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Isolation and Identification of Secondary Metabolites from *Etlingera rubroloba* Rhizome and Their Biological Activity as Antimicrobial and Antioxidant

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ABSTRACT. Etlingera is a genus known for its potential medicinal properties. *Etlingera rubroloba* A.D. Poulsen is a recently identified species within the Etlingera genus found in Southeast Sulawesi.
This study aims to isolate and identify secondary metabolite compounds from the methanol extract of the *E.rubroloba* A.D. Poulsen rhizome and its activity as an antimicrobial and antioxidant. The compound was isolated by using Thin Layer Chromatography (TLC), Vacuum Liquid Chromatography (VLC), and Radial Chromatography (RC) techniques. Isolated compounds were identified by ^{1}H and ^{13}C NMR spectroscopy techniques and compared the data with reference. Antimicrobial tests were carried out on *Escherichia coli*, *Streptococcus mutans,* and *Candida albicans* by broth microdilution method. The antioxidant test was carried out using the 1,1-dipheny1-2-picrylhydrazyl (DPPH) method. Four isolated compounds were successfully isolated : stigmasterol **(1)**; 1,7-diphenyl-6-hepten-3-on; **(2)**; and 1,7-diphenyl-heptan-3-on **(3);** and sinapyl alcohol diacetate **(4)**. Antimicrobial activity test results extract were active against all bacteria and *C. albicans*. The results of antioxidant activity test showed that extract, compound 2, 3, and 4 had strong and medium antioxidant activity with IC50 values at 75.05 µg/mL; 201.4 µg/mL; 207.88 µg/mL, and 190.59 µg/mL, respectively.

Keywords: antioxidant, antimicrobial, DPPH, *Etlingera*, isolation

INTRODUCTION

Infection is a major problem that attracts worldwide attention. Many infectious diseases are caused by pathogenic microorganisms and are dynamic. Based on data from the Southeast Sulawesi Provincial Health Office in 2016, Upper Respiratory Tract Infection (URTI) is the most common disease suffered by the community, especially in children with a total of 114,137 cases. Infectious diseases by bacteria and fungus are often found in tropical countries, caused by humid air that supports the development of fungal infections on the skin (Ahmed et al., 2015). Treatment for infectious diseases caused by antimicrobial-resistant microbes requires new products that have potential, one alternative is derived from medicinal plants (Novaković et al., 2015). Furthermore, the number of degenerative disorders caused by free radicals is rising annually (Phanm-Huy et al., 2019). The free radicals are highly reactive, destructively through oxidation reactions, and triggering various diseases such as coronary heart disease, premature aging and cancer (Ehiobu et al., 2021). Thus, the exogenous antioxidant compounds act to counteract these free radicals by preventing adverse effects that lead to excessive oxidation of the biological systems. Phenolics, flavonoids, carotenoids, and vitamins are examples of natural exogenous sources of antioxidants that come from plants (Shanti et al., 2023).

One of the medicinal plants that is already known to have pharmacological activity comes from the Zingiberaceae family. Zingiberaceae is the largest family of Zingiberales consisting of 52 genera and more than 1300 species (Jabbar et al., 2021). Previous studies have shown that Etlingera genus is abundant in phytochemical compounds, including phenolics, flavonoids, steroids, terpenoids, alkaloids, and diarylheptanoids. One of the Etlingera species found recently in Southeast Sulawesi in an expedition conducted by Poulsen (2012) is *Etlingera rubroloba* A.D. Poulsen. Research has demonstrated that diarylheptanoid (Wahyuni et al., 2024; Jabbar et al., 2022) tannins, flavonoids, saponins, alkaloids, polyphenols, terpenoids, triterpenoids, and phenolics (Ilyas et al., 2022), exhibit antioxidant and antimicrobial properties. Another pharmacological activities of this plant have been reported, such as *E. rubroloba* stems which act as antioxidants and anti-inflammatory (Jabbar et al., 2021). These finding suggest that this species has a strong pharmacological potential of *E. rubroloba* as a reliable source of bioactive compounds. However, there are limited information about chemical compounds and biological properties of *E. rubroloba.* Therefore,

research on this plant needs to be carried out to explore the pharmacological activities and secondary metabolite compounds from this species.

EXPERIMENTAL SECTION

General

This research was conducted in the Laboratory of Faculty of Pharmacy Halu Oleo University, and BRIN Research Center for Chemistry performed identification using NMR (JEOL ECP 500, 500 MHz for ^{1}H and 125 MHz for^{13}C).

Extraction

A total of 20 kg of rhizome of *E. rubroloba* A.D. Poulsen were collected from Laiwoi Village, Laeya District, South Konawe Regency. The sample was determined at the Research Center for Biology, LIPI, Cibinong, Bogor, Indonesia with registered number registered 1535/IPH.1.01/If.07/VIII/2019. The samples were cleaned, dried under direct sunlight and then powdered. Then extracted with 96% ethanol (12 L, 3×24 hrs), using the maceration method. The obtained filtrate was evaporated with an evaporator (50°C) and a thick extract was obtained.

Isolation

A total of 37.17 g thicked extract *E. rubroloba* rhizome was fractioned by vacuum liquid chromatography (VLC) and a mixture of *n*-hexane: ethyl acetate (7:3 v/v). The fraction was performed till obtained 7 fractions, which were Fraction A (1.371 g); fraction B (4.9 g); fraction C (4.7 g); fraction D (94 mg); fraction E (94 mg); fraction F (77 mg); and fraction G (5.8 g). Non polar fraction was continued fractioned using VLC with eluent *n*-hexane: ethyl acetate (7: 3 v/v) and obtained 20 fractions. The fraction 11 then purified to obtain compound **1**. Other fractions were combined and obtained 8 main fractions, namely fractions 1-8. Fraction 6 (95 mg) was separated with radial chromatography (RC) with eluent dichloromethane: methanol (9:1 v/v) and obtained 14 fractions. Fraction 11 (32.2 mg) purified to obtain a yellow oil as compound **2**. Furthermore, fraction 12 (56.73 mg) further refined by using dichloromethane: methanol (8:2 v/v) to obtain compound **3**. Fraction C was separated by the VLC method using the *n*-hexane:ethyl acetate (8:2 v/v); The result showed a good separation, and the the isolates was tested using three eluent systems: *n*-hexane: ethyl acetate (7:3), *n*-hexane: acetone (7:3), and *n*-hexane:chloroform: acetone (7:2:1), which showed that the chromatogram obtain by TLC showed a single spot. Thus, compound **4** was obtained for 30 mg.

Structure Determination

The structure of isolates was determined with ^{1}H NMR and ^{13}C NMR. The obtained data were compared to existed references.

Antimicrobial Properties Test

A total of 100 μL of media was added to all wells of a 96-well microplate. The first column was filled with 200 μL of the isolate concentration of 512 μg/mL. Then, 100 μL of the isolate solution from the first column was transferred to the second column and mixed well, until 100 μL of the solution from the tenth column was set aside. A total of 100 μL of the bacteria suspension (10^{6}–10^{8} CFU/mL) was added to each well. The eleventh column contains media with only microbial suspension used as a growth control, while the twelfth column contains only 200 μL of media as a media control. The mixture of microbial suspension and isolates in the media is incubated for 1x16-20 hours at 37°C for bacteria and 3x24 hours for fungi at 35°C. Chloramphenicol was used as positive control. The MIC value is determined by observing the turbidity of the mixture at each concentration (CLSI, 2012).

Antioxidant Properties Test

Antioxidant properties were carried out based on those previously reported, the 2,2 diphenil-1-picrylhidrazil (DPPH) method (Imran et al., 2022) with slight modifications. Samples were diluted in ethanol solvent at various concentrations (100 to 3.3 g/mL). DPPH radical solution (HIMEDIA) 0.05 mM (1.98 mg DPPH) was prepared in 100 mL ethanol (Merck). A total of 4 mL of radical solution was mixed with 1 mL of the sample at all concentrations, shaken and incubated for 30 minutes. Absorbance was determined using a UV-Vis spectrophotometer (Thermo Scientific) at 517 nm (DPPH) against the blank. The inhibitory activity of DPPH is expressed according to equation:

Inhibitory activity (%) = (ADC-ATS)/ADC x 100%

where ADC is the absorbance of the DPPH control and ATS is the absorbance of the test sample in radicals. Test samples showing antioxidant activity with % inhibition above 50% were then determined for IC_{50} values using Graphpad Prism 5 (Graphpad Software®, La Jolla, Canada, USA). The positive control used in this test is ascorbic acid (Jabbar et al., 2021)

RESULTS AND DISCUSSION

Structure Determination

The ^{1}H NMR spectra of compound **1** is very identical to the steroid spectra characterized by the presence of distinctive features. **Figure 1** showed namely the overlapping of proton peaks at δ 0-3 ppm, thus strengthening the conjecture. **Table 1** exhibit the ^{1}H NMR spectra of steroid is characterized by the presence of 6 typical high intensity methyl peaks at δ 0.69-0.71, 0.83, 0.85, 0.87, 0.94 and 1.02 ppm. Thus, these charactristics located by another proton at δ 5.36 (H-6a & H-6b) and 3.54 (H-3a & H-3b) ppm. This indicates that the proton is representative of compound cyclic compounds. **Table 1** showed aliphatic alkene groups are also shown at δ 5.03 (H-23a) and 5.16 (H-22a) ppm with an integration value each other. This indicates that one of the compounds refers to stigmasterol (Piere and Moses, 2015) (**Figure 2**).

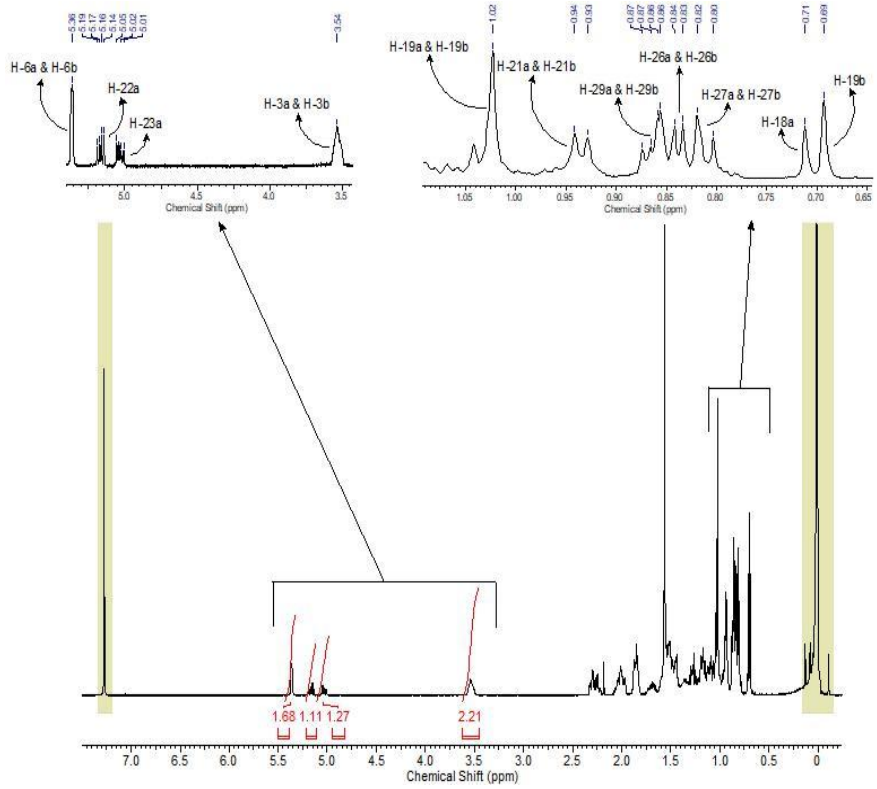

Figure 1. ^{1}H-NMR spectrum of compound **1**

Table 1. ^{1}H-NMR spectrum of compound **1**

No. H	Compound **1** δH (ΣH, *m*, J Hz)	No. H	Stigmasterol δH (ΣH, *m*, J Hz) (Forgo & Kövér., 2004)
H-3	3.54 (1H, *m*)	H-3	3.51 (1H, *m*)
H-6	5.36 (1H, *m*)	H-6	5.34 (1H, *m*)
H-18	0.71 (3H, *s*)	H-18	0.70 (3H, *s*)
H-19	1.02 (3H, *s*)	H-19	1.01 (3H, *s*)
H-21	0.94 (3H, *d*, 6.8)	H-21	1.03 (3H, *d*)
H-22	5.17 (1H, *dd*, 15.2)	H-22	5.17 (1H, *dd*, 15.2)
H-23	5.03 (1H, *dd*, 8.6)	H-23	5.04 (1H, *dd*, 8.6)
H-26	0.85 (3H, *d*, 7.3)	H-26	0.85 (3H, *d*)
H-27	0.83 (3H, *d*, 6.8)	H-27	0.80 (3H, *d*)
H-29	0.87 (3H, *m*)	H-29	0.81 (3H, *t*)

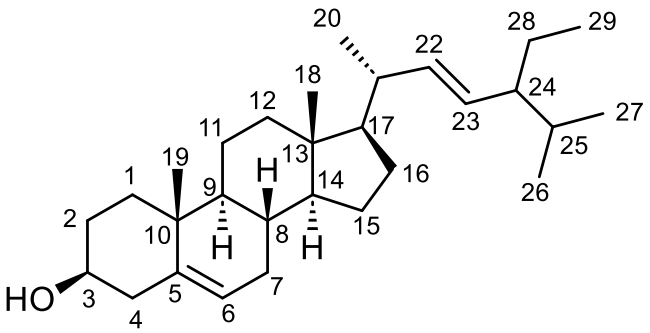

Figure 2. Compound **1**, stigmasterol

Compound **2** was obtained as aromatic yellow oil. This compound showed black spots under UV 254 nm, invisible spots under UV 366 nm and indigo spots after derivatization using cerium sulfate followed by heating (orange spots when observed again under UV 366 nm). Based on NMR spectra, this compound has molecular formulas $C_{19}H_{20}O$ with 10 DBEs derived from 1 carbonyl C=O bond, 8 from aromatic double bonds (C=C), and 1 from olefin. **Table 2** The ^{1}H-NMR spectrum showed protons with 11 types of protons with a total of 20 protons. There is aromatic groups at δ 7.14 ppm with very large intensity indicating the presence of two aromatic rings as characterized from diaryl compound. The ^{13}C NMR spectrum shows 10 types of carbon characterized by the presence of carbonyl groups (209.34 ppm). The spectrum at 128.42-128.61 ppm shows considerable one olefin near of carbonyl group (**Figure 3**). This indicates that in that area there is an aromatic ring from compound. Chemical shifts that appear indicated the compound 2 has the same structural framework as the diaryl heptanoid compound called 1,7-diphenyl-6-heptene-3-on (**Figure 4)**.

The ^{1}H-NMR spectrum of compound **3** showed total of 22 protons and molecular formula $C_{19}H_{22}O$ (**Figure 5**). Signal at 7.33 ppm with very large intensity indicating the presence of 2 aromatic rings as typical of diaryl compounds. The ^{13}C-NMR spectrum shows presence of carbonyl group (210.23 ppm) and aliphatic chains (23.48-44.54 ppm). **Table 3** reveals chemical shifts that appear indicate that the compound 2 has slight difference between compound 3 without presence of olefin group. The isolated compound named 1,7-diphenyl-heptane-3-on (**Figure 6**).

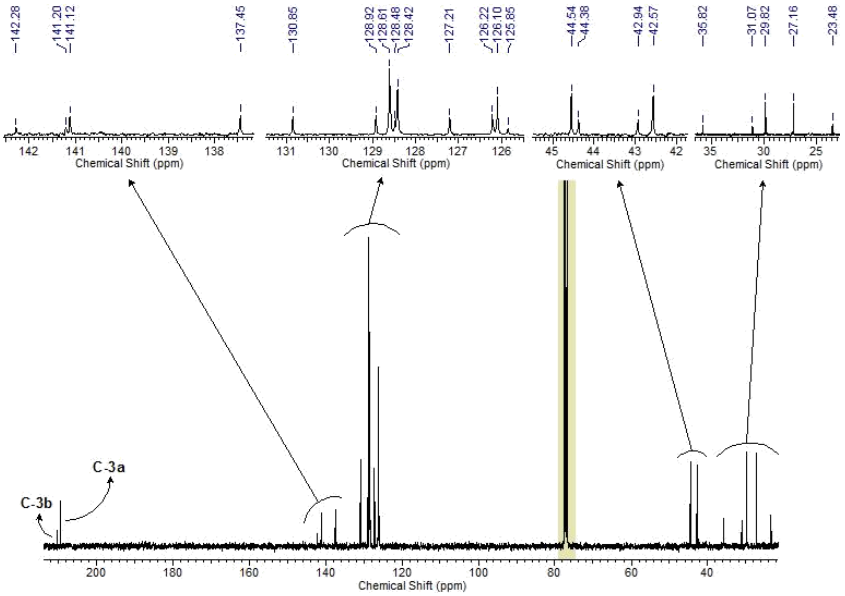

Figure 3. ^{13}C-NMR spectrum of compound **2**

Table 2. ^{1}H-NMR and ^{13}C-NMR spectrum of compound **2**

No. C	δC (ppm)	Compound **2** δH (ΣH, *m*, J Hz)	No. C	1,7-diphenyl-6-heptene-3-on, δH (ΣH, *m*, J Hz) (Jurgens et al., 1994)
1	29.82	2.89 (*m*)	1	29.98
2	44.54	2.73 (*m*)	2	44.57
3	209.34	-	3	209.17
4	42.57	2.46 (*m*)	4	42.49
5	27.16	2.27 (*m*)	5	27.38
6	130.85	6.16 (*dt*, 15.6)	6	130.79
7	128.92	6.38 (*d*, 15.9)	7	129.52
1'	141.20	-	1'	141.68
1"	137.45	-	1"	137.88
2' & 6'	128.48		2' & 6'	128.73
4'	127.21		4'	127.35
2" & 6"	126.10	7.14-7.22 (*m*)	2" & 6"	126.26
4"	126.22		4"	126.31
3' & 5'	128.61	7.27-7.33 (*m*)	3' & 5'	128.81
3" & 5"	128.42		3" & 5"	128.63

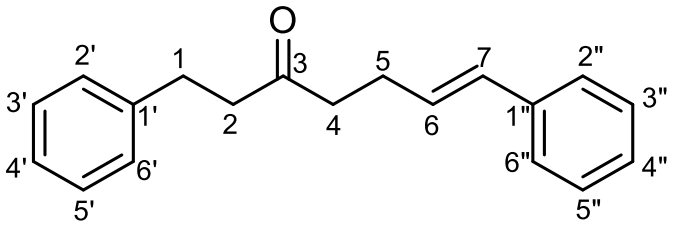

Figure 4. Compound **2**, 1,7-diphenyil-6-hepten-3-on.

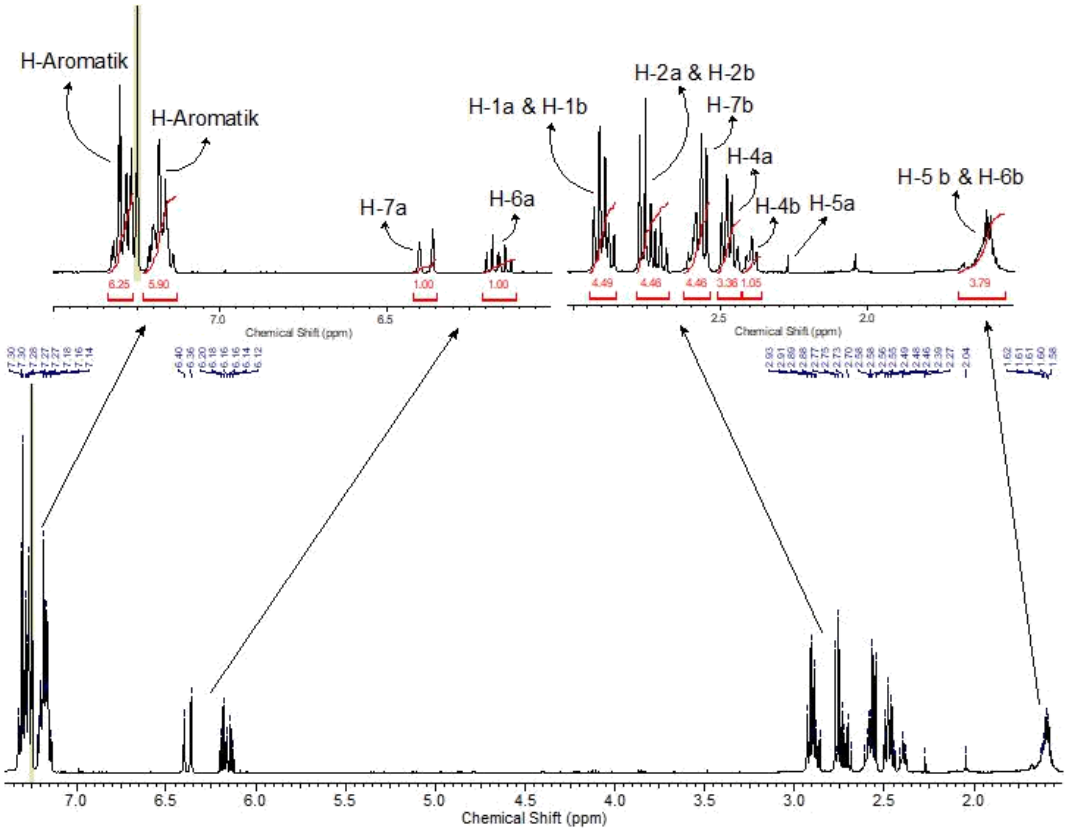

Figure 5. ^{1}H-NMR spectrum of compound 3

Table 3. ^{1}H-NMR and ^{13}C-NMR spectrum of compound **3**

Compound **3**			1,7-diphenynil-heptane-3-on (Suksamrarn et al., 2008)	
No. C	δC (ppm)	δH (Σ*H*, *m*, J Hz)	δC (ppm)	δH (Σ*H*, *m*, J Hz)
1	29.82	2.89 (*m*)	29.7	2.87 (2H, *t*, 7.8)
2	44.38	2.73 (*m*)	44.2	2.68 (2H, *t*, 7.8)
3	210.23	-	209.9	-
4	42.94	2.39 (*t*, 6.7)	42.7	2.37 (2H, *t*, 6.5)
5	23.48	1.60 (*m*)	23.3	1.58 (4H, *m*)
7	35.82	2.58 (*m*)	35.6	2.58 (2H, *t*, 6.9)
1'	141.12	-	141.0	-
1"	142.28	-	142.1	-
2" & 6"	128.42	7.14-7.22 (*m*)	128.3	7.10-7.20 (6H, *m*)
4"	126.10		126.0	
3', 5', 3", 5"	128.61	7.27-7.33 (*m*)	128.4	7.22-7.30 (4H, *m*)

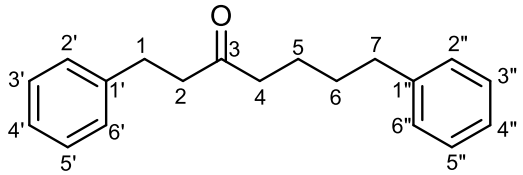

Figure 6. Compound **3**, 1,7-diphenyl-heptane-3-on.

The compound **4** was obtained in green oil form with a sharp aroma. This compound shows black spots under 254 nm UV light, invisible spots under 366 nm UV light and purple spots are visible after derivatization using cerium sulfate followed by heating. This compound has a molecular formula of $C_{15}H_{18}O_6$. The 1H-NMR showed seven types of protons representing 18 protons, consisting of methine protons (δ 6.62, 6.58, 6.22 ppm), methylene protons (δ 4.71 ppm), methoxy protons (δ 3.82), and methyl protons (δ 2.32, 2.10 ppm). Te data indicated coupling constant of H-6 to H-5 at 15.87 Hz, revealed that both protons are in the trans position. Meanwhile, H-5 and H-7 are in the cis position, with a constant coupling constant of 6.71 Hz. The ^{13}C-NMR indicated 15 types of carbon, where two carbon peaks in this compound are the carbonyl carbon at δ_C 170.98 and 168.86 ppm (C-10, C-12), the quaternary carbon at δ_C 152.25 (C-3, C-15) and δ_C 128.64 (C-4) is the quaternary carbon that binds oxygen (C-O) in the aromatic ring, while δ_C134.73 (C-1) is the quaternary carbon found in the aromatic ring (**Table 4**). The methine carbon can be seen in three signals seen at δ_C134.07 (C-7), 123.71 (C-8), and the methylene carbon at δ_C 64.97 ppm (C-9). The methyl carbon shifted to the 56.21 ppm region (C-14 & C-15) is a characteristic of methoxy (-OCH_3). Another 2 carbon signals are also seen at δ_C 21.13 (C-11) and 20.57 (C-13) ppm, which are methyl carbons bonded to the carbonyl group. The data obtained were then compared with the literature namely sinapyl alcohol diacetate (**Figure 7)**

Table 4. ^{1}H-NMR and ^{13}C-NMR spectrum of compound **4**

Compound **4**			Sinapyl alcohol acetate (Ralph et al., 2004)		
No. C	δ_C (ppm)	δH (ΣH, *m*, J Hz)	No. C	δ_C (ppm)	δH (ΣH, *m*, J Hz)
1	134.73	-	1	134.71	-
2	103,3	6.62 (2H, s)	2	103.38	6.84 (2H, s)
3	152.25	-	3	152.28	-
4	128.64	-	4	128.75	-
5	152.25	-	5	152.28	-
6	103,3	6.62 (2H, s)	6	103.38	6.84 (2H, s)
7	134.07	6.58(1H, d, 15.87)	7	134.01	6.66 (1H, d, 15.9)
8	123.71	6.22 (1H, dt, 15.87,6.71)	8	123.71	6.38 (1H, dt, 15.8, 6.2)
9	64.97	4.71 (2H, dd, 6,10)	9	64.90	4.69 (2H, dd, 6.2, 1.2)
10	170.98	-	10	170.85	-
11	21.13	2.32 (3H,*s*)	11	21.05	2.22 (3H, s)
12	168.86	-	12	168.71	-
14	56.21	3.82 (6H, s)	14	56.21	
15	56.21		15	56.21	

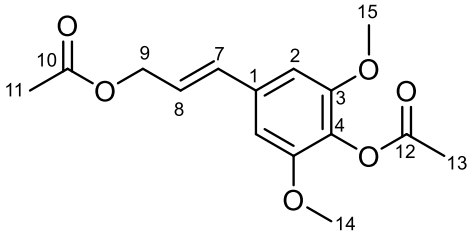

Figure 7. Compound **4**, sinapyl alcohol diacetate

The present work provides the first documentation of these compounds being isolated from *E. rubroloba* rhizome. Earlier studies on this species have focused on stem and as general phytochemical profiles with the purification and structural identification of specific constituents (Jabbar et al., 2021). Their successful isolation here expands the known chemical diversity of *E. rubroloba* and offers new material for examining the biological roles and potential applications of this underexplored species.

Antimicrobial Activity

The antimicrobial activity test were performed on *S. mutans* ATCC 25175 as Gram positive bacteria, *E. coli* ATCC 35218 as Gram negative bacteria, and *C. albicans* as fungal. The selection of these bacteria and fungi aims to see the spectrum of the isolate compounds tested. The results obtained were compared with the positive control and negative control. The positive control used was chloramphenicol for bacteria, nystatin for fungi, while the negative control used 10% DMSO. The results of the antibacterial activity test showed the weak antibacterial activity of all compounds compared to chloramphenicol with IC_{50}>32 μg/mL (CLSI, 2012) (**Table 5**). The result impict that most steroid and diaryl heptanoids show weak to moderate antibacterial potency due to poor membrane permeability and limited binding into the target (Sun et al., 2016; Novaković et al., 2015).

The isolate component from *E. rubroloba* rhizome extract was tested for antifungal activity using a method identical to the antibacterial test to obtain the Minimum Inhibitory Concentration (MIC). The primary distinction in this test is that the measurement parameters were taken twice, first for 24 hours and 48 hours. The result showed all the compounds were not active againts *C. albicans* ATCC 10231, while nystatin strong MIC value of 16 μg/mL at 24 hours and increases to 256 μg/mL at 48 hours (**Table 5**). This indicates that nystatin's activity acts as a fungistatic. Nystatin's activity depends on the presence of sterol bonds in the fungal or yeast cell membrane, especially ergosterol. As a result of the formation of bonds between sterols and antifungals, there will be changes in cell membrane permeability so that the cells will lose various small molecules. Pure nystatin compound has an MIC value of 0.625 μg/mL after 24 hours and 1.25 μg/mL after 48 hours (Nenoff et al., 2016).

Phenolic compounds, especially diary heptanoids show lower antifungal activity compared with their antibacterial effects because their structural features and chemical behavior do not efficiently disrupt fungal cells, which are more complex than bacterial cells. Fungi possess a multilayered wall composed of chitin, β-glucans, and proteins. Furthermore, ergosterol increases membrane rigidity, making it harder for small or moderately lipophilic phenolics to insert into or destabilize the membrane (Daglia, 2012).

Antioxidant Activity

This test was performed on the extracts and pure compounds obtained and compared with ascorbic acid as a positive control. The concentration of DPPH solution used was 0.2 mM. The measurement results were obtained through observation of color change/decolorization by monitoring the absorbance at the optimum wavelength of DPPH which is 540 nm in DMSO solvent (Clarke et al., 2013). The measurement value is expressed as IC_{50}. The smaller the IC_{50} value means the greater the ability of the compound to reduce the radicals.

Table 5. Antibacterial activity of extract and isolated compound

Samples	MIC (μg/mL)	
	S. mutans	*E. coli*
E. rubroloba rizhome extract	>512	256
Compound 1	>256	>256
Compound 2	>256	>256
Compound 3	>256	>256
Compound 4	256	>256
Chloramphenicol	8	8

Table 6. Antifungal activity of extract and isolated compounds

Sampels	MIC (μg/mL)	
	24 hrs	48 hrs
E. rubroloba rizhome extract	>256	>256
Compound 1	>256	>256
Compound 2	>256	>256
Compound 3	>256	>256
Compound 4	>256	>256
Nystatin	16	256

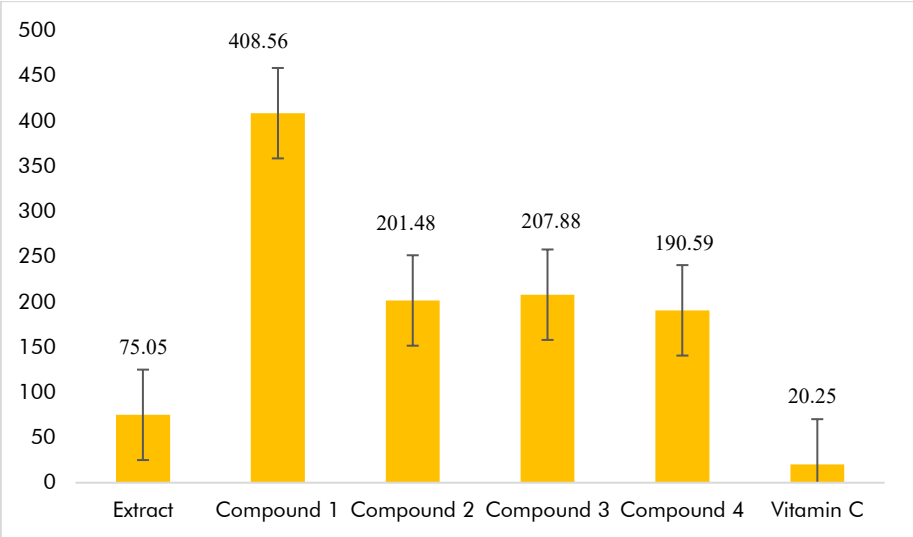

Figure 8. IC_{50} values of ethanolic extract, positif control, and compounds 1-4

The pharmacological properties as antioxidants of the three compounds were tested using the DPPH method, and the results are shown in **Figure 8**. The ethanolic extract showed the activity in neutralizing free radicals with IC_{50} values >50–100μg/mL. Compound 2, 3, and for exhibited medium activity with IC_{50} values >100-250 μg/mL, meanwhile compound 1 showed the weak activity with IC_{50} values >400 μg/mL. The presence of hydroxyl groups in the aromatic ring is likely responsible for its action. Compounds with hydroxyl groups can prevent cellular damage by inhibiting oxidation and reducing free radicals. In the presence of OH groups free radicals absorb hydrogen atoms and create oxygen radicals. These radicals are subsequently delocalized through resonance, resulting in stable radicals (Sutariya et al., 2023). The identified compounds have promising pharmacological properties and could be effective antioxidants agent.

CONCLUSIONS

This study found that the ethanol extract of *E. rubroloba* A.D. Poulsen rhizome and all isolated compounds have weak activity as antimicrobial, but they have antioxidant activity with a strong and medium category by radical scavenging parameters. This research could lead to developmentof traditional the medicine, especially for the treatment degenerative disease and natural antioxidants.

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CONFLICT OF INTERESTS

The author states that there is no conflict of interest, all authors agree that manuscripts are confidential and will not be submitted, or published elsewhere.

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