IDENTIFICATION OF PHYTOCHEMICAL COMPOUNDS OF ETHYL ACETATE EXTRACT OF BULUNG ANGGUR (*Caulerpa* sp.) BY GC-MS AND TOXICITY TEST ON *Artemia salina* Leach SHRIMP LARVAE

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ABSTRACT

Sea grape (*Caulerpa* sp.) is one of the potential types of seaweed. Sea grape potential as a natural ingredient for making medicine. Balinese people use sea grape for consumption. The content of bioactive compounds in sea grape is suspected to be toxic at certain doses. The purpose of this study was to determine the compound content of ethyl acetate extract of sea grape (*Caulerpa* sp.) and the toxicity on *Artemia salina* Leach. The identification of the bioactive compounds was carried out by Gas Chromatography-Mass Spectrophotometry and the toxicity test of extracts was carried out using the Brine Shrimp Lethality Test (BSLT) method. The results showed that the ethyl acetate extract of sea grape (*Caulerpa* sp.) contained 27 chemical compounds, 7 of which had a quality value > 70%, namely *Propanoic acid, ethyl ester*; *n-Propyl acetate*; *sec-Butyl acetate*; *Toluene*; *Acetic acid, butyl ester*; *Bicyclo [4.2.0] octa-1, 3, 5-triene*; and *Styrene*. The results of the toxicity test of *Artemia salina* Leach are toxic which had an LC_{50} value of 44,070 ppm so that they can be used as a larvicide.

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INTRODUCTION

Indonesia is known as a country which has a sea area with a high biodiversity county. Seaweed is one of biological resources there are available abudant in Indonesian waters. In generally, seaweed well-known source of important food hydrocolloids, such as agar, alginates, and carrageenan. In addition, secondary metabolites found in seaweed potential for activity antimicrobial such as antiviral, antibacterial, and antifungal (Suptijah, 2002). Several species of seaweed is potentially developed as a medicine. Sea grape (*Caulerpa* sp.), locally known as bulung anggur in Bali has potensial nutrient such as

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carbohydrates, crude fiber, high ash, and low fat content (Tapotubun, 2018). Balinese people have been consuming sea grape as a fresh vegetable and salad.

The previous study reported several beneficial phytochemicals components in the ethyl acetate extract of *Caulerpa* sp., which exhibited strong antibacterial activity and antioxidant (Marraskuranto *et al*, 2021). According to Anggadiredja (2006), sea grape has been widely used as traditional medicine because it has plant chemical compound that are active substances. The content of bioactive compounds in sea grape is suspected to be toxic at certain doses. Therefore, it is necessary to study the identification of the bioactive components in the ethyl acetate extract of *Caulerpa* sp. through gas chromatography to determine the chemical compositions, especially the biological activity with toxicity test use Brine Shrimp Lethality Test (BSLT) method. Plant bioactive components can be obtained by extraction using solvent. Ethyl acetate solvent is reffered to as semipolar solvent.

Toxicity testing of the extract can be perfomed through *Brine Shrimp Lethality Test* (BSLT) using larvae *A. Salina*. The BSLT test has a spectrum of pharmacological activity that is easy to perform, simple, fast, and does not require large costs with a 95% confidence level. This method can identify the toxicity of natural ingredients and be seen from the number of dead larvae *A. Salina* with observed after 24 hours. The toxicity test was assessed by determining the LC_{50} score. The LC_{50} score is defined as the concentration of compound causing 50% mortality of shrimp larva with using the solvent of ethyl acetate.

The objective of this study was to analyzed the bioactive components in the ethyl acetate extract of *Caulerpa* seaweeds using GC-MS analysis and the toxicity test with BSLT method to determine the biological activity using larvae *A. Salina*.

MATERIALS AND METHODS

Preparation of Sample

Samples of fresh bulung anggur (*Caulerpa* sp.) macroalgae used in this study was collected at Serangan, Bali. Bulung anggur was cleaned from impurities and washed under running water. The sample bulung anggur is thoroughly rinsed with water, dried with air at room temperature for 4 days, then oven-dried at a temperature of 45^0 C for 3 days until a constant weight was obtained.

Extraction with Macerated Method

The dried sample was blended and sieved resulting in powder. 250 g of sample powder was macerated by 3 L of ethyl acetate for a period of three days with regular shaking. The extract is roughly filtered with filter paper and funnel. The total filtrate with extraction

carried out in double, then evaporated at 35^0 C until the viscous extract obtained.

Identification Bioactive Compounds Using *Gas Chromatography-Mass Spectrometry* (GC-MS)

Analysis of sample bulung anggur was performed by using GC-MS machine in Denpasar Police Forensic Denpasar. A volume of 1µl was injected in injector with temperature was set at 290^0 C for 27 minutes. Nitrogen was used as a carrier gas at a constant flow of 1.0 ml/min.

Preparation of Larvae *A. Salina*

Synthetic sea water was prepared by dissolving 40 g of salt without iodine in 2000 mL of water which was irradiated with 25 watt fluorescent lamp. At the same time, aeration is regulated by utilizing an aerator. Then enter the amount of approximately 1 mg of *A.salina* eggs into the vessel that already contains seawater. The eggs hatch after 24-36 hours. A period of 2 to 3 days, larvae into instar level 2 and larvae can be used as test animals.

Toxicity Test

The test solution is made with a concentration of 500 ppm, 250 ppm, 125 ppm, 50 ppm, 10 ppm, and 0 ppm as a control or without added extract. The sampel was added Dimethyl sulfoxide (DMSO) as much 10 µl to dissolve in 5 mL of a suitable solvent into each test tube. Put 10 larvae of *A. salina* into the test tube and add seawater to a final volume of 5 ml so that the final results of the test solution are obtained with concentrations of 500 ppm, 250 ppm, 125 ppm, 50 ppm, and 10 ppm. The control group was only given 5 ml of seawater and added DMSO without added extract. Each concentration had five replications. The total number of *A. salina* was used 300 larvae. The percentage of dead *A. Salina* observed after 24 hours an was calculated. The standard criteria for assessing the mortality of *A. salina* larvae is that the larvae do not show movement of observation. The toxicity test was assessed by determining the LC_{50} score. Eq. 1 shows the % larvae mortality equation. (Nurhayati et al. 2006)

$$\% \text{ Mortality} = \frac{\text{Total larvae mortality}}{\text{Total larvae}} \times 100\% \quad \text{.....(1)}$$

The LC_{50} score is defined as the concentration of a compound causing 50% mortality of larvae *A. Salina.* Data were analyzed by probit in linear regression y = mx + b was carried out using the Microsoft office excel. The level of toxicity of a compound was classified according to Hamidi *et al.,* 2014. It was toxic with high to low intensity when the LC_{50} of < 1000 mg/L and it was non toxic when the LC_{50} > 1000 mg/L.

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RESULTS AND DISCUSSION

Identification of the bioactive compounds in the ethyl acetate extract of *Caulerpa*

The chromatogram of gas chromatography analysis is shown in Figure 1 Information on compound names, retention time (Rt) and the area under the curve (AUC) is shown in Table 1, sorted from the highest to the lowest quality. Bulung anggur extract contains *Propanoic acid, ethyl ester* as the dominant compound. *Propanoic acid, ethyl ester* shows the activity as antimicrobial (Wang *et al.*, 2014) and food flavouring (Vidya, 2010). Figure 1 shows the chromatogram of bulung anggur ethyl acetate extract.

The relative amount of each component was calculate by comparing its avarage peak area to the total areas. The spectrum component was compared with the spectrum of the component stored in the library.

The GC-MS analysis of ethyl acetate extract of *Caulerpa* revealed 27 chemical compounds, which has 7 compounds with quality value > 70%. The highest peak (RT 2.476) indicating the presence of *Propanoic acid, ethyl ester* as the most abundance compound of the ethyl acetate extract of bulung anggur. Table 1 shows the bioactive compounds in ethyl acetate extract of *Caulerpa*.

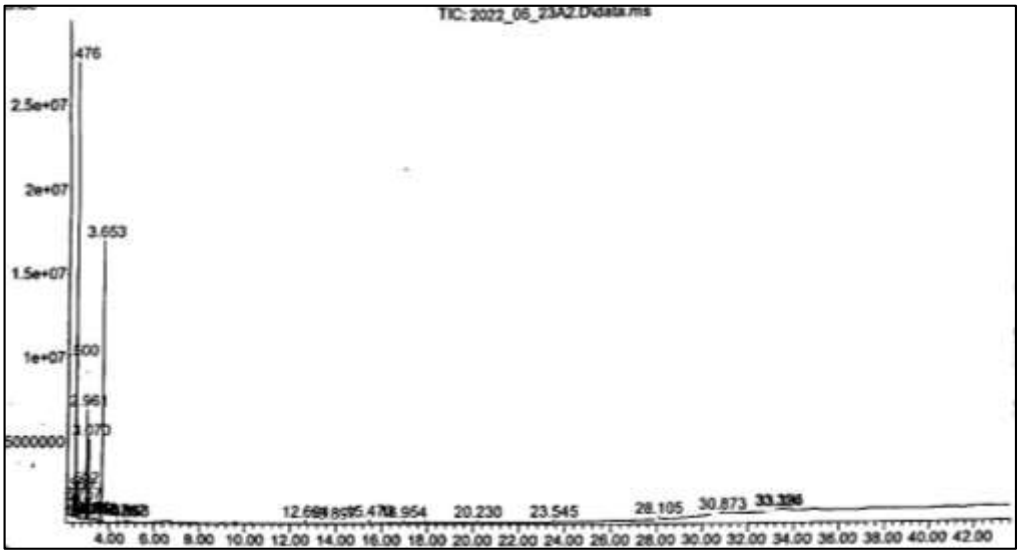

Figure 1. The chromatogram of bulung anggur ethyl acetate extract

In Table 1, the GC-MS analysis of ethyl acetate extract of *Caulerpa* revealed 3 chemical compounds, which has a quality value > 90% such as *Toluene, Bycyclo {4.2.0} octa-1, 3, 5-triene,* and *Styrene*. *Toluene* compound has a quality value 94%, *Bicyclo {4.2.0} octa-1, 3, 5-triene* compound has a quality value 92%, dan *Styrene* compound has a quality value 92%.

There are two main groups of phytochemical components are ester functional groups and aromatic hydrocarbon compounds. Ester functional groups are reported as larvacide agent on *Aedes Aegypti* larvae (Suirta *et al.,* 2007). The presence of compounds such as *Propanoic acid, ethyl ester* which has the highest AUC value and *Acetic acid, butyl ester* are include in the Ester functional groups. On the other hand, *Toluene* and *Styrene* compound was identified as aromatic hydrokarbon compounds. Aromatic hydrokarbon compounds reported that in low levels can reduce the rate of growth and development of aquatic animals (Marsaoli, 2010).

Table 1. Bioactive compounds in ethyl acetate extract of *Caulerpa*

Rt (min)	AUC (%)	Compound
2.476	37.71	*Propanoic acid, ethyl ester*
2.500	10.00	*n-Propyl acetate*
2.961	8.63	*sec-Butyl acetate*
3.070	6.41	*Toluene*
3.653	24.33	*Acetic acid, butyl ester*
4.868	0.22	*Bicyclo [4.2.0] octa-1, 3, 5-triene*
4.868	0.22	*Styrene*

Analysis GC showed that, other compounds of ethyl acetate extract of bulung anggur, for instance *n-Propyl acetate*, *sec-Butyl acetate,* and *Bicyclo [4.2.0] octa-1, 3, 5-triene* compound. *N-Propyl acetate* was observed can be use as food flavoring, fragrance ingredients, and solvent (PubChem, 2005). *Sec-Butyl acetate* is used in extraction solvent in the process of petroeum and pharmaceuticals (Norliana and Rabiah, 2021). *Bicyclo [4.2.0] octa-1, 3, 5-triene* can be used as medicine mixture (Firdouse, 2019). Table 2 shows the result of *Caulerpa* extract on *A. salina*.

Table 2. The result of *Caulerpa* extract on *A. salina*

Concentration	Replication					Total death	Avarages of death	Percentage of death (%)
	I	II	III	IV	V			
500	10	10	10	10	9	49	0,98	98
250	8	8	8	9	7	40	0,8	80
125	5	6	6	6	5	28	0,56	56
50	4	5	4	4	3	20	0,4	40
10	3	3	2	4	2	14	0,28	28
0 (Control)	0	0	0	0	0	0	0	0

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Toxicity Test with the BSLT Method

As shown in Table 2, the test result showed that the amount of extract concentration in the media could kill *A. salina* with concentrations of 500, 250,125, 50, 10, 0 ppm, respectively. The number of *A. salina* deaths in each test tube at various concentrations of *Caulerpa* extract treatments is shown in Table 1. It can be seen from the table that variations in the concentration of *Caulerpa* extract in this experiment showed different effects on the mortality of *A. salina.*

Total larva mortality was obtained by adding up the larvae that died at each concentration. The highest number of deaths occured at a concentration of 500 ppm an the lowest occured at a concentration of 10 ppm. Meanwhile in the control batch there is no the death of the larvae shrimp, it means caused by substance contained in extract, not from factors beyond control such as temperature, humidity, light intensity and the lack of other food sources.

The result of each organic solvent partitioning revealed a different effect on the mortality rate of the tested *Artemia salina* larvae shrimp. This was caused by the different extractive substance content in each solvent. Toxicity evaluation via BSLT method required high precision, because many factors can affect the mortality of larvae shrimp *Artemia salina* L. The shrimp larvae are very sensitive to any substance presence within their habits. Their skin is a thin membrane circumstances which allow diffusion of substances from environment, affecting their metabolism. In addition to their sensitivity to the environment. Figure 2 shows the graph of the log relationship between the concentrations of bulung anggur extract.

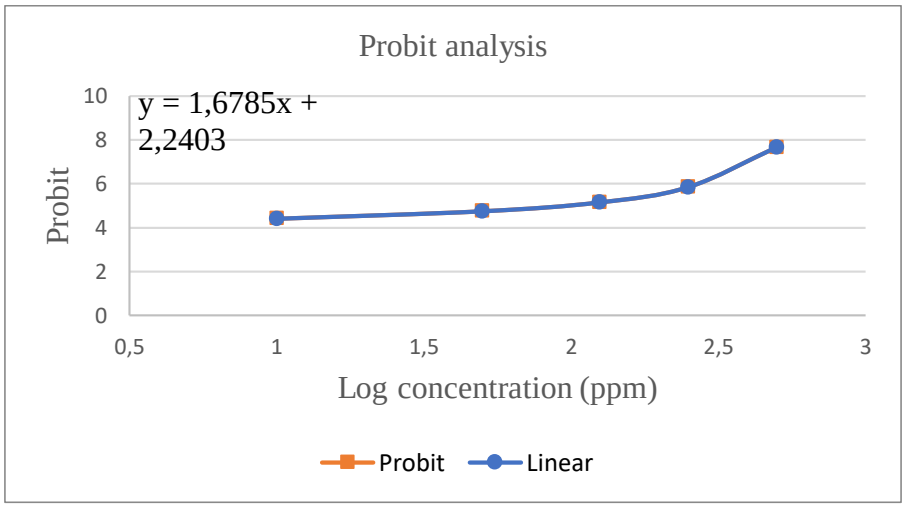

Figure 2. Graph of the log relationship between the concentrations of bulung anggur extract

The result of probit analysis using *Microsoft office excel* showed that the LC_{50} value of *Caulerpa* extract was 44,07 ppm. The extract showed toxic activity in a toxicity test if the extract caused the death of 50% of test animals at concentration < 1000 ppm. Based on this statement, the *Caulerpa* extract is toxic. The mechanism of death of *A. salina* is related to the function of ester functional groups and aromatic hydrokarbon compounds inhibiting larvae feeding power (antifedant).

The way these compounds work is to act as stomach poisoning or stomach poison. Therefore, when these compounds enter the larva's body, the digestive system will be disturbed. This compound will block the taste receptors in the mouth area of the larvae. This resulted in the larvae failing to get a taste stimulus so they were unable to recognize their food and consequently the larvae starved to death. According to aromatic hydrokarbon compounds are plant defense compounds that suspected to be toxic at certain doses and can inhibit insect eating.

CONCLUSIONS

Based on this study it can be concluded that ethyl acetate extract of bulung anggur revealed 27 chemical compounds which has 7 compounds with quality value of > 70% such as *Propanoic acid, ethyl ester; n-Propyl acetate; sec-Butyl acetate; Toluene; Acetic acid, butyl ester; Bicyclo [4.2.0] octa-1, 3, 5-triene;* and *Styrene*. Ethyl acetate extract of bulung anggur was determined to be moderately toxic to *A. Salina* larvae with an LC_{50} value of 44,070 ppm. Hence, it has the potential to be developed as a larvacide.

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