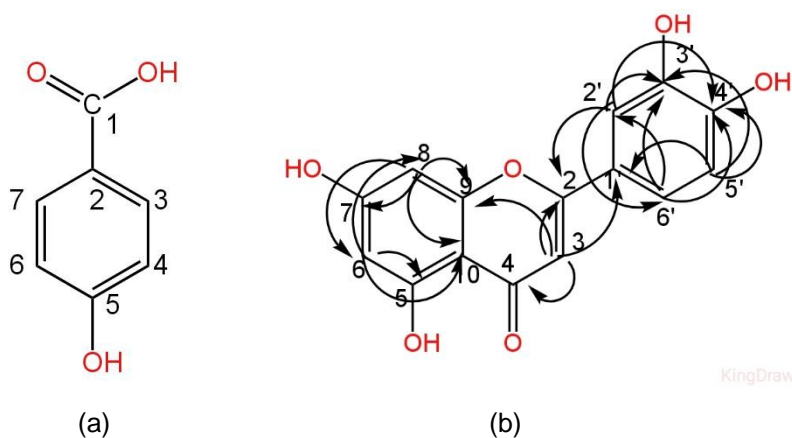


Figure 4. Prediction structure for p-hydroxybenzoate (a); and luteolin with key HMBC (H→C) (b)

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 IC₅₀ 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 IC₅₀ 18.3 ± 0.2 µg/mL [64], EC₅₀ 49.36 ± 0.22 µM [64], and IC₅₀ 2.099 ± 0.0587 [65] for DPPH assay, EC₅₀ 15.69 ± 0.23 µM [66] and IC₅₀ 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].

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: -CH₂COOH > -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.

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None

Competing interests

Authors have declared that no competing interests exist.

Authors' Contributions

All authors read and approved the final manuscript.

Consent (where ever applicable)

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

REFERENCES

1. Ávila-Román J, Soliz-Rueda JR, Bravo FI, et al. Phenolic compounds and biological rhythms: Who takes the lead? *Trends Food Sci Technol.* 2021;113. doi:10.1016/j.tifs.2021.04.050
2. Nollet LML, Gutierrez-Urbe JA. *Phenolic Compounds in Nature. Phenolic Compounds in Food: Characterization & Analysis.* (Leo M.L. Nollet JAGU, ed.). Taylor & Francis; 2018. doi:https://doi.org/10.1201/9781315120157
3. Anantharaju PG, Gowda PC, Vimalambike MG, Madhunapantula S V. An overview on the role of dietary phenolics for the treatment of cancers. *Nutr J.* 2016;15(1). doi:10.1186/s12937-016-0217-2
4. Günes-Bayir A, Kiziltan HS, Kocyigit A, Güler EM, Karataş E, Toprak A. Effects of natural phenolic compound carvacrol on the human gastric adenocarcinoma (AGS) cells in vitro. *Anticancer Drugs.* 2017;28(5). doi:10.1097/CAD.0000000000000491
5. Berrani A, Marmouzi I, Bouyahya A, et al. Phenolic Compound Analysis and Pharmacological Screening of Vitex agnus-castus Functional Parts. *Biomed Res Int.* 2021;2021. doi:10.1155/2021/6695311
6. Haghghi TM, Saharkhiz MJ. Phytotoxic potential of Vitex pseudo-negundo leaf and flower extracts and analysis of phenolic compounds. *Biocatal Agric Biotechnol.* 2021;34. doi:10.1016/j.bcab.2021.102018
7. Itam A, Wati MS, Agustin V, Sabri N, Jumanah RA, Efdi M. Comparative Study of Phytochemical, Antioxidant, and Cytotoxic Activities and Phenolic Content of Syzygium aqueum (Burm. f. Alston f.) Extracts Growing in West Sumatera Indonesia. *Sci World J.* 2021;2021. doi:10.1155/2021/5537597
8. Sahare K. Antifilarial Screening of Vitex negundo L. Leaves Compound. *J Sci Res.* 2021;65(6). doi:10.37398/jsr.2021.650622
9. Liu X, Tian R, Tao H, et al. The cardioprotective potentials and the involved mechanisms of phenolic acids in drug-induced cardiotoxicity. *Eur J Pharmacol.* 2022;936:175362. doi:10.1016/j.ejphar.2022.175362

10. Tirado-Kulieva VA, Hernández-Martínez E, Choque-Rivera TJ. Phenolic compounds versus SARS-CoV-2: An update on the main findings against COVID-19. *Heliyon*. 2022;8(9). doi:10.1016/j.heliyon.2022.e10702
11. Luo Y, Shang P, Li D. Luteolin: A Flavonoid that has multiple cardio-protective effects and its molecular mechanisms. *Front Pharmacol*. 2017;8. doi:10.3389/fphar.2017.00692
12. Tungmunnithum D, Thongboonyou A, Pholboon A, Yangsabai A. Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. *Medicines*. 2018;5(3). doi:10.3390/medicines5030093
13. Alotaibi BS, Ijaz M, Buabeid M, Kharaba ZJ. Therapeutic Effects and Safe Uses of Plant-Derived Polyphenolic Compounds in Cardiovascular Diseases: A Review. Published online 2021:4713-4732.
14. Kowalczyk M, Golonko A, Świsłocka R, et al. Drug Design Strategies for the Treatment of Viral Disease. Plant Phenolic Compounds and Their Derivatives. *Front Pharmacol*. 2021;12(July):1-21. doi:10.3389/fphar.2021.709104
15. Zeb A. Concept, mechanism, and applications of phenolic antioxidants in foods. *J Food Biochem*. 2020;44(9):1-22. doi:10.1111/jfbc.13394
16. Chen J, Yang J, Ma L, Li J, Shahzad N, Kim CK. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. Published online 2020:1-9.
17. Islam N. Investigation of comparative shielding of Morin against oxidative damage by radicals: A DFT study. 2015;(December). doi:10.1080/23312009.2015.1078272
18. Gershoni-Poranne R, Rahalkar AP, Stanger A. The predictive power of aromaticity: Quantitative correlation between aromaticity and ionization potentials and HOMO-LUMO gaps in oligomers of benzene, pyrrole, furan, and thiophene. *Phys Chem Chem Phys*. 2018;20(21):14808-14817. doi:10.1039/c8cp02162g
19. Van Die M., Burger H., Teede HJ, Bone K. *Vitex agnuscastus* extracts for female reproductive disorders: a systematic review of clinical trials. *Planta Med*. 2013;79:562–575. doi:10.1007/s00044-017-1937-3
20. Kamal N, Mio Asni NS, Rozlan INA, et al. Traditional medicinal uses, phytochemistry, biological properties, and health applications of *Vitex* sp. *Plants*. 2022;11(1944):1-33. doi:10.3390/plants11151944
21. Tewari M, Mahawer SK, Kumar R, Prakash O. A comparative study of selected vitex species for phenolics estimation along with their antioxidant and herbicidal activities. *J Indian Chem Soc*. 2022;99(11):100723. doi:10.1016/j.jics.2022.100723
22. Dwivedi MK, Shukla R, Sharma NK, et al. Evaluation of ethnopharmacologically selected *Vitex negundo* L. for In vitro antimalarial activity and secondary metabolite profiling. *J Ethnopharmacol*. 2021;275(December 2020). doi:10.1016/j.jep.2021.114076
23. Huang Y, Ding GY, Hu P. Vitexnegheteroin M, a new phenolic glycoside from *Vitex negundo* var. *heterophylla*. *Nat Prod Res*. 2021;35(9):1518-1524. doi:10.1080/14786419.2019.1656628
24. Djimabi K, Li, B.; Chen XH, Su P., et al. Chemical constituents from the fruits of *Vitex trifolia* L. (Verbenaceae) and their chemotaxonomic significance. *Biochem Syst Ecol*. 2021;97(104305):1-5. doi:10.1016/j.bse.2021.104305
25. Vo G Van, Nguyen THT, Nguyen TP, et al. In silico and in vitro studies on the anti-cancer activity of artemetin, vitexicarpin and penduletin compounds from *Vitex negundo*. *Saudi Pharm J*. 2022;30(9):1301-1314. doi:10.1016/j.jsps.2022.06.018
26. Ata A., Mbong N., Iverson CD., Samarasekera R. Minor chemical constituents of *Vitex pinnata*. *Nat Prod Commun*. 2009;4:1-4. doi:10.1177/1934578x0900400102
27. Kamal N, Clements C, Gray AI, Ebel RE. Discovery of bioactive metabolites from the leaves of *Vitex pinnata* using high-throughput flash chromatography. *Planta Med* 2011, 77, <https://doi.org/10.1055/s-0031-1282552>

28. Anwar L, Efdi M, Ninomiya M, et al. Labdane diterpene lactones of *Vitex pubescens* and their antileukemic properties. *Med Chem Res.* 2017;26(10):2357-2362. doi:10.1007/s00044-017-1937-3
29. Dewanjee S., Gangopadhyay M., Bhattacharya N., Khanra R., Dua TK. Bioautography and its scope in the field of natural product chemistry. *Pharm Anal.* 2015;5(2):75-84. doi:10.1016/j.jpha.2014.06.002
30. Purkait S, Bhattacharya A, Bag A, Chattopadhyay RR. Correction to: TLC bioautography-guided isolation of essential oil components of cinnamon and clove and assessment of their antimicrobial and antioxidant potential in combination (*Environmental Science and Pollution Research*, (2021), 28, 1, (1131-1140), 1. *Environ Sci Pollut Res.* 2022;29(49):75101. doi:10.1007/s11356-022-23261-9
31. Praptiwi, Ilyas M, Palupi K., et al. Rapid Screening Of Antibacterial And Antioxidant Metabolites From Endophytic Fungi Isolated From *Papuacedrus papuana* By Tlc-Bioautography. *J Kim dan Kemasan.* 2021;43(2):110-116. <http://dx.doi.org/10.24817/jkk.v43i2.6980>
32. Januarti R, Santoni A, Efdi M. Isolation Roxbof Flavonoid Compound and Antioxidant Activity of *Salix tetrasperma*. *Indones J Fundam Appl Chem.* 2019;4(2):42-46. doi:10.24845/ijfac.v4.i2.42
33. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Med (United Kingdom).* 2018;13(1):1-26. doi:10.1186/s13020-018-0177-x
34. Maghsoudlou Y, Asghari Ghajari M, Tavasoli S. Effects of heat treatment on the phenolic compounds and antioxidant capacity of quince fruit and its tisane's sensory properties. *J Food Sci Technol.* 2019;56(5):2365-2372. doi:10.1007/s13197-019-03644-6
35. Mahasuari NPS, Paramita NLPV, Yadnya Putra A. GR. EFFECT OF METHANOL CONCENTRATION AS A SOLVENT ON TOTAL PHENOLIC AND FLAVONOID CONTENT OF BELUNTAS LEAF EXTRACT (*Pulchea indica* L.). *J Pharm Sci Appl.* 2020;2(2):77. doi:10.24843/jpsa.2020.v02.i02.p05
36. Ramos MT, Fort JB, Watson NJ, Lopez IIR, Galicia GC, Jimenez EC. Effect of solvent composition and its with ultrasonic energy on the ultrasound-assisted extraction of phenolic compounds from mango peel (*Mangifera indica* L.). *Food Bioprod Process.* 2020;122:41-54. doi:10.1016/j.fbp.2020.03.011
37. Sánchez-Valdepeñas V, Barraón E, Vegara S, et al. Effect of instant controlled pressure drop (DIC) pre-treatment on conventional solvent extraction of phenolic compounds from grape stalk powder. *Ind Crops Prod.* 2015;76(December 2015):545-549. doi:10.1016/j.indcrop.2015.04.033
38. Pintaća D, Majkića T, Torovićb L, et al. Solvent selection for efficient extraction of bioactive compounds from grape Pomace. *Ind Crop Prod.* 2018;111:379-390. doi:10.1016/j.indcrop.2017.10.038
39. Shafie NA, Suhaili NA, Taha H, Ahmad N,. Evaluation of antioxidant, antibacterial and wound healing activities of *Vitex pinnata*. *F1000Research.* 2020;9:1-17. doi:10.12688/f1000research.21310.2
40. De Brum TF, Zadra M, Piana M, et al. HPLC analysis of phenolics compounds and antioxidant capacity of leaves of *Vitex megapotamica* (Sprengel) moldenke. *Molecules.* 2013;18(7):8342-8357. doi:10.3390/molecules18078342
41. Gothai S, Muniandy K, Zarin M., et al. Chemical composition of *Moringa oleifera* ethyl acetate fraction and its biological activity in diabetic human dermal fibroblasts. *Phcog Mag.* 2017;13:462-469. doi:10.4103/pm.pm_368_16
42. Lasboi E. R, Rissyelly, Katrin K. Angiotensin i-converting enzyme inhibitory activity, total phenolic and flavonoid content of extract and fraction of jam fruit leaves (*Muntingia calabura* L.). *Pharm Clin Res.* 2017;10(17):166-168. doi:10.22159/ajpcr.2017.v10s5.23123

43. Okselni T, Santoni A, Dharma A, Efdi M. Determination of Antioxidant Activity, Total Phenolic Content, and Total Flavonoid Content of Roots, Stem Bark, And Leaves of *Elaeocarpus mastersii* KING. *Rasayan J Chem.* 2018;11(3):1211-1216. doi:10.31788/RJC.2018.1133058
44. Tinco-Jayo J, Aguilar-Felices E., Enciso-Roca E., Arroyo-Acevedo J., Herrera-Calderon O. Phytochemical Screening by LC-ESI-MS/MS and Effect of the Ethyl Acetate Fraction from Leaves and Stems of *Jatropha macrantha* Müll Arg. on Ketamine-Induced Erectile Dysfunction in Rats. *Molecules.* 2022;27(115). doi:10.3390/molecules27010115
45. Pretsch E, Bhulmann P, Badertscher M. *UV/Vis Spectroscopy.* 5th ed. Springer Berlin Heidelberg; 2020. doi:10.1007/978-3-662-62439-5
46. Thompson JM. *Some Fundamentals of Infrared Spectroscopy.* *Infrared Spectroscopy.* Pan Stanford Publishing; 2018. <https://doi.org/10.1201/9781351206037-1>
47. Field L., Li L., Magill A. *Organic Structure from 2D NMR Spectra.* John Wiley & Sons; 2015. <https://www.pdfdrive.com/organic-structures-from-2d-nmr-spectra-e184624679.html>
48. Jacobsen N. *NMR Data Interpretation Explained: Understanding 1D and 2D NMR Spectra of Organic Compounds and Natural Product.* John Wiley & Sons; 2017. <https://lccn.loc.gov/2016009193>
49. Yurkanis BP. *Organic Chemistry.* 8th ed. Pearson Education; 2015. <https://www.pdfdrive.com/organic-chemistry-e182031466.html>
50. Field L., Sternhell S, Kalman J. *Organic Structure from Spectra, 5th Ed.* 5th ed. Wiley & Sons; 2013. <https://www.pdfdrive.com/organic-structures-from-spectra-5-ed-e186418058>
51. Silverstein R., Webster F., Kiemle D., Bryce D. *Spectrometric Identification of Organic Compounds.* 8th ed. Wiley & Sons; 2015.
52. Anwar L, Santoni A, Putra DP, Efdi M. JCNaR Journal of Chemical Natural Resources Structure Elucidation of aPentacyclicTriterpenoid and Phenolic from Stem Bark of *Vitexpubescens*Vahl. *J Chem Nat Resour.* 2019;01(01):68-74.
53. Mastura M, Barus T, Marpaung L, Simanjuntak P. Isolation and Antioxidant Activity of Phenolic Compounds from Halban Leaves (*Vitex pinnata* Linn) in Aceh. *Elkawnie.* 2020;6(2):213. doi:10.22373/ekw.v6i2.5532
54. Rudrapaul P, Sarma IS, Das N, De UC, Bhattacharjee S, Dinda B. New flavonol methyl ether from the leaves of *Vitex peduncularis* exhibits potential inhibitory activity against *Leishmania donovani* through activation of iNOS expression. *Eur J Med Chem.* 2014;87:328-335. doi:10.1016/j.ejmech.2014.09.076
55. Badgujar N., Mistry K., Rank D., Joshi CG. Screening of antiproliferative activity mediated through apoptosis pathway in human non-small lung cancer A-59 cells by active compounds present in medicinal plants. *Asian Pac J Trop Med.* 2018;11(12):666-675. <https://doi.org/10.4103/1995-7645-248338>
56. Baranovic G, Segota S. infrared spectroscopy of flavones and flavonols. Reexamination of the hydroxyl and carbonyl vibrations in relation to the interactions of flavonoids with membrane lipids. *Spectrochim Acta Part A Mol Biomol Spectrosc.* 2018;192:473-486. <https://doi.org/10.1016/j.saa.2017.11.057>
57. Zhang Y., Gan F., Shelar S., Ng K., Chew EH. Antioxidant and Nrf2 inducing activities of luteolin, a flavonoid constituent in *Ixeris sonchifolia* Hance, provide neuroprotective effects against ischemia-induced cellular injury. *J Food Chem Toxicol.* 2013;59:272-280. <http://dx.doi.org/10.1016/j.fct.2013.05.058>
58. Lin LC, Pai YF, Tsai TH. Isolation of Luteolin and Luteolin-7-O-glucoside from *Dendranthema morifolium* Ramat Tzvel and Their Pharmacokinetics in Rats. *J Agric Food Chem.* 2015;63(35):7700-7706. doi:10.1021/jf505848z
59. Rufi T, Aditi P, Pankaj J, Jayant S, Gauresh S, Sadhana S. Assessment of luteolin

- isolated from *Eclipta alba* leaves in animal models of epilepsy. *Pharm Biol.* 2016;55(1):264-268. <http://dx.doi.org/10.1080/13880209.2016.1260597>
60. Kusriani RH, Rosandhy SM, Elfahmi E. Luteolin, a flavonoid from *Syzygium myrtifolium* Walp. *Curr Res Biosci Biotechnol.* 2019;1(1):31-33. doi:10.5614/crbb.2019.1.1/fkan4064
 61. Cuong DTD, Dat HT, Duan NT, et al. Isolation and characterization of six flavonoids from the leaves of *Sterculia foetida* Linn. . *Vietnam J Chem.* 2019;57(4):438-442. doi:10.1002/vjch.201900084
 62. Bhardwaja P, Naryala A, Thakura M., et al. Comparative antioxidant, antibacterial, and GC-MS analysis of methanol extract's fractions and isolation of luteolin from leaves of trans-Himalayan *Codonopsis clematidea*. *Crop Prod.* 2020;144(112046). <https://doi.org/10.1016/j.indcrop.2019.112046>
 63. Vonja S, Hartati R, Insanu M. In Vitro Alpha-Glucosidase Inhibitory Activity and the Isolation of Luteolin from the Flower of *Gymnanthemum amygdalinum* (Delile) Sch. Bip ex Walp. *Molecules.* 2022;27(7). doi:10.3390/molecules27072132
 64. Ahmadi SM, Farhoosh R, Sharif A, Rezaie M. Structure-Antioxidant Activity Relationships of Luteolin and Catechin. *J Food Sci.* 2020;85(2):298-305. doi:10.1111/1750-3841.14994
 65. Aruwa C., Amoo S., Koorbanally N, Kudanga T. Enzymatic dimerization of luteolin enhances antioxidant and antimicrobial activities. *Biocatal Agric Biotechnol.* 2021;35:102-105. <https://doi.org/10.1016/j.bcab.2021.102105>
 66. Tiana C, Liua X, Changa Y, et al. Investigation of the anti-inflammatory and antioxidant activities of luteolin, kaempferol, apigenin and quercetin. *South African J Bot.* 2021;137:257-264. <https://doi.org/10.1016/j.sajb.2020.10.022>
 67. Lingli Z, Mengfei Z, Hao C, et al. The activity comparison of six dietary flavonoids identifies that luteolin inhibits 3T3-L1 adipocyte differentiation through reducing ROS generation. *J Nutr Biochem.* Published online 2022. <https://doi.org/10.1016/j.jnutbio.2022.109208>
 68. Kongpichitchoke T, Hsu JL, Huang TC. Number of hydroxyl groups on the B-ring of flavonoids affects their antioxidant activity and interaction with phorbol ester binding site of PKC δ C1B domain: In vitro and in silico studies. *J Agric Food Chem.* 2015;63(18):4580-4586. doi:10.1021/acs.jafc.5b00312
 69. Zheng Y., Deng G, Guo R, Chen D., Fu Z. DFT Studies on the antioxidant activity of naringenin and its derivatives: effects of the substituents at C3. *Int J Mol Sci.* 2019;20(6). <https://doi.org/10.3390/ijms20061450>
 70. Lee C., Anamoah C, Semenya J, et al. Electronic (donating or withdrawing) effects of ortho-phenolic substituents in dendritic antioxidants. *Tetrahedron Lett.* 2020;61(151607). <https://doi.org/10.1016/j.tetlet.2020.151607>
 71. Moazzen A, Öztinen N, Ak-Sakalli E, Koşar M. Structure-antiradical activity relationships of 25 natural antioxidant phenolic compounds from different classes. *Heliyon.* 2022;8(9). doi:10.1016/j.heliyon.2022.e10467
 72. Oroian M, Esriche I. Antioxidants: Characterization, natural sources, extraction and analysis. *Food Res Int.* 2015;74:10-36. <https://doi.org/10.1016/j.foodres.2015.04.018>
 73. Farhoosh R, Johnny S, Asnaashari M, Molaahmadibahraseman N, Sharif A. Structure-antioxidant activity relationships of o-hydroxyl, o-methoxy, and alky ester derivatives of p-hydroxybenzoic acid. *Food Chem.* 2016;194:128-134. doi:10.1016/j.foodchem.2015.08.003