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A SPECTROSCOPIC ANALYSIS OF ACTIVE COMPOUND ISOLATED FROM THE ETHYL ACETATE FRACTION OF *Calophyllum macrophyllum* SCHEFF AND TOXICITY TESTS USING THE BRINE SHRIMP LETHALITY TEST

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ABSTRACT

The objective of this study was to analyse the spectroscopic data of active compound isolated from the ethyl acetate fraction of *Calophyllum macrophyllum* Scheff which included UV, IR, GC-MS, ^{1}HNMR, and ^{13}CNMR. The bioactivity testing was conducted using the Brine Shrimp Lethality Test (BSLT) with *Artemia salina* Leach larvae. The results of the spectroscopic analysis showed the chemical formula of derivative of fatty acid methyl ester (methyl-octadec-14 enoic), in the form of a yellow liquid with a boiling point of 173-175° C. The initial screening for bioactivity through the toxicity test using BSLT method revealed an LC50 value of 141.2 g/mL. Based on the results of the spectroscopy data, it can be seen that the isolate of Calophyllum macrophyllum Sheff plant was a compound derived from fatty acid methyl esters. This fatty acid compound had an LC50 of 141.2 g/mL based on the BSLT, indicating that this compound has the potential as a bioactive substance.

Key words: BSLT, *Calophyllum* macrophyllum Scheff, fatty acids

ABSTRAK

Penelitian ini bertujuan menganalisis data spektroskopi senyawa aktif yang diisolasi dari fraksi etil asetat Calophyllum macrophyllum scheff meliputi UV, IR, GC-MS, ^{1}HNMR, dan ^{13}CNMR. Pengujian bioaktivitas dilakukan dengan uji toksisitas menggunakan metode Brine Shrimp Lethality Test (BSLT) terhadap larva udang Artemia salina Leach. Hasil analisis spektroskopi menunjukkan adanya rumus kimia turunan metil ester asam lemak (methyl-octadec-14 enoic), berupa cairan berwarna kuning dengan titik didih 173-175° C. Skrining bioaktivitas melalui uji toksisitas metode BSLT terhadap larva udang Artemia salina Leach menunjukkan nilai LC_{50} sebesar 141,2 g/ml. Berdasarkan data hasil spektroskopi dapat diketahui bahwa isolat tanaman Calophyllum macrophyllum Sheff merupakan senyawa turunan dari metil ester asam lemak. Senyawa asam lemak ini memiliki LC_{50} sebesar 141,2 g/ml berdasarkan uji BSLT yang menunjukkan bahwa senyawa ini berpotensi sebagai zat bioaktif.

Kata kunci: BSLT, Calophyllum macrophyllum Scheff, asam-asam lemak

INTRODUCTION

Selection of plants in the search for new bioactive compounds can be done through ethnopharmacology and chemotaxonomic approaches. The ethnopharmacology approach is a searching method based on the use of natural ingredients by a particular ethnic group for medicinal purposes, while the chemotaxonomic approach is a way to search by finding plants with familial relationship, with the assumption that related plants have the same chemical content or at least have the same framework or core of active compounds (Simanjuntak 2003).

Calophyllum from the Guttiferae/Clussiaceae tribe is one of the Indonesian high-level plant genera that can be a source of bioactive chemical compounds. The Calophyllum genus is almost evenly distributed throughout the major islands of the archipelago and is also found in Vietnam, Cambodia, Thailand, Singapore, Southern Australia, Andaman Islands, and Sri Lanka with 200 species being identified (Backer and Brink 1963; Lemmens *et al*. 1995). There have been many reports of chemical compounds with unique bioactivity obtained from this plant genus, including xanthones, coumarins, flavonoids, biflavonoids, triterpenoids, organic acids, and benzophenone compounds (Cao *et al*. 1998; Yimdjo *et al*. 2004; Taher *et al*. 2005).

The xanthone group (+) calanolide A from *Calophyllum lanigerum* was reported as anti-HIV (Linuma *et al*. 1996; harmaratne *et al*. 1999). Compounds of the coumarin group such as brasimarin A, B, and C, 4-phenylcoumarin from the bark of *Calophyllum brasiliense* were shown to have antitumor activity (Ito *et al*. 2003). A polyisoprenyl ketone compound, namely enervosanone isolated from the bark of *Calophyllum enervosum*, was found to be an active antibacterial against *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* and *Staphylococcus aureus* (ATCC 6538) bacteria (Taher *et al*. 2005). Gunatilaka *et al.* (1984) and Linuma *et al.* (1994) discovered the biflavonoid compounds (amentoflavone and merelloflavone) from *Calophyllum calaba* and epikatechin from *Calophyllum inophyllum*, respectively. Likewise, triterpene compounds and organic acids were found by Reyes-Chilpa *et al.* (2004) who isolated triterpenes from *Calophyllum brasiliense* leaves. Friedelin as an active anti-HIV and acidic compound (protocatestrong acid and shikimic acid) were found in *Calophyllum brasiliense* leaves.

Frengki *et al.* (2011) have attempted to explore one of the Callophyllum, namely *Calophyllum macrophyllum* Scheff to find compounds with bioactivity that can be made into drugs. Frengki *et al.*

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(2011) also succeeded in isolating flavan-3-ol one from the ethyl acetate extract obtained at the beginning of the extraction process. The current study aimed to report the structure of this compound through the analyses of UV, IR, GC-MS, ^{1}HNMR, and ^{13}CNMR spectroscopic data, which were amplified by HMQC and HMBC spectra. In addition, the bioactivity test was carried out through a toxicity test, which employed the Brine Shrimp Lethality Test (BSLT) method using *Artemia salina* Leach shrimp larvae.

MATERIALS AND METHODS

Materials

The sample was an isolate from the bark of the *Calophyllum macrophyllum* Scheff plant obtained from the Kerinci mountain forest, Jambi Province. The equipment used to identify the structure of the isolate compounds was Ultra Violet-Visible (UV-VIS) spectrophotometer (Hitaci U 2000, Japan), infrared spectrophotometer (Prestige-21 Shimadzu, Japan), Core Magnetic Resonance (Jeol JNM-ECA 500 Mhz, USA), Liquid Chromatography-Mass Spectrometry (LC-MS), and Gas Chromatography-Mass Spectrometry (GC-MS) Mariner Biored (70 ev) (Perkin Elmer Clarus 5000, USA).

Research Procedure

Spectroscopy

The identification of isolates using the spectroscopy method included UV-Vis, Infrared, LC-MS Mass spectrum, Nuclear Magnetic Resonance Spectophotometry (^{1}H-NMR and ^{13}C-NMR) as well as amplification by 2D NMR techniques (HMQC and HMBC).

Preparation of test samples

The stock solution was prepared by dissolving 4 mg of the sample with 10 mL of dimethyl sulfoxide (DMSO) and adding sea water with a salt content of 3.8% to 2 mL. The concentration of the liquor was 2000 g/mL. The fractions to be tested were prepared at concentrations of 1000, 500, 100, and 10 g/mL. As a blank, 10 L DMSO was added to 2 mL of seawater.

Toxicity test by Meyer method

Toxicity test of ethyl fractions of the stem bark of *Calophyllum macrophyllum* Scheff and the isoalte was carried out using the BSLT that utilized *Artemia salina* Leach larvae. The *Artemia salina* Leach hatchery was performed in a box that had been divided into two parts with a perforated bulk head inserted with sufficient seawater. One side of the box was covered with aluminum foil. Then, the box was placed under a UV lamp for 48 hours. Larvae that penetrated bright areas after 48 hours were ready for the toxicity test.

A total of 10 *Artemia salina* Leach larvae in 100 L of seawater were put into a test vial; then, 100 L of sample solution was added. For each concentration, three repetitions were performed. As a control, 100 L of the blank solution was added and mixed with 200 L of seawater. Observations were made after 24 hours by counting the number of live and dead shrimp larvae. Then, the mortality was calculated using the following formulae:

$$\% \text{ Mortality} = \frac{\text{Accumulated dead}}{\text{Accumulated dead} + \text{Accumulated lives}} \times 100\ \%$$

The LC_{50} value was determined through a regression equation (the x-axis is [log k], while the y-axis was % mortality with the help of a simple computer program Mic. Excel. A fraction or extract is said to be active if it has an LC_{50} value of 1000 g/mL. For a pure compound, it is considered active if it contains an LC_{50} value of 200 g/mL (Alam 2002).

RESULTS AND DISCUSSION

Spectroscopic Data of *Calophyllum macrophyllum* Scheff Plant Isolates

Spectroscopic data of isolates started with the observations of UV, IR, LC/GC-MS, ^{1}H-NMR, and ^{13}C-NMR spectra. The UV spectrum of the isolate in methanol showed maximum absorption at wavelengths (λmax) of 221 and 281 nm. The IR spectrum showed absorption at wave numbers (ύ) 2922, 2850, 1462, 1739, and 1166 cm1. The examination of the molecular weight of compound S1 with GC-MS revealed the relative molecular weight at m/z 296 with a retention time of 20.95 minutes in methanol solvent. The ^{1}H-NMR spectrum in $CDCl_3$ solvent discovered a proton signal at a chemical shift (δH) of 5.34 doublets, 3.67 triplets, and 0.88 triplets (ppm). Meanwhile, the ^{13}C-NMR spectrum in the $CDCl_3$ solvent indicated a carbon signal at a chemical shift (δC) of 174.4; 130.1; 129.8; 51.5, and 14.9 ppm. The details are shown in Figure 1 and Table 1.

UV-Vis spectroscopy showed maximum absorption at wavelengths (λmax) 221 nm and 281 nm, indicating the presence of double bonds and carbonyl groups. GC-MS analysis showed this compound has a molecular weight of 296. From the infrared spectrum, it indicated a sharp absorption band at wave numbers (ν) 2922 and 2850 cm-1, proving the existence of stretching vibrations from the aliphatic -CH group. This was reinforced by the presence of a strong absorption peak. Sharp in the wave number region (ν) 1462 cm-1 proved the buckling vibration of the aliphatic -CH group. The presence of an absorption peak at wave number (ν) 1739 cm-1 was evidence of the presence of an ester group. This was confirmed by the presence of the -C-O group, with an absorption band peak at a wave number of 1166 cm-1. Based on the infrared spectral data, it can be estimated that this molecule is a straight-chain fatty acid compound.

Determination of the structure was then carried out by utilizing ^{1}H-NMR spectrum data. The presence of a proton signal (2H) on a chemical shift (δH) of 5.34 ppm indicated a non-aromatic double bond, which was confirmed from the ^{13}C-NMR spectrum data showing the presence of a methine (2C) bond (-CH) in a

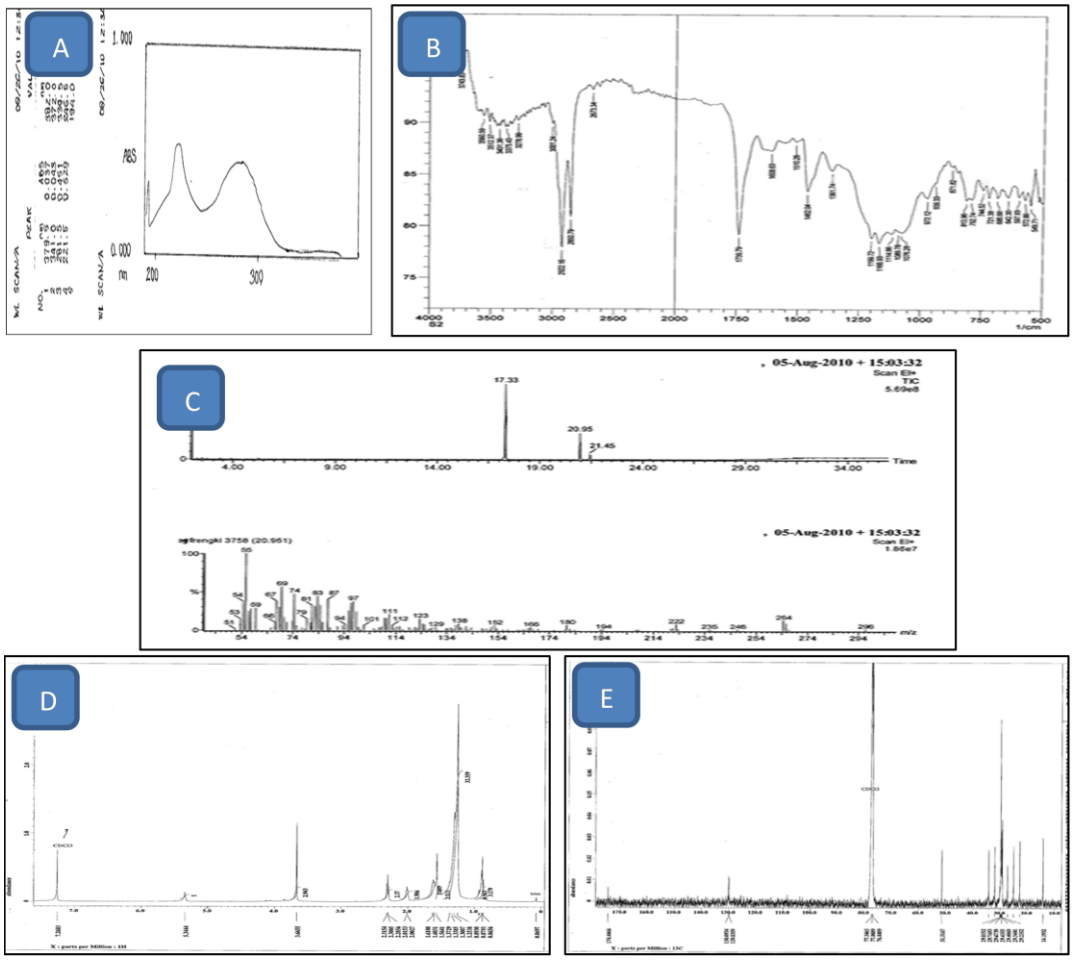

Figure 1. Spectroscopic data of compounds isolated from *Calophyllum macrophyllum* Scheff. A= UV-Vis, B= Infra-red, C= LC-MS, D= ^{1}H-NMR, E= ^{12}C-NMR

Table 1. Spectroscopic data of isolates

Spectroscopy	Data results
UV-Vis	(λmaks) 221 and 281 nm
Infra-Red	(ύ) 2922, 2850, 1462, 1739, 1166 cm^{-1}
LC/GC-MS	MW 296 m/z
^{1}H-NMR	[(δH) 5.34; 3.67; 2.4-1; 0.88] ppm
^{13}C-NMR	[(δC) 174.4; 130.1; 129.8; 51.5; 25-35; 14.9] ppm

chemical shift (δC) of 130.09 and 129.84 ppm. The presence of methoxy was indicated by the presence of a proton (3H) signal at a chemical shift (δH) of 3.66 ppm, and supported by the ^{13}C-NMR spectral data indicating the presence of carbon (CH_3) bound to an Oxygen atom (-O-CH_3) in a chemical shift (C) 51.52. The presence of a proton (3H) signal on a chemical shift (δH) of 0.88 ppm was apparent from the ^{13}C-NMR spectrum data indicating the presence of a methyl bond (-CH_3) on a chemical shift (δC) of 14.19 ppm. There was a presence of a proton signal collection in the chemical shift (δH) between 1 to 2.4 ppm, which was confirmed by the ^{13}C-NMR spectrum data with a chemical shift (δC) between 25 to 35 ppm indicating the presence of methylene bonds (-CH_2) and confirmed by the DEPT spectrum. From the explanation above, it can be concluded that the molecule has ester, methoxy, methyl, methylene, and methine where the fragmentation of the molecule can be described in Figure 2.

From the sum of the peaks of the DEPT carbon spectrum, it shows 1 quaternary C atom, 2 –CH (methin) groups, 2 CH_3 (methyl) groups, and with 2 O atoms. It is estimated that the molecular formula of the compound was $C_5H_8O_2$ with the molecular weight being approximately 100. The result of GC-MS measurement of the molecular weight of this compound was 296; hence, the difference was 196, which was the weight of the 14 methylene (CH_2) group, so the actual molecular formula was $C_{19}H_{36}O_2$. The number of double bonds (F=2) has been calculated using the same formula as reported by Frengki *et al.* (2011). If five fragments (a), (b), (c), (d), and (e) were combined, a possible structure was obtained in the form of a fatty acid methyl ester compound. This compound was similar to that reported by Yu *et al.* (2013). To strengthen the alleged structure above, a 2D analysis was carried out using the HMQC and HMBC techniques as shown in Table 2. The following results

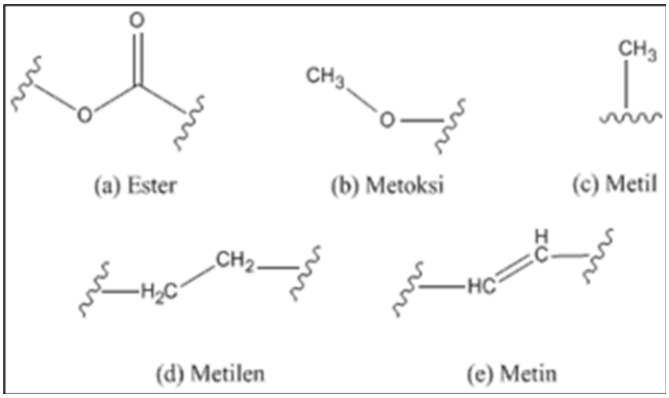

Figure 2. Fragments that make up the fatty acid methyl ester molecule

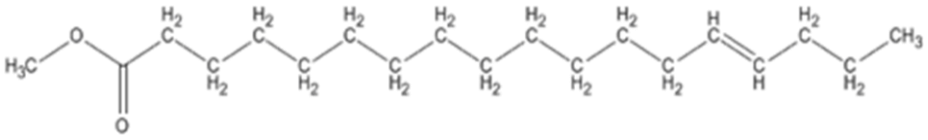

Figure 3. Molecular structure of fatty acid methyl ester

Table 2. 2D analysis of fatty acid methyl esters using HMQC, HMBC, and DEPT techniques

H-NMR	HMQC	HMBC (δ ppm)					DEPT
	δ ppm	1	2	3	4	5	
3.67	51.52	174.45					CH_3
	22.77						CH_2
	32,01						CH_2
5.33	129.84						CH
5.33	130.09						CH
2.01	27.29	29.35	130.09				CH_2
1.65	25.05	29.34	34.21	29.41			CH_2
2.30	34.21	25.05	29.41	174.45			CH
0.88	14.19	22.77	32.01				CH_3

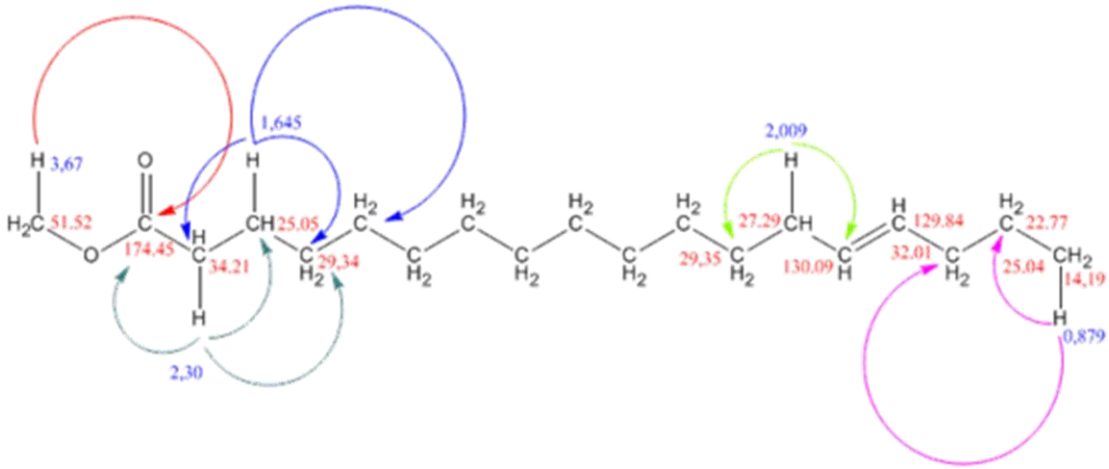

Figure 4. Molecular structure of fatty acid methyl esters from 2D analysis using HMQC, HMBC, and DEPT techniques

of the 2D analysis using the HMQC and HMBC techniques (Table 2) confirmed the above assumption.

Toxicity Test (BSLT)

BSLT is a screening method to determine the toxicity of an extract or compound. This method is often used to find anticancer compounds in plants (Meyer *et al.* 1982). The observation parameter to detect the presence of toxicity of an extract or compound is the mortality of *Artemia salina* Leach expressed in LC_{50} value. LC_{50} is the concentration of compounds that can give a mortality rate of 50% *Artemia salina* Leach. The LC_{50} value is a value that indicates the toxic nature of the test material obtained

Table 3. Toxicity test results on *Artemia Salina* Leach larvae

Samples	Concentration (ppm)	Log C	Early life			End of life			Total dead	Total lives	% Mortality	LC_{50} (µg/mL)
Ethyl acetate fraction	10	1	10	10	10	10	10	9	1	71	1.39	269.2
	100	2	10	10	10	3	6	8	14	42	25	
	500	2.7	10	10	10	5	3	5	31	25	55.36	
	1000	3	10	10	10	4	5	3	49	12	80.33	
Isolate	10	1	10	10	10	10	10	10	0	59	0	141.2
	100	2	10	10	10	9	9	8	4	29	12.12	
	500	2.7	10	10	10	2	1	0	31	3	91.18	
	1000	3	10	10	10	0	0	0	61	0	100	

using a linear regression analysis between the concentration log and the probit percent mortality. The smaller the LC_{50} value, the stronger the toxic properties. A plant extract is considered to have a cytotoxic effect if the toxicity (LC_{50}) to *Artemia salina* Leach larvae is 1000 g/mL and 200 g/mL for pure compounds (Alam 2002).

The toxicity of *Calophyllum macrophyllum* Sheff was carried out on the ethyl acetate fraction because from this fraction, a substance that was easily purified was obtained. The results of this toxicity test obtained the LC_{50} value of the ethyl acetate fraction of 269.15µg/mL. Meanwhile, the fatty acid compounds isolated from this fraction obtained an LC_{50} value of 141.2 g/mL. Based on the criteria proposed by Alam (2002), the ethyl acetate fraction and fatty acid compounds are still quite toxic to *Artemia salina* Leach larvae; hence, both of them still have the potential to have certain bioactivity that may be useful as drug candidates. The toxicity data are shown in Table 3.

CONCLUSION

Based on the results of the spectroscopy data, it can be seen that the *C. macrophyllum* Sheff plant isolate was a compound derived from fatty acid methyl esters. This fatty acid compound had an LC_{50} of 141.2 g/mL based on the BSLT test on *Artemia salina* Leach larvae, indicating that this compound has the potential as a bioactive substance.

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