ISOLATION AND ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS FROM HALBAN LEAVES (*Vitex pinnata* Linn) IN ACEH

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Abstract: An isolation and identification of phenolic compounds from Halban Leaves (*Vitex pinnata* Linn), a traditional medicinal plant in Aceh, has been done. Halban leaves were extracted through methanol maceration followed by isolation. The methanol extract went into n-hexane and ethyl acetate partition consecutively resulting in 3 different fractions of ethyl acetate and n-hexane extract. The ethyl acetate fraction demonstrated the most promising antioxidant and cytotoxic activity. Therefore, purification of ethyl acetate fraction was done by column chromatography [SiO_2; (i) *n*-hexane-ethyl acetate 10:1, 1:1 ; (ii) *n*-hexane-ethyl acetate = 2 : 1); (iii) n-hexane-acetone = 1:1]. The pure isolate as identified by using UV, IR, NMR, and MS spectra afforded 2 phenolic compounds, namely (1) 4-hydroxymethyl benzoate and (2) *p*-hydroxymethyl benzoic acid (PHBA). The antioxidant activity of compound 1 and cytotoxicity activity of 2 expressed in IC_{50} and LD_{50} was 41.08 ppm and 59.41 ppm, respectively.

Keywords: *V. Pinnata* Linn; Halban leaves; phenolic compound; Aceh; bioactivity

Abstrak: Isolasi dan identifikasi senyawa fenolik dari Daun Halban (*Vite xpinnata* Linn) tanaman obat tradisional asal Aceh telah dilakukan. Isolasi ini dilakukan dengan cara mengekstraksi daun halban dengan cara maserasi menggunakan pelarut metanol, Ekstrak metanol kemudian dipartiisi dengan *n*-heksan dan etilasetat secara berturut-turut sehingga diperoleh tiga ekstrak yaitu ekstrak etil asetat, *n*-heksan dan air. Ekstrak etil asetat memiliki bioaktivitas sebagai antioksidan dan juga toksisitas. Pemurnian dilanjutkan dengan cara kromatografi kolom [SiO_2; (i) *n*-heksan-etilasetat 10:1, 1:1 ; (ii) *n*-heksan-etilasetat = 2 : 1); (iii) n-heksan-aseton = 1:1] dan diperoleh isolat murni. Isolat murni tersebut diidentifikasi berdasarkan interpretasi data spektra UV, IR, NMR dan MS. Hasil interpretasi data menunjukkan daun halban (*V. pinnata* Linn) mengandung dua senyawa fenolik yaitu (1) 4-hidroksi metil benzoat dan (2) asam para hidroksi benzoat (PHBA). Senyawa 1 memiliki aktivitas antioksidan dengan nilai IC_{50} = 41,08bpj dan senyawa 2 memiliki aktivitas toksisitas dengan nilai LC_{50} = 59,41.

Kata Kunci: *V.pinnata* Linn; daun Halban; senyawa fenolik; Aceh; bioaktivitas

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Introduction

Vitex pinnata L is known as laban, halban or "Mane" in Acehnese term which means Asian tropical plants with huge potential as a medicinal plant. Almost all parts of the plants can be applied as medicine. As a traditional medicine, the leaves are widely used to treat wounds and fever also to increase appetite. The stem bark is reported to be able to cure stomach ache, wounds and be used as a colouring agent while the roots can be used as stomach pain medicine (Nuraskin *et al.*, 2019). Nuraskin *et al.*, 2019 reported that the decoction of *V. pinnata* bark could cure stomach pain and the leaves can be used as fever and wounds treatment. People in Aceh use Halban leaves as anti-cholesterol, gastric and high blood pressure medications. The fruits are used for carbuncle and fever (Mastura *et al.*, 2018).

The ethnobotany approach allows us to assume that *V. pinnata* contains active compounds to treat fever, carbuncle, stomach ache and wounds (Nuraskin *et al.*, 2019). Generally, Vitex contains chemical compounds like *para*-hydroxymethyl benzoic acid (PBHA) and agnuside (AGN) in which they can be found in genus *Vitex negundo* and *V. trifolia* (Bello *et al.*, 2017). Roy *et al.*, 2015 also reported that *Vitex negundo* Linn exhibited other herbal activities such as anti-inflammatory, antiarthritic, anti-analgesic and hepatoprotective. In Brunei, the young leaves are consumed to treat hypertension and fever. Tea made of the root of the plants is used to treat back pain, body aches and fatigue (Goh *et al*., 2017). This compound is important to be isolated to determine its chemical structure. The *Vitex pinnata* L. plant is important for testing its antioxidant activity because the compound to be selected for its chemical structure must be biologically active. The objective of the study is to isolate and identify chemical compounds in Halban leaves (*V. pinnata* L) from Aceh and the bioactivity test.

Research Methodology

Materials

Materials used in this study include leaves of *Vitex pinnata* L., methanol, water, n-hexane, ethyl acetate.

Extraction

5 kg of Halban leaves is macerated with methanol for 48 hours in triplicate, filtered and evaporated in a rotary evaporator to obtain dense methanol extract. Water was added to the methanol extract, then filtered. The filtrate was partitioned using n-hexane with 4 repetitions. The fraction was evaporated with a rotary

evaporator, and it gave 3 different types of extract, which are n-hexane, ethyl acetate and water extract (Mastura *et al*., 2017).

Bioactivity: Antioxidant and Toxicity Activity Test

Antioxidant activity was tested based on Molynes procedure in which DPPH concentration has been modified (Molyneux, 2004) whereas toxicity test was done using Brine Shrimp Lethality Test (BSLT). BSLT method is very suitable for the isolated bioactive compounds from plant extracts. Toxicity test was based on Meyer procedure with modifications using live animals (Yang *et al.*, 2015). Extract solutions were made with concentrations of 2000 ppm, 1000 ppm, 200 ppm, 100 ppm, 20 ppm and 10 ppm. The extract used was 40 mg and 5-20 mL of seawater. If the sample is insoluble, 5 drops of 1% DMSO are used. The toxicity test was carried out by inserting 15 larvae of Artemia Salina Leach shrimp for 48 hours into a bottle containing the extract solution and seawater, with the concentration as above. For each concentration carried out 3 times (triple). As a control, seawater was not given a sample extract. The test vials were stored under a TL lamp. Observations were made after 24 hours. The number of shrimp larvae that died was recorded then the percentage of death was calculated. The data obtained were processed using probit analysis.

Purification

Ethyl acetate fraction (9 gr) was purified by column chromatography (SiO_2; n-hexane : ethyl acetate = 10 : 1 ~ 1 : 1) and produced 10 sub-fractions, VDH-1 ~ VDH-10. Next, all fractions were tested for bioactivity, antioxidant and toxicity. From the 10 sub-fractions, VDH-8 and VDH-9 have the highest cytotoxic and antioxidant activity, respectively. VDH-9 sub-fraction was then purified by column chromatography (SiO_2; *n*-hexane : ethyl acetate = 2 : 1 and n-hexane : acetone = 1 : 1) to produce pure compound VDH 9-3-1 so that 9 fractions were obtained. Similarly, VDH-8 was also purified by column chromatography (SiO_2 ; *n*-hexane : ethyl acetate = 5 : 1, 2 : 1 and 1 : 1) until a pure compound VDH 8-3-2 was produced so that 15 fractions were obtained.

Identification

Identification was made to both pure VDH 9-3-1 and VDH 8-3-2 compounds by taking spectra data from Ultra Violet (UV), Infrared (IR), Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) and Mass Spectra. At the characterization stage, the equipment used was the Variant Conc.100 ultraviolet spectrophotometer, the Perkin Elmer Spectrum One FTIR spectrophotometer, the JEOL JNM A5000 NMR spectrometer which operates at 500 MHz NMR (1H) and 125 MHz (13C) frequencies using TMS as the internal standard.

Discussions

The extract of Halban leaves (*Vitex pinnata* Linn) from Aceh gives more water fraction (%) compared to the others (n-hexane and ethyl acetate fraction). Halban leaves are assumed to contain more polar compounds than non-polar or semipolar compounds (Breda *et al*., 2016). The yield of Halban leaves extract can be seen in Table 1.

Table 1. The weight of methanol, n-hexane, ethyl acetate and water fraction from Halban leaves

Fraction Type	Weight (gr)	Yield (%)*)
Methanol	48.8	0.81
n-hexane	4.01	0.06
Ethyl acetate	9.98	0.16
Water	32.91	0.54

*): calculated from 6 kg of dry weight Halban leaves

Bioactive cytotoxic test for VDH 8-3-2 with Brine Shrimp Lethality Test (BSLT) shows LC_{50} (Lethal Concentration) value of 59.41 at 1000 ppm concentration. VDH 3-8-2 compound is a yellowish amorphous crystal (Ntie-Kang *et al*., 2016). The results of the toxicity test can be seen in Table 2.

Table 2. Cytotoxicity Test for VHD-1 to VHD-10 fraction

Fraction Number	Fraction	LC_{50} (mol/L)
1	VDH-1	1,630
2	VDH-2	1,253
3	VDH-3	9,230
4	VDH-4	1,242
5	VDH-5	1,123
6	VDH-6	-2,570
7	VDH-7	1,040
8	VDH-8	59.41
9	VDH-9	-2,310
10	VDH-10	-2,410

An extract is called active and has toxic properties if it causes 50% death of test animal at the concentration below 1,000 ppm and does not have toxic properties if it is found at above 1,000 ppm (Sobolewska *et al.*, 2016; Molyneux, 2004).

Fraction VDH 9 -3-13 obtained is a transparent white crystal. The result of the antioxidant activity test from free radical scavenging method using DPPH (1,1-diphenyl-2-picrylhydrazyl) reagent. The results of antioxidant activity test for VDH 9-3-13 in resistance percentage and IC_{50} values can be seen in Table 3 (Iqbal *et a*l., 2015).

Table 3. Antioxidant activity test of VDH 9-3-13

Fraction/ Sample	Concentration (ppm)	Average Absorbance	%Resistance	IC_{50} (mol/L)
VDH 9-3-13	5	0.660	34.42	41.087
	10	0.428	12.83	
	25	0.182	62.93	
	50	0.039	92.06	
	100	0.026	94.71	

Based on antioxidant activity test shown in Table 3 above, VDH 9-3-13 has IC_{50} (Inhibition Concentration) value of 41.087. This indicates that the compound has a very strong antioxidant activity.

Identification of VDH 8-3-2 and VDH 9-3-13 Compounds based on Spectra Data

Based on UV spectra, VDH 8-3-2 gives a peak at 251.5 nm wavelength. According to (Shah, 2016), the peak at 230 nm wavelength is a carboxylic acid which binds with benzene functional groups (λ230 nm) and OH attached at para position (25 nm). It is predicted that the functional group appears at 255 nm wavelength. While VDH 9-3-13 gives a peak at λ255.5 nm. According to (Shah, 2016), the peak at 230 nm is methyl functional group that is attached with carboxylic acid and benzene functional group (λ230 nm), and OH is binded at para position (25 nm). Infrared spectra show VDH 8-3-2 contains OH functional groups at 3429.43 cm^{-1} wavenumbers, carboxylic acid carbonyl group at 1726.29 cm^{-1} and C=C aromatic benzene group at 1600.02 cm^{-1} wavenumber. Hydroxyl group in Infrared spectra for pure VDH 9-3-13 is shown in 3350 cm^{-1} wavelength, carboxyl group at 1787 cm^{-1} and benzene aromatic is shown at 1606 cm^{-1} (da Silva *et al.*, 2016).

The analysis on NMR from ^{13}C-NMR for VDH 8-3-2 gives 5 signal of C-$sp^{2,}$ which represents 7 carbon atoms. The 5 signals consist of one conjugated carbonyl (carboxylate) (C-7), which is very specific at carbon shifting (δ_C 168.10 ppm). The other 4 signals (C-2, C-6) and (C-3, C-5) have carbon shifting (δ_C137.2 ppm) and (δ_C116.00 ppm), respectively. Benzene at C4 carbon shifts (δ_C162.7 ppm) is oxi aryl carbon. Whereas for C-1 carbon shift is (δ_C122.4 ppm). Spectra data ^{1}H-NMR for VDH 8-3-2 shows that there are two signals for the aromatic area with 2H integration each. Both signals, H-2 and H-6, are found in the shifts, (δ_H 7.93 ppm) and they have doublet multiplicity while H-3 and H-5 have proton shift (δ_H 6.92 ppm). The signals with those characteristics are specific for 1,4-disubstituted benzene. The chemical shift values of ^{1}H-NMR and ^{13}C-NMR from VDH 8-3-2 compound is concluded in Table 4 (Juvik *et al.*, 2016).

Table 4. Chemical shift ^{1}H-NMR and ^{13}C-NMR correlation for VDH 8-3-2 compound (^{1}H-NMR with acetone solvent at a 500 MHz, ^{13}C-NMR with TMS solvent at 125 MHz)

No	^{1}H-NMR			^{13}C-NMR
	δ (ppm)	*I*	*M*	
1	-	-	-	122.40
2	7.93	2H	*D*	132.70
3	6.92	2H	*D*	116.00
4	-	-	-	162.70
5	6.92	2H	*D*	116.00
6	7.93	2H	*D*	132.70
7	-	-	-	168.10

Because the chemical structure of compounds is very simple, only 1-D NMR was used. Based on UV, IR, proton and carbon NMR spectra data, the chemical structure of the pure VDH 8-3-2 compound can be determined as para hydroxybenzoic acid (PHBA). The chemical structure of the compound in Halban leaves from Aceh (*V. pinnata* L) is shown in Figure 1 (Mastura *et al*., 2017).

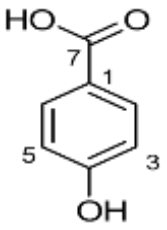

Figure 1. Chemical structure of para hydroxybenzoic acid from Halban leaves isolation

Proton NMR spectra analysis of VDH 9-3-13 shows proton shift at δ_H 3.81 (OCH_3), δ_H 7.89 (2 H) dan δ_H 6.91 (2H) (aromatic functional groups). Carbon NMR gives 8 carbon atoms which consist of five -CH= (d); one (s) -C, one C=O and one OCH_3. The shift value of ^{1}H-NMR ^{13}C-NMR of VDH 9-3-13 is concluded in Table 5.

Table 5. Chemical shift ^{1}H-NMR and ^{13}C-NMR correlation for VDH 9-3-13 compound (^{1}H-NMR with acetone solvent at a 500 MHz, ^{13}C-NMR with TMS solvent at 125 MHz)

No	^{1}H-NMR			^{13}C-NMR
	δ (ppm)	*I*	*M*	
1	-	-	-	122.52
2	7.89	2H	*D*	131.85
3	6.91	2H	*D*	115.48
4	-	-	-	158.67
5	6.91	2H	*D*	115.48
6	7.89	2H	*D*	131.85
7	-	-	-	167.11
8	3.81	3H	*S*	51.81

Based on Ultra Violet (UV) data, there is benzene chromophore functional groups. From infrared (IR), there is OH, C=O and aromatic functional groups. Proton NMR shows OCH_3 functional groups and CH aromatic while carbon NMR gives OCH_3, carbonyl and aromatic. Therefore the chemical structure of VDH 9-3-13 isolated from Halban leaves (*V. pinnata* L) from Aceh can be determined as 4-hydroxy methyl benzoate compound (Mastura *et al*., 2017) (Figure 2).

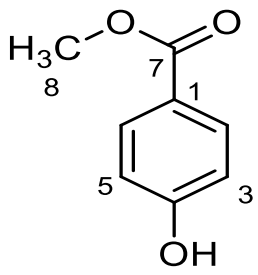

Figure 2. Chemical structure of 4-hydroxy methyl benzoate from Halban leaves isolation.

Conclusions

From the research, it can be concluded that Halban leaves (*V. pinnata* Linn) from Aceh. Ethyl acetate fraction contains phenolic compounds such as para hydroxybenzoic acid (PHBA) and 4-hydroxy methyl benzoate. Based on the test results of all fractions, only PHBA has antioxidant activity, and only 4-hydroxy methyl benzoate has cytotoxic activity. Bioactivity test for both compounds shows that PHBA has toxicity with LC_{50} = 59.41 and 4-hydroxy methyl benzoate has antioxidant activity with IC_{50} = 41.807.

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