SECONDARY METABOLITES AND CYTOTOXIC ACTIVITIES OF HEXANE EXTRACT GRACILARIA SALICORNIA SEAWEED AGAINST MCF-7 BREAST CANCER CELLS

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Abstract

This study aims to assess the cytotoxic activity against MCF-7 breast cancer cells and the content of secondary metabolites from the hexane extracts of Gracilariasaliconia. Samples of G. salicornia were taken from Hari Island waters, Southeast Sulawesi, Indonesia, and then extracted by maceration using hexane. Chemical content was analyzed using phytochemical tests and Gas Chromatography-Mass Spectrometry (GCMS). Toxicity potential was evaluated using the Brine Shrimp Lethality Test (BSLT) method, and cytotoxicity to MCF-7 cells using the MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This study showed that the hexane extract showed a cytotoxic potential of 248.40 mg/L (BSLT) and 325.34 mg/L (MTT). This activity was supported by the phytochemical screening of hexane extracts containing alkaloid, phenolic, and steroid/terpenoid compounds. GCMS data of hexane extract contains 16 secondary metabolites with the main compounds 1-(1-(Methylthio) propyl)-2-propyldisulfane; N-Methoxydiacetamide; Hydrazine, 1,1-dimethyl-2-propyl. This study concludes that the hexane extracts from G. salicornia contain many secondary metabolites, which may be responsible for the cytotoxic activity against MCF-7 breast cancer cells.

Keywords*: G. salicornia, cytotoxic, BSLT, MTT, MCF-7*

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Introduction

Breast cancer is the most common cancer worldwide and continues to impact cancer deaths significantly. In 2020, there were more than 2.3 million new cases and 685,000 deaths from breast cancer. The high number of people living with breast cancer worldwide (Arnold et al., 2022) has motivated researchers to research drugs to treat breast cancer. There are limitations in using chemotherapeutic agents in treating cancer caused by the high level of toxicity, and tumour resistance to chemotherapeutic medications after continued therapy is still unavoidable. Therefore, it is imperative to continue to search for new drugs that improve or maintain efficacy while minimising toxicity and delaying the development of drug resistance. One alternative is to search for new medicinal compounds derived from seaweed (Fristiohady et al., 2021).

Seaweed is very abundant in Indonesia (Rugebregt et al., 2021). In Southeast Sulawesi, especially in Hari Island waters, the composition of seaweed for the Chlorophyta class is 44%, and Rhodophyta and Phaetophyta each are 28%. In the Rhodophyta class, the genus Gracilaria is most commonly found, namely 43% compared to other genera. The species found in the genus Glacilaria are G. salicornia, G. edulis, and G. verrucose (Ira et al., 2018). Rhodophyta contains rich secondary metabolites such as sulfated polysaccharides, acrylic acid, polyunsaturated fatty acids, phytol and polyphenols which have biological activities as antioxidants, antifungal, anti-tumour, antimicrobial, anti-inflammatory and spasmolytic activity (Gowdhami et al., 2019). The genus Gracilaria is one of the seaweeds from the Rhodophyta class used for traditional medicine, including antihypertensive, digestive systems treatment such as diarrhoea, dysentery, intestinal inflammation, haemorrhoids, constipation, and jaundice, thyroid system medicine, toroid tumours, treatment of the reproductive system such as libido stimulant, vaginal discharge and bleeding, respiratory system treatment such as pneumonia, cough, throat irritation and lung complications, urinary system treatment such as bladder complications, difficulty urinating and diuretic properties, as well as other diseases such as beriberi berries, diabetes, obesity, wounds and swelling (Torres et al., 2019).

Secondary metabolites and extracts from the genus Gracilaria have biological effects, including phytol from G. edulis inhibits A549 cancer cells with an lC50 of 24.5 ± 19.1 µg/mL (Sakthivel et al., 2016), MCF-7 cancer cells with lC50 125 µg/mL (Sheeja et al., 2016), Stigmasterol, β-Stigmasterol, Brassicasterol and Campesterol from Gracilariaspp (Kasanah et al., 2015), G. corticate methanol extract inhibited cancer cells with HeLa with lC50 27 µg/mL, HepG2 with lC50 92 µg/mL, HT-29 with lC50 130 µg/mL, MCF-7 with lC50 25 µg/mL, MDA-MB-231 with lC50 45 µg/mL (Namvar et al., 2014). Methanol extract from G. changii has anti-inflammatory activity (Shu et al., 2013), methanol and acetone extract from G. verrucosa, methanol:acetone extract of G. multipartita has antibacterial activity (Oumaskour et al., 2013).

G. salicornia is a genus of Gracilaria, which has limited research reports on the bioactivity of the extract and its secondary metabolites. The new secondary metabolites found from G. salicornia originating from the coast of India having activity as antioxidants and anti-inflammatories are terpenoid secondary metabolites (Antony† and Chakraborty†, 2018; Antony and Chakraborty, 2020a; Chakraborty et al., 2019), spiro derivatives (Chakraborty and Antony, 2019) and chromene derivatives (Antony and Chakraborty, 2020b). Several studies on extracts and secondary metabolites of G. salicornia included the methanol extract of G. salicornia as an anticancer inhibiting the growth of HT-29 cancer cells with lC50 68.2 µg/mL, HeLa with lC50 125.9 µg/mL and MCF-7 with lC50 185.8 (Ghannadi et al., 2016). The water extract of G. salicornia is an anti-inflammatory (Paramsivam et al., 2016).

In this study, the BSLT and MTT cytotoxic tests were carried out on MCF-7 cancer cells, secondary metabolite profiles, and secondary metabolite content in hexane extracts of G. salicornia which was the first report originating from Indonesia.

Research methods

Materials collection: Sampling G. salicornia in Hari Island Waters, South Konawe Regency, Southeast Sulawesi Province (S 04o02.270' and E 122o46.555') and identified at the Oceanography Laboratory, BRIN. Samples of G. salicornia were dried at room temperature in an aerated manner, protected from direct sunlight for five days, and then pollinated with a Hammer Mill grinding machine.

Sample extraction: G. salicornia dry powder is macerated use hexane solvent. Soaking using extracting solvent was carried out for 3 x 24 hours, and the macerate obtained was concentrated with an evaporator until a thick extract was obtained.

Phytochemical screening assay: It was carried out using the colourimetric method to detect the presence of secondary metabolites in the hexane extract of G. salicornia in the analysis of secondary metabolites of alkaloids, flavonoids, tannins, and steroids/terpenoids following the procedure of Yadav et al., (2014). The content of secondary metabolites from G. salicornia was identified using GCMS by following standard instrument procedures used by Khadijah et al., (2021).

Cytotoxic Test: The toxicity of hexane extract of G. saliconia was analysed using a modified Brine Shrimp Lethality Test (BSLT) following the procedure Wahyuni et al. (2021). The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess the cytotoxicity of hexane extract of G. saliconia against MCF-7 breast cancer cells in vitro following procedure Vivek et al., (2022).

BSLT assay: It was used to study the general toxicity of hexane extracts. Artemia salina eggs are incubated in a transparent container filled with seawater. To keep the temperature from 25-30°C during the incubation process, a 40-60 Watt bulb is placed. The blower will supply oxygen for toxicity studies using larvae aged 48 hours. Concentrations of 10, 100, 500, and 1000 mg/L and negative controls were used in BSLT. A stock solution of solid extract was prepared by equilibrating 5 mg of extract and dissolved in 5 mL of methanol up to 1000 ppm. The stock solution is then pipetted into the vial. Each concentration had three replicates (triplicates). The vial is ventilated to dry the sample placed in it. 1% DMSO was added to the bottle for the water-insoluble fraction. 5 milliliters of seawater is added to the bottle. The process was continued by adding samples to the vial with concentrations of 10, 100, 500, and 1000 mgL-1. The control contains no samples. Ten larvae of Artemia salina were added to each vial. Observations were made for 1x24 hours. A toxicity test was determined based on the number of dead larvae. The LC50 score determines the toxicity studies evaluated. The LC50 score was first performed to assess larval mortality after 24 hours of exposure.

Mortality (%) = (Total larvae mortality/Total larvae) x 100%.

Probit analysis was performed using mortality to determine the LC50 score. The LC50 score is the concentration at which a compound causes 50% mortality. After probit analysis, LC50 uses the linear regression formula y = a+bx. The toxicity of the compound was classified using the LC50 score. LC50 with low toxicity (LC50>1000 mg/L), medium toxic (31-1000 mg/L) and highly toxic (≤30 mg/L) (Sami et al. 2019).

MTT Assay: The dry hexane extract was weighed as much as 7.81; 15.63; 31.25; 62.50; 125 mg is dissolved in 1 mL of dimethylsulfoxide (DMSO) to make 7.81; 15.63; 31.25; 62.50; 125 mgL-1 stock solution. A 0.2 m filter was used to filter the sample stock solution and stored at 26°C until use. The solution used must be fresh. Doxorubicin was used as a positive control. The solution is prepared at four °C and protected from light for each work. MCF-7 cells in RPMI-1640 medium with 10% FBS, 90 µL, were inserted into a 96-well plate with a density of 1 x 104 cells per well, incubated for 24 hours at 37oC with 5% CO2. Add 10 µL of G. salicornia hexane extract solution (7.81; 15.63; 31.25; 62.50; 125 mgL-1) and Doxorubicin as a positive control, incubate for 72 hours under the same conditions. The medium was discarded and replaced with 100 µL MTT in RPMI-1640 with 10% FBS (0.5 mg/mL); the cells were then incubated for three h at 37oC with 5% CO2. After 3 hours, the MTT solution was discarded, the formazan crystals were dissolved in DMSO, and the incubation was 15 minutes. The absorption absorbance was measured with an Elisa reader

at 570 nm. Cell cytotoxicity of each extract was calculated using the following equation:

%cytotoxicity = 100 − (ABStest/ABScontrol × 100%).

Where ABStest is the average absorbance of cells treated with algal extracts, ABScontrol is the average absorbance of corresponding DMSO control; the data were analysed using Microsoft Excel. Microsoft Excel is used to find out the lC50 value in the sample.

Results and Discussion

Phytochemical Screening Assay

The results of maceration of 127 kg of G. salicornia dry powder after the solvent had been evaporated yielded 2.0 g (0.018%) of hexane extract. The results of the phytochemical tests for the two extracts obtained are presented in Table 1. Based on Table 1, the hexane extract contains an alkaloid, phenolic, and steroid/terpenoid.

Table 1. Phytochemical screening of hexane extract of G. Salicornia

Sample (s)	Weight (g)	Chemical Contents			
		Flavonoid	Alkaloid	Phenolic	Steroid/ Terpenoid
Hexane Extract	2.0 (0.018 %)	-	+	+	+

Note: (+): detected to contain chemicals, (-): not detected to contain chemicals

From several studies, the results of phytochemical tests from the water extract of G. salicornia originating from the coast of India contain tannins, flavonoids, phenol, and steroids (Paramsivam et al., 2016), the methanol extract of G. salicornia originating from the Indonesian Seribu Islands contains flavonoids, saponins, and steroids (Widowati et al., 2021), the methanol extract of G. salicornia originating from the Persian Gulf contains tannins, alkaloids, saponins, sterols and flavonoids (Ghannadi et al., 2016). The difference is caused by dissolution extraction and different environmental and climatic conditions (Barbouchi et al., 2020).

Analysis of secondary metabolite content using the GCMS instrument. The GCMS data from the hexane extract in Table 2 shows the presence of 16 secondary metabolites belonging to the compound esters, alkenes, ketones, and fatty acids. Based on the relative percentage of secondary metabolite areas, the order is as follows: Hexadecanoic acid, methyl ester; 3-methyl-1-pentene; Butyl isocyanatoacetate; 1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester; Eicosane; Hexadecanoic acid, 2-methyl-, methyl ester; 10-Nonadecenoic acid, methyl ester; l-(+)-Ascorbic acid 2,6-dihexadecanoate; (E)-methyl 11-methyloctadec-12-enoate; Methyl 13-methyltetradecanoate; 6,10,14-trimethyl-2-pentadecanone; Methyl tetradecanoate; 6,10,14-trimethyl-2-pentadecanone; trans-13-Octadecenoic acid, methyl ester; cis-13-Octadecenoic acid, methyl ester; Methyl stearate; and 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester.

Table 2. Compounds of hexane of G. salicornia based on GC-MS/MS data

No.	Retention Time (min)	Relative Area (%)	Similarity Index (%)	Compound
1	1.377	20.85	93.1	Butyl isocyanatoacetate
2	1.49	22.67	89.4	3-methyl-1-pentene
3	20.57	4.05	91.0	Eicosane
4	21.059	1.45	92.3	Methyl tetradecanoate
5	22.287	1.54	88.3	Methyl 13-methyltetradecanoate
6	23.304	1.53	92.4	6,10,14-trimethyl-2-pentadecanone
7	24.852	27.42	94.7	Hexadecanoic acid, methyl ester
8	25.518	1.68	84.8	l-(+)-Ascorbic acid 2,6-dihexadecanoate
9	26.032	2.47	90.9	Hexadecanoic acid, 2-methyl-, methyl ester
10	27.841	1.39	93.1	trans-13-Octadecenoic acid, methyl ester
11	27.936	1.39	92.8	cis-13-Octadecenoic acid, methyl ester

12	28.28	1.23	92.0	Methyl stearate
13	28.341	1.57	81.7	(E)-methyl 11-methyloctadec-12-enoate
14	29.668	1.74	87.4	10-Nonadecenoic acid, methyl ester
15	34.449	1.22	85.7	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
16	38.578	7.14	90.3	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester

Cytotoxic Test

The hexane extract of *G. salicornia* was followed by a preliminary anticancer screening test using a toxicity test using *Artemia salina Leach* shrimp larvae. This method is known as the Brine Shrimp Lethality Test (BSLT). The BSLT method is often used for the initial screening of active compounds contained in extracts which can determine the toxicity of a compound (Wahyuningsih, 2022). As an early anticancer screening test, the BSLT method is insufficient to determine bioactive substances' mechanism of action and is not specific to the anticancer activity. The BSLT toxicity value of the hexane extracts of *G. salicornia* was obtained through a linear regression equation between log concentration and the probit value. The graph can be seen in **Figure 1**. It can be seen that the linear regression equation of hexane extract, and potassium dichromate as positive controls is y = 0.5107x +3.7768, and y = 1.0027x + 3.8519. From the linear equation, the value of x is obtained as the toxicity of LC_{50}.

The BSLT method is insufficient to determine the mechanism of action of the bioactive agent and is not specific to its anticancer activity. However, the BSLT method provided data that a more specific bioassay could support after the compound tested was toxic to *Artemia salina*, suggesting that the compound may be a potential candidate for cancer research. Further evaluation to demonstrate the cytotoxicity of *G. salicornia* extract was carried out in vitro using the3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT) assay (Aksano et al., 2022). The MTT toxicity value of the hexane extracts of *G. salicornia* was obtained through a linear regression equation between concentration and inhibition. The graph can be seen in **Figure 2**. The linear regression equation of hexane extracts, and doxorubicin as positive controls is y = 0.1255x + 9.1699, and y = 1.4306x + 44.147. The MTT toxicity value was obtained from the x value of the linear equation as IC_{50} toxicity.

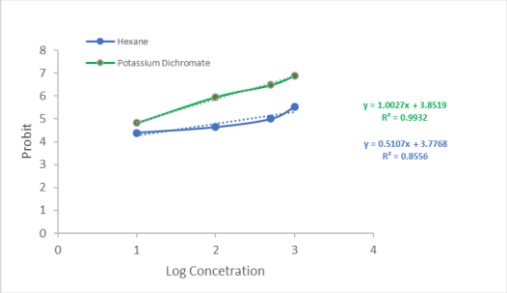

Figure 1. Diagram of the relationship between the probit value and the log extract concentration

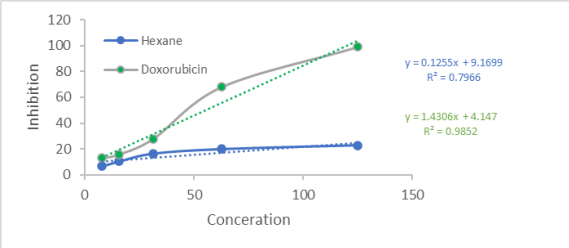

Figure 2. Diagram of the relationship between the inhibition value and the extract concentration.

The LC_{50} and IC_{50} toxicity values of the hexane extracts of *G. salicornia* are shown in Table 4. The BSLT values of hexane extracts with LC_{50} values of 248.40 mg/L were included in the medium toxic category (31-1000 mg/L) using a positive control potassium dichromate with LC_{50} values of 13.96 mg/L included in highly toxic category (≤ 30 mg/L). The results of this study are similar to previous studies. The hexane extracts of *G. salicornia*

originating from the Indonesian Island of Selayar have an LC_{50} value of 662.23 mg/L in the medium toxic category (Bahrun et al., 2021). Data from anticancer test results of hexane *G. salicornia* extract against MCF-7 breast cancer cells with an IC_{50} value of 325.34 mg/L in the moderately active cytotoxic category (100-500 mg/L) in active cytotoxic category (10-100 mg/L) (Khotimchenko et al., 2020) with doxorubicin positive control with IC_{50} 4.09 mg/L. Previous studies for the anticancer test against MCF-7 breast cancer in the species *G. salicornia* only came from the Persian Gulf with an IC_{50} of 185.6 mg/L in the moderately active category (Ghannadi et al., 2016).

Table4. Cytotoxic Activity of hexane extract of *G. Salicornia*

Sample	**LC_{50}/ IC_{50} Value (mg/L)**	
	BSLT	**MTT**
Hexane Extract	248.40	325.34
Potassium Dichromate	13.96	-
Doxorubicin	-	4.09

From the cytotoxic values of BSLT and MTT, it appears that the hexane extract of G. salicornia containing the main compound Hexadecanoic acid, methyl ester which has antibacterial and anti-fungal activity (Krishnamoorthy and Subramaniam, 2014). In addition, the combination of various groups of compounds will have a synergistic effect which can cause a cytotoxic effect from the hexane extracts of G. saliconia as potential anticancer agents against MCF-7 breast cancer cells (Sami, 2020).

Conclusion

The hexane extracts of G. salicornia contain many secondary metabolites with the potential to anticancer breast against MCF-7 cells with moderately categories.

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