

**ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES IN ETHYL ACETATE FRACTION FROM INDONESIAN *EUCHEUMA SPINOSUM***Nurul Inayah¹, Masruri Masruri^{2*}¹ Chemistry Department, Institute of Science and Technology (IST) Annuqayah, Sumenep Madura, Indonesia, 69463² Chemistry Department, Brawijaya University, Jl. Veteran Malang, Indonesia, 65145**ARTICLE INFO****ABSTRACT****Article History**

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Eucheuma spinosum is a type of red algae (*Eucheuma sp.*) which is used as a food that has a high source of carrageenan. Information on secondary metabolites in *E. spinosum* is known to have bioactive properties as an antioxidant and antibacterial. The purpose of this study was to isolate and characterize secondary metabolites in *E. spinosum*. The study was started by isolating secondary metabolites from *E. spinosum* using Soxhlet extraction followed by solvent partitioning, fractionation and characterization. The yield from methanol extract was 6.07% while the highest partition result was in the ethyl acetate fraction, which was 1.25%. Fractionation was carried out by column chromatography resulting in 2 single spots F401 and A402. Pure isolate F401 has an R_f value of 0.71 in the form of a white solid, A401 has an R_f value of 0.67 in the form of a brown gel. The results of characterization of isolates with a UV-Vis spectrophotometer obtained maximum absorption and absorbance values of F401 218.50 nm 4.79 M⁻¹ cm⁻¹ 273 nm 1.06 M⁻¹ cm⁻¹ respectively. Isolate A402 233.50 nm 4.1 M⁻¹ cm⁻¹, 407 nm 1.03 M⁻¹ cm⁻¹. Identification using FT-IR found that both isolates showed the presence of functional groups =C-H stretching alkene, O-H bending of alcohol, C=O stretching of ester, C-H sp² stretching of alkane, C-Cl stretching of halogen. According to the result of the UV and FT-IR spectra, two isolates can be identified as terpenoid compounds.

Keywords: *E. spinosum*, Secondary Metabolites, maceration, Ethyl acetate fraction, characterization.

1. Introduction

Spinosum is a genus of the red seaweed group which is the family of solieriscae [1]. This seaweed grows attached to rocks, coral reefs, hard objects and shells. *E. spinosum* usually grows in coral reef areas such as the islands of Sumatra, Bali and Java. *E. spinosum* is one of the seaweeds that is widely traded, both for domestic and export industrial raw materials such as pharmaceutical and food industries[2].

Research on *E. spinosum* have been reported by Al-Hajj, et al.,[3] using *E. spinosum* from Malaysia which is active as an antibacterial against *S. aureus* and *S. pyogenes*. Fattah, et al.,[4] using *E. spinosum* samples from Makassar were also prevent inhibit the growth of *S. aureus* and *Vibrio cholera* by 40%. Damongilala, et al.,[5] used *E. spinosum* from Sulawesi as an antioxidant. Total phenol analysis obtained 5.87 ± 0.15 mg GAE/g and 75.27 ± 0.29% DPPH. Inayah, et al[6] used *E. spinosum* from Sumenep which was reported to be active as an antioxidant.

Metabolites of *E. spinosum* are classified into primary metabolites and secondary metabolites. The primary metabolites of *E. spinosum* are polysaccharide compounds such as carrageenan agar and alginate[7] [8]. Polysaccharide compounds are widely used in various industries, including the food, cosmetic and pharmaceutical industries[9] [10]. Preliminary information on the content of secondary metabolites in *E. spinosum* from Sumenep is alkaloid, saponin and terpenoid

compounds[6]. Where as in other references it is known that *E.spinosum* contains flavonoid compounds[11] [4], alkaloids and phenols[12], and terpenoids[13].

The existence of preliminary information regarding the bioactivity and content of secondary metabolites in *E.spinosum* encouraged the identification of its constituent compounds through the process of fractionation and data interpretation. Fractionation to determine the constituent compounds of secondary metabolites from *E.spinosum*, especially Indonesian samples, has not been widely reported.

Fractionation is a technique used to reduce the fraction contained in an extract. Fractionation can use column chromatography using a certain ratio of eluent or mobile phase. Fractionation aims to obtain pure isolates or single spots through a screening process using thin layer chromatography (TLC). Polzin et al 2003 isolated monoterpenes from the red algae *Ochodes secundiramea* using the column chromatography method. Guella et al 2006 with the same method isolated alkaloid compounds in red algae. Meanwhile, secondary metabolites in *E.spinosum* are known to contain alkaloids, saponins and terpenoids[6], flavonoids[11] [4], alkaloids[12]. Damongilala et al,[5] 2013 also reported the presence of phenol in *E. spinosum*.

The information regarding the high bioactivity and content of secondary metabolites in *E.spinosum* encourages us to find out its constituent compounds through fractionation to obtain pure extracts. Meanwhile, the compound characterization was carried out using an ultraviolet-visible spectrophotometer (UV-Vis) and functional groups were characterized using an infrared spectrophotometer (FT-IR).

2. Materials and Methods

2.1. Materials

2.1.1. Sample

E.spinosum washed with fresh water and dried in the sun. Dried *E.spinosum* which was red in color was mashed to obtain reddish brown *E.spinosum* powder.

2.2. Methods

2.2.2. Soxhlet Extraction

As much as 500 g of sample powder the *E.spinosum* was extracted with 1L methanol using a soxhlet extractor. Extraction was carried out until the solvent which soaked the sample looked clear. The same treatment was carried out up to 5 repetitions using the new sample powder. The green extract was concentrated using a rotary evaporator. Crude extract of red algae (*E.spinosum*) was then partitioned with ethyl acetate, dichloromethane, and n-hexane solvents.

2.2.3. Fractionation by Column Chromatography

The partition results obtained were analyzed using TLC to find the best eluent in column chromatography. The length of the chromatography column was 30 cm with a diameter of 10 cm. The eluent used was gradient starting from n-hexane : ethyl acetate with the ratio (9:1), (6:4), (4:6), (8:2), ethyl acetate:methanol (8:2), (1 :1) (2:8) and 100% methanol. Each solution collected in a bottle was monitored by Thin Layer Chromatography (TLC) using n-hexane:ethyl acetate (1:1) eluan. Fractions that have the same R_f value were combined and then concentrated using a rotary evaporator. Pure compounds were indicated by the appearance of 1 spot on the TLC results.

2.2.4. Structure Elucidation

Structural elucidation was carried out using UV-VIS and FT-IR on isolates resulting from separation and purification from the ethyl acetate fraction to determine the resulting spectrum. This data was then used to predict the compounds contained in *E. spinosum*.

3. Result and Discussion

Extraction of *E.spinosum* was carried out by Soxhlet extraction using methanol as solvent. The advantage of this method was that the process takes place continuously, so it did not require a large amount of solvent. In this study, the extraction process took place optimally at the 18th circulation which was marked by the clear color of the mixture and it was assumed that all the compounds contained in the *E.spinosum* sample had all been extracted. The extract obtained was concentrated using a rotary evaporator with reduced pressure. The results showed that the crude extract weight was 303.44 gr with a blackish green color. Based on the results of the calculation of the sample result, it was obtained at 6.07%.

In the fractionation process, extraction was used with several solvents that had different levels of polarity, namely ethyl acetate, dichloromethane and n-hexane. The results of the extraction and fractionation were shown in Table 1. The highest

result of fractionation was obtained from the ethyl acetate extract. This process shows that in the *E.spinosum* sample the main components are polar.

Table 1. Result of extracts and fractions of *E.spinosum*

Solvent	Color of Filtrate	Color of concentrated extract	Weight of Extract	Result % b/b
Methanol	Dark green	Blackish green	303,44 gr	6,07
Ethyl acetate	Dark brown	Blackish green	62,23 gr	1,25
Dichloromethane	Brownish yellow	yellowish brown	7,44 gr	0,15
n-hexane	Yellowish clear	Yellowish brown	5,73 gr	0,12

3.1. Fractionation Using Column Chromatography

The separation and purification of the components was carried out on the extract which had the highest result, namely ethyl acetate extract. Prior to the separation by column chromatography, several eluent selection mixtures were first carried out which were able to separate the compounds contained in the ethyl acetate extract using thin layer chromatography. The eluents used included ethyl acetate, ethyl acetate-n-hexane (1:1), ethyl acetate-n-hexane (2:8) and ethyl acetate-n-hexane (1:9). The determination of the solvent was carried out to obtain a solvent ratio that showed the best separation pattern with the formation of spots/stains on a thin layer chromatography plate. This solvent serves to screen the resulting column solution. The solvents used in the chromatography column were n-hexane-ethyl acetate (9:1-8:2-6:4-4:6-2:8), ethyl acetate, n-hexane:methanol (8 :2-6:4-4:6-2;8) and 250 ml of methanol each.

The results of purification with columns that had been accommodated were screened using thin layer chromatography (TLC). The eluent used was obtained from the results of the comparison of solvents that had been done previously. Eluean n-hexane:ethyl acetate 1:1 was chosen because the results of separation as eluent in TLC obtained the best separation. The TLC results of purified isolates can be seen in Figure 1.

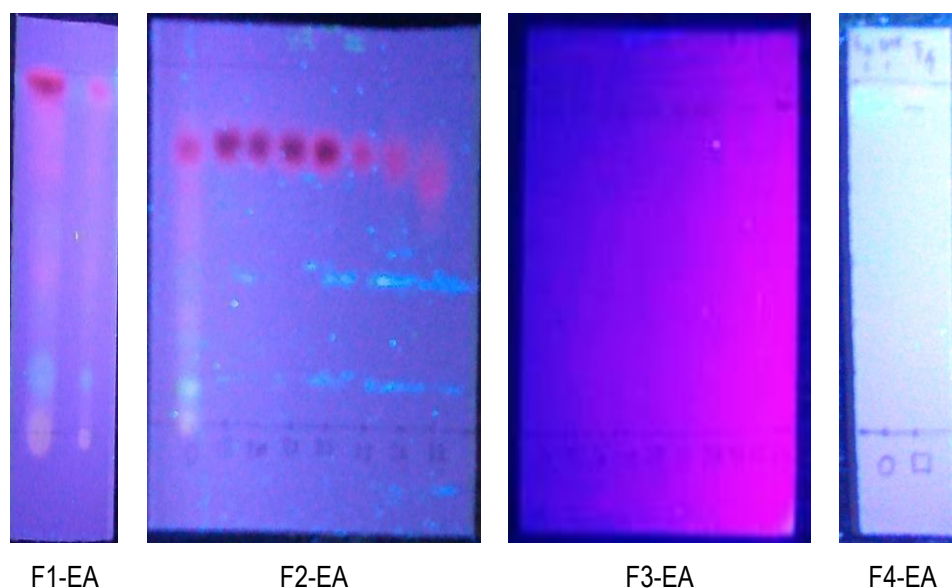


Figure 1. TLC of isolated fractions

3.2. Identification of pure isolates by TLC

The pure isolates obtained from column chromatography were then identified using TLC with various eluents. In this study, 4 variations of the mobile phase were used to ensure a single spot. The results of the TLC can be seen in Figure 2. From the picture above it is known that the color spots formed are only one spot, namely bright blue (F401) and purplish red (A402) at 365 nm UV light. The number of colored spots formed on the TLC plate is thought to be pure isolate obtained from the column results. Each isolate will then be characterized by UV-Vis and FT-IR to predict its molecular structure. In Table 2, data for calculating the Rf value is presented based on a comparison of the distance traveled by the color spot and the solvent.

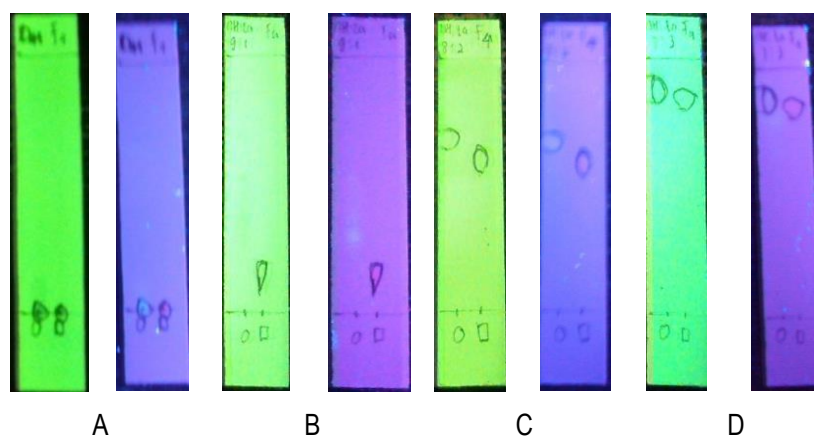


Figure 2. TLC of pure isolates with various solvents

- A : Eluent n-hexane 100%
 B : Eluent n-hexane:ethyl acetate (9:1)
 C : Eluent n-hexane:ethyl acetate (8:2)
 D : Eluent n-hexane:ethyl acetate (7:3)
 ○ : Pure Isolate F401
 □ : Isolate Pure A402

Table 2. RF value of each pure isolate using TLC

Isolat	RF isolates from each eluent				UV 254	UV 356
	n-hexane 100%	n-hexane: ethyl acetate (9:1)	n-hexane: ethyl acetate (8:2)	n-hexane: ethyl acetate (7:3)		
F401	0	0,22	0,53	0,71	colorless	Bright blue
A402	0	0,1	0,49	0,67	colorless	Purplish red

3.3. Structural elucidation of pure isolates F401 and A402 using UV-Vis and FT-IR.

3.3.1. Pure isolate F401 *E. spinosum*

Characterization of isolates using a UV-Vis spectrophotometer obtained maximum absorption with a wavelength of 218.50 nm and an absorbance value of 4.79 M⁻¹ cm⁻¹. Absorption at these wavelengths is thought to be due to the π - π^* transition where organic molecules have unsaturated functional groups. Based on the results of a study conducted by Jongaramruong, et al., [14] reported the presence of monoterpenes in the red algae *Plocamium cartilagineum* absorption appeared in the 234 nm and 249 nm regions. Likewise in the study of Wang, et al., [15] who isolated alkaloid compounds, absorption appeared at 220 nm. When compared with the UV value of the isolate which is worth 218.50 nm, see Figure 3. There is a blue shift, namely a shift in the absorption band towards shorter wavelengths.

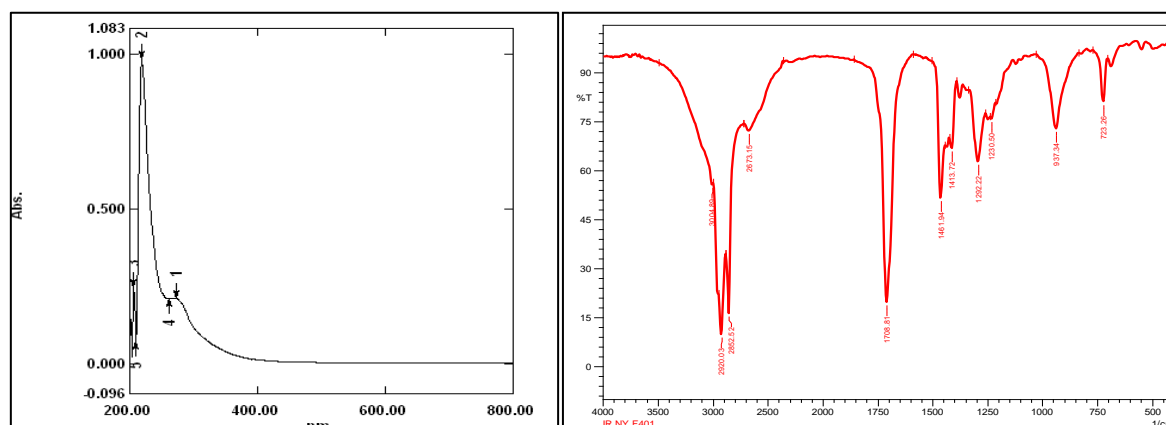


Figure 3. The UV-VIS and FT-IR spectra of isolate F401 *E. spinosum*

The results of UV-Vis analysis were supported by FT-IR spectrum analysis data to show possible functional groups present in isolate F401. The spectrum data showed that there was a sharp absorption in the wave number region 2852-2923 cm⁻¹ which was thought to be absorption from the C-H aldehyde group. This data were strengthened by the absorption of wave number 1708cm⁻¹ which indicated the presence of a C=O carbonyl group present in aldehydes. However, the

absorption of this carbonyl group was relatively low to be said as an aldehyde carbonyl compound because it shifted closer to 1700 cm^{-1} . At 2852-2923 cm^{-1} indicates the presence of a C-H group which can be methyl (CH_3) and methylene (CH_2). This was reinforced by the presence of bending vibrations seen at 1461-1463 cm^{-1} for the methylene group (CH_2). The C-C functional groups were seen at wave numbers 1170-1236 cm^{-1} . At 3010 cm^{-1} there was an absorption for stretching vibrations =C-H. bending (bending vibration) of H-C = out of plane vibration (out of plane vibration or deformation vibration) appears at the absorption value at 937 cm^{-1} . At 723 cm^{-1} there is a stretching vibration of the C-Cl halogens. These data supported research conducted by Suzuki et al[16] (2009), Cabrita et al[17] (2010), Kokkotou et al[18] (2014) which stated that *E.spinosum* has a functional group in the form of halogens.

3.3.2. Pure isolate A402 *E.spinosum*

The results of the analysis on isolate A402 using a spectrophotometer gave maximum absorption at a wavelength of 233.50 nm with an absorbance value of 0.82 and 407.50 nm with an absorbance value of 0.206. This absorption maximum is thought to be due to the $n-\pi^*$ transition by a C=O chromophore group. This conjecture is supported by the appearance of absorption at IR with wavelengths 1710.74 and 1743.53 which were characteristic of the wavelength region of the carbonyl group. The appearance of two absorption bands in the UV spectrum suggests that some transitions occur in these compounds. The formation of UV-Vis spectral bands were caused by the occurrence of electronic excitation of more than one kind in the molecular groups of a compound. UV-Vis spectrum data can be seen in Figure 4 below.

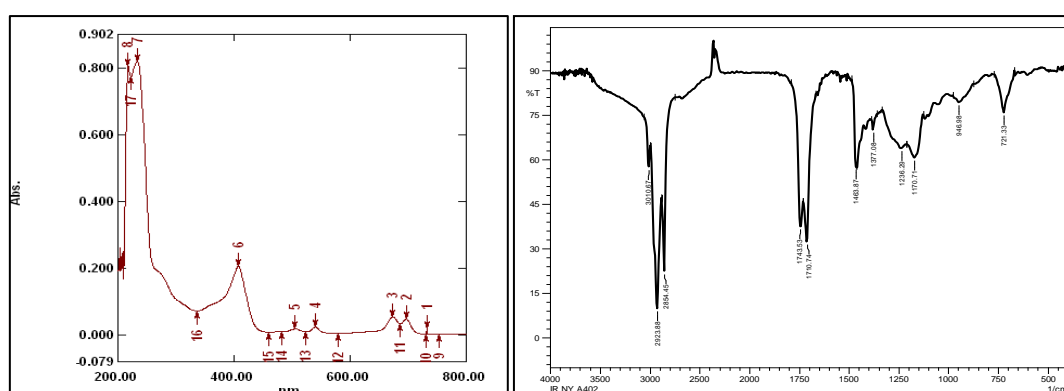


Figure 4. The UV-VIS and FT-IR spectra of isolate A402 *E.spinosum*

The results of absorption spectrum data from analysis with the IR spectrophotometer on pure isolate A402 showed absorption at wave numbers 2854.45 and 2923.88 indicating the presence of aliphatic C-H stretching vibrations which may be the presence of CH_3 and CH_2 methyl groups. This data were reinforced by the emergence of absorption at 1463.38 and 1377.08. Besides that, there was a stretching vibration at 1170-1236 which indicated the presence of C-C groups in the isolate.

At wave numbers 1710-1743 it is known that it was an absorption for C=O carbonyl which was usually typical for aldehydes and ketones. For C=C absorption in the spectrum it was possible that this group was isolated by other groups, but at wave number 3010 a stretching vibration absorption appeared which was the =C-H region as well as 721 and 946 bending vibrations from =C-H. Based on both UV and NMR data, the wavenumber absorption data supported each other. In UV it is known to have the C=O functional group, as well as IR which appears absorption at 1710 and 1743. The appearance of two absorptions at this wave number is suspected in isolate compound A402 having two C=O groups.

Based on the UV-Vis and FTIR of compound identification in the two pure isolates indicated the presence of terpenoid compounds, each of which was bound to a halogen group. as a study conducted by Kokkotou et al [18] 2014 which stated that red algae have a functional group in the form of halogens. This was also previously confirmed through initial screening on *E. spinosum* by Inayah et al, [6] 2021 that the *E. spinosum* sample positively contained terpenoid compounds. Gressler et al.[13] 2011, Jongaramrourng & Blackman [14] 2000 also succeeded in isolating terpenoid compounds in *E. spinosum*.

4. Conclusion

Isolation of secondary metabolites in *E.spinosum* was carried out using column chromatography technique. The isolates obtained were white solids (F401) and brown gels (A402). Based on the thin layer chromatography qualitative test with four different types of solvents, it was stated that the stain formed was a pure compound. Each has R_f 0.71 with a bright blue color (F401) and R_f 0.67 with a purplish red spot color (A402). Interpretation of data using UV-VIS and FT-IR data for the presence of terpenoid compounds in *E.spinosum*.

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