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Reviewers Recommendation

Reviewer 1	EJABF-2403-4072	Minor Revision

Reviewer Comment For Author:

This study aimed to investigate cytotoxic activity of C racemosa against breast cancer cells.

- The locations season and the year of the algae collection is not indicated. It is very important because of the seasonal variations that may occur in chemical composition and consequently in biological activities.

- It should indicate the reasons why authors use methanol instead of other methods and or solvents.

Reformat Figure 3, it is unclear, provide standard error and statistical analysis
Rewrite conclusions.

Original Manuscript Status	Manuscript Needs Revision (Minor Revision)
Revision Due Date	2024-12-08

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Cytotoxic activity of *Caulerpa racemose* nanoparticles on breast cancer cells

Sri Haryanti, Mutmainah, Dewi Ramonah, Lia Kusmita*

STIFAR Yayasan Pharmasi Semarang Letjend Sarwo Edhie Wibobo Km 1, Semarang, Indonesia
***Corresponding Author: lia_kusmita@yahoo.com**

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ABSTRACT

Indonesia has abundant marine biological resources, so that it can be utilized as the basic material of a search for medicinal materials or treatment. Among these resources is *Caulerpa racemosa*, a species of green algae commonly known as sea grape, which harbors a diverse array of secondary metabolites containing bioactive compounds with cytotoxic properties against cancer cells. Cancer remains a formidable global health challenge, particularly impacting women, with factors such as uncontrolled cell division and metastasis contributing to its severity.*Caulerpa racemosa* is one type of green algae that can be utilized as anti-cancer. The caulerpenin content in *Caulerpa racemosa* shows bioactivity against human cell lines and has anticancer, antitumor, and antiproliferation properties. This study aims to determine the anticancer effect of methanol extract and *Caulerpa racemosa* nanoparticles on MCF-7 breast cancer cells. The formulation of extract nanoparticles utilized the ionic gelation technique. The results showed that the concentrations of nanoparticle preparations used were successively: 15; 30; 60; 120; 240; and 480 µg/mL with incubation time for 48 hours. The results showed that methanol extract has cytotoxic activity with IC50 of 38.29 ± 3.2µg/mL in the active category. And nanoparticle preparations have cytotoxic activity against MCF-7 cancer cells with an IC50 value of 12.35 ± 2.8 µg/mL in the very active category. Based on these results, it appears that *Caulerpa racemosa* nanoparticle preparations have higher cytotoxic activity than methanol extracts against MCF-7 breast cancer cells.

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INTRODUCTION

Breast cancer is one of the most common cancers affecting women, with an incidence rate of approximately one in 8-10 women **(Fard *et al.*, 2018)**. Based on World Cancer Report data, it is predicted that there will be an annual increase of 22 million cases **(Chia *et al.*, 2015)**. This disease ranks among the deadliest illnesses for women globally, driven by uncontrollable factors such as cell division and metastasis **(Amalina *et al.*, 2021)**. Presently, the medical approach to cancer treatment involves chemotherapy, radiotherapy, and surgery. However, these methods can induce hypoxia and cell death, potentially harming healthy non-cancerous cells **(Zeichner *et al.*, 2015)**.

Therefore, it is necessary to develop effective anticancer drugs as potential alternatives to chemotherapy. One of them is with natural plant bioactive compounds that increase effectiveness but have mild side effects. Nanotechnology is known as a branch of engineering that deals with the identification and control of materials in the range of 1 to 100 nm, thus providing unusual physical, chemical, and biological properties of nanoparticles **(Stoica *et al.*, 2013).** This technology provides benefits by increasing the bioavailability of active ingredients, controlling the release of active ingredients and possibly improving sensory properties. With a nanometer size, the active ingredient particles are more easily absorbed by the small intestinal wall thus increasing their bioavailability. Absorption of active ingredients is increased due to increased particle solubility and large particle surface area.

Caulerpa racemosa is one type of green algae that lives in some Indonesian waters. Based on the research of **Uddin *et al.*, (2020)**, it shows that the cytotoxic activity of *Caulerpa racemosa* has an IC50 value of 119.62 µg/mL. The content contained in *Caulerpa racemosa* alkaloids, flavonoids, glycosides, phenols, saponins, steroids and tannins **(Uddin *et al.*, 2020)**. *Caulerpa racemosa* also contains caulerpenin which shows bioactivity against human cell lines and has anticancer, antitumor, and antiproliferation properties **(Chew *et al.*, 2008)**. Afftan *et al* (2020) reported that the cytotoxicity of ethanol extract of green seaweed *C. racemosa* is less cytotoxic with a value (LC50 = 929 µg/ml) **(Villegas Vílchez *et al.*, 2020).** According to **Chew *et al* (2008)** hexane extract showed the highest cytotoxicity against breast cancer cells, followed by ethyl acetate and ethanol extracts (IC50 23.7 ± 2.0, 66.7 ± 5.8 and 182.7 ± 14.3 µg/mL). In this study, the formulation of extract nanoparticles with ionic gelation technique using chitosan, tripolyphosphate is carried out in the hope of improving the physical characteristics of the nanoparticles formed so as to increase the bioavailability of its active compounds.

MATERIALS AND METHODS

Equipment and Materials

Samples were *Caulerpa racemosa* from Panjang Island, Jepara, Central Java, Indonesia. *Caulerpa racemosa* were taken in July 2023 during bright daylight hours. The materials used in this study were chitosan (pharmaceutical grade), ethanol p.a. (Merck), tripolyphosphate (technical), CH3COONa (technical), glacial acetic acid (technical), ethyl acetate (Merck), ethanol 96% (technical), HCl 37% (Merck), NaCl (Sigma-Aldrich), NaOH (Merck), K_2HPO_4 (Merck) and distilled water. Equipments used in this study include UV-Vis spectrophotometer (Jenway 6800), shaker incubator (Stuart SI500), centrifuge (Boeco Zentrifugen D-78532), analytical balance (Precisa XB 220A), vortex mixer (Stuart SA8), hotplate and magnetic stirrer (Stuart CB162), pH meter (Jenway 370), micropipette (Smart Gen-nex) and glassware (Pyrex). Formulation was carried out at the Pharmaceutical Technology Laboratory of Stifar Yaphar Semarang. Determination of particle size and zeta potential using Particle Size Analyzer (PSA) (Beckman Coulter).

Extraction of *Caulerpa racemose*

Fresh *Caulerpa racemosa* is selected with green color, shaped like seaweed with a small round and a bit flat. Washed using sea water (salt water), then washed with fresh water until clean, removing dirt that sticks. Then it was cut into small pieces and pounded. A total of 1 kg of fresh *Caulerpa racemose* was cut into small pieces then pounded and put into a maceration vessel and added 5 liters of methanol solvent. Then it was mixed homogeneously while occasionally stirred. The extract was macerated for 3 x 24 hours. The results were filtered using kola cloth. Then the extract was concentrated with a rotary evaporator with a temperature of 40 ℃ and 50 rpm **(Hainil *et al.*, 2022).**

Phytochemical Screening of *Caulerpa racemose* Extracts

Phytochemical screening was conducted to determine the class of compounds contained in *Caulerpa racemose* extract. The screening process was initiated by using color reaction and precipitation. Screening was carried out on alkaloids, flavonoids, saponins, tannins, terpenoids.

Preparation of Nanoparticles

A total of 2.5 mL of *Caulerpa racemosa* extract solution of 100 mg/mL concentration variation was then added with 2.5 mL of tween 80 (a). The next step was mixing using a magnetic stirrer for 5 minutes at 1200 rpm. A solution of 0.3% chitosan in acetate buffer pH 4 was added to a solution of tripolyphosphate (TPP) in distilled water (concentration 00.1%) in a ratio of 1:5 and then mixed using a magnetic stirrer again for 5 minutes at 1200 rpm (b). 3 mL of mixture a and 3 mL of mixture b were mixed and homogenized using a magnetic stirrer for 5 minutes at 1200 rpm **(Hussain and Sahudin, 2016)**.

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Evaluation of physical characteristics of Nanoparticles

The particle size, zeta potential and polydispersity index obtained after ultracentrifugation were resuspended in distilled water. The average particle size, zeta potential and PDI were then measured using a Particle Size Analyzer (PSA) (Beckman Coulter) **(Hussain and Sahudin, 2016)**.

Cytotoxic Test of *Caulerpa racemose* nanoparticles with MTT method

a. Cell culture

This study used test cells, namely MCF-7 (breast) cells. MCF-7 cells were grown using Dulbecco's Modified Eagle Medium (DMEM) containing 5% Fetal Bovine Serum (FBS); 1% penicillinstreptomycin; and 0.5% amphotericin A then incubated at 37°C in a 5% CO2 incubator. Cell harvesting was performed by the addition of trypsin-EDTA after cell confluence.

b. Cytotoxic test

The cytotoxic test of nano preparations of seaweed extracts was carried out by the MTT method as described by **Pakki *et al*. (2019)** and **Tanumihardja *et al*. (2020)** with slight modifications **(Pakki *et al*., 2019; Tanumihardja *et al*., 2020)**. A total of 100 µL of cell suspension (104 cells/mL) was put into a 96-wellplate and incubated for 24 hours then 100 µL of nano-preparation with various concentrations (6.25 - 500 µg/mL) was added. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) with a concentration of 0.5 mg/mL was added to each well and incubated for 4 hours. The reaction was stopped by the addition of 10% sodium dodecyl sulfate (SDS). The absorbance of each well was measured using a microplate reader (Thermo) at a lambda of 595 nm. Percent inhibition was calculated by the formula:

$$\text{Inhibition (\%)} = \frac{OD\ of\ cell\ control - OD\ of\ sample}{OD\ of\ cell\ control} x100$$

RESULTS AND DISCUSSION

The extraction process in this research uses methanol. The choice of methanol solvent was based on the fact that methanol is universal (can attract all compounds). The extract obtained from the extraction process is carried out methanol-free test first. Methanol-free test results are carried out to ensure that the concentrated extract obtained is free from methanol solvents. The results of the ethanol-free test are shown in the table 1.

Table 1. Methanol Free Test Results of *Caulerpa racemose* Extract

Reagent	Positive result (literatur)	Research	Keterangan
+ potassium dichromate + H_2SO_4	The color of the solution does change	 Colour does not change	(-) negative

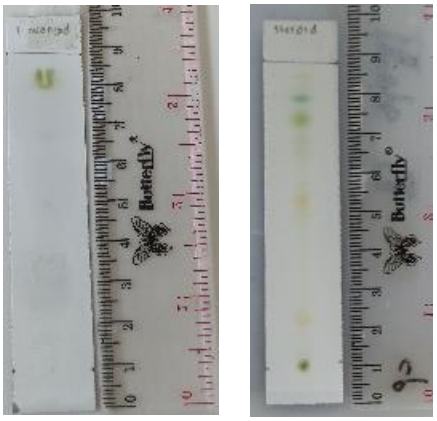
The test results showed that *Caulerpa racemose* extract was free from methanol solvents because there was no color change after the addition of potassium dichromate and sulfuric acid. After the methanol-free test, the extract obtained was then subjected to phytochemical screening test. The results of the screening test are shown in Table 2.

Table 2. Phytochemical screening of *Caulerpa racemose* extract

Uji	Reagent	Literature (Harbone, 1987)	Research	Conclusion
Flavonoid	Magnesium powder + $HCl_{(p)}$ + amyl alcohol	The solution is red, yellow, or orange in the amyl alcohol layer	The solution is red	Positive
Tannin	Gelatin	White precipitate	Brown solution	Negative
Alkaloid	HCl 2N + *Dragendorff*	Orange precipitate	no precipitate is formed	Negative
	HCl 2N + *Mayer*	White precipitate	no precipitate is formed	Negative
	HCl 2N + *Wagner*	Brown precipitate	no precipitate is formed	Negative
Saponin	shaken + $HCl_{(p)}$	No foam	Stable foam	Negative
Terpenoid	Ether + acetat acid	Green	Blue or green	Positive (steroid)

+ $H_2SO_{4(p)}$	(Steroids), Purple (Terpenoids), 2007)

Screening results showed that *Caulerpa racemose* extract was positive for flavonoids and terpenoids. The test was confirmed by KLT test, the results of KLT test of Caulerpa racemose extract for terpenoid group are shown in Fig 1.



(a) (b)

Fig 1. TLC results of confirmatory tests on flavonoids (a) and steroids (b)

The KLT results were positive for flavonoids with yellow stains. After ammonia evaporation, a brownish yellow stain is formed on the extract sample. According to the literature, the sample contains flavonoids if it gives a brownish stain after ammonia evaporation **(Harborne, 1987)**. Likewise, terpenoids showed positive stain results after being sprayed with 10% H_2SO_4 spot. The presence of steroids is indicated by the appearance of greenish yellow, blue, purplish red, reddish blue, yellowish white, purplish blue spots **(Wafa *et al*., 2014)**.

The extract obtained was then made into nanoparticle preparations. In this study, several stages were carried out to obtain nanoparticle preparations. The initial stage is the preparation of a formula that refers to the research of **Hussain and Sahudin (2016)**. The use of the ratio between chitosan and tripolyphosphate (TPP) is based on the research of **Stoica *et al.*, (2013)**, chitosan and TPP are used because they can produce different nanoparticle sizes. The increasing ratio of TPP chitosan, it will produce nanoparticles with a smaller size. The results of the comparison used in this study chitosan: TPP is 1:5. The next stage is homogenization. One of the parameters that affect particle size is intensity and homogeneity **(Gupta, 2006)**. The results of nanoparticle preparation are shown in Fig 2.

Fig 2. *Caulerpa racemosa* extract nanoparticle preparation

The initial characteristics of nanoparticle preparations were carried out by physical observation. The results of physical observations in the form of clarity show that the *Caulerpa racemose* extract nanoparticle preparation is clear greenish in color. The results of the evaluation of *Caulerpa racemose* extract nanoparticles are further shown in table 3.

Table 3. Evaluation results of Caulerpa racemose extract nanoparticles

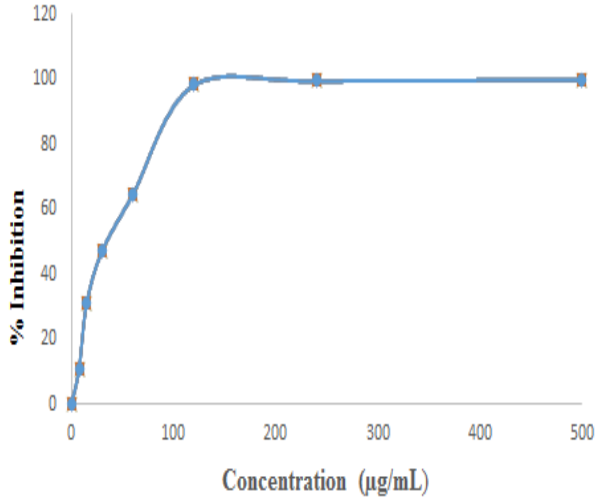
Nano Particle Size (nm)	Zeta Potensial (mV)	Polidispersitas Indeks
83.7 ± 0.65	+29.15 ±0.55	0.37 ± 0.01

The size of the resulting nanoparticles is still in the range of 10-1000 nm (**Stoica *et al.*, 2013)**. Based on **Colonna *et al.*, (2008)** nanoparticles with TPP chitosan made by ion gelation method so that it will produce particles with sizes ranging from 200 to 500 nm. The zeta potential value of nanoparticles is generally used to characterize the surface charge properties of these nanoparticles. The particle surface characteristics of a nanoparticle system affect stability. Particles with potential zeta values more positive than +30mV or more negative than -30mV are predicted to be stable during storage and no aggregation occurs between particles **(Mohanraj and YChen, 2006)**. The potential zeta value obtained from the nanoparticle preparation is close to +30 mV so it can be predicted that the nanoparticle preparation is quite stable.

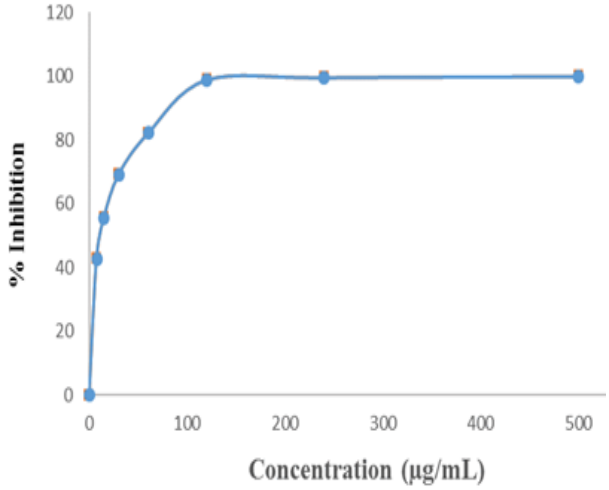
The polydispersity index value is a parameter that expresses the particle size distribution of a nanoparticle system. A polydispersity index value of less than 0.3 indicates that the size distribution is very narrow. This can be seen from the small particle size and homogeneous particle size distribution. While the polydispersity value of more than 0.3 (PI>0.3) indicates that the particle size distribution is very broad. The polydispersity index value states the stability of a nanoparticle system, the greater the polydispersity index value indicates the more particles that aggregate so that the preparation is increasingly unstable **(Feng Lin Yen *et al.*, 2010)**.

The extracts and nanoparticle preparations were then subjected to cytotoxic testing against MCF-7 cells. The ability of a substance to kill cancer cells can be measured through cytotoxic test using MTT method. The principle of this method is the change of yellow color from MTT tetrazolium to formazan which is purple. MTT will

only be absorbed by living cells and reduced by the reductase enzyme in the mitochondria to formazan salt which is insoluble in water. To dissolve the formazan salt and stop the reaction, 10% SDS is added. The more purple color means the more cells are alive, and vice versa **(Benov, 2019; Rai *et al*., 2018)**. The results of the cytotoxic test of *Caulerpa racemose* extract nanoparticle preparations against MCF-7 cells are shown in Fig 3.



(a)



(b)

Fig 3. Cytotoxic effect of methanol extract (a) and Caulerpa racemose nanoparticle preparations on MCF-7 cells

The higher the concentration of *Caulerpa racemose* extract nanoparticle preparation used, the greater the cytotoxic effect or known as dosedependent manner. The cytotoxic effect began to appear at a concentration of 7.5 µg/mL and reached maximum mortality at a concentration of 240.00 µg/mL (Figure 3). These results indicate that the nanoparticle preparation of *Caulerpa racemose* extract has a cytotoxic effect on cancer cells. Good cancer cell candidates must be toxic to cancer cells but safe (non-toxic) to normal cells **(Andreani *et al.*, 2017)**.

The cytotoxic effect of a substance is assessed by the value of half maximal inhibitory concentration or better known as IC_{50}. IC_{50} is the concentration that can kill 50% of cancer cells. The smaller the IC_{50} value, the greater the cytotoxic activity. The IC_{50} value of the methanol extract was 38.29 ± 3.2µg/mL and the nanoparticle preparation was 12.35 ± 2.8 µg/mL. Methanol extracts made into nanoparticle preparations have better cellular uptake ability than in the form of extracts. According to **Nordin *et al*. (2018)** the cytotoxic activity of a substance can be categorized based on the IC_{50} value. IC_{50} value ≤ 20 µg/mL is very active category, 20-100 µµg/mL is active category, 100-1000 is very weak category, and ≥1000 is inactive category. Based on these results, the methanol extract is included in the active category and the nanoparticle preparation is included in the very active category against MCF-7 cancer cells.

CONCLUSION

Caulerpa racemose algae has secondary metabolite compounds that have cytotoxic activity against MCF-7 cells. The results showed that the methanol extract was included in the active category with an IC_{50} of 38.29 ± 3.2µg/mL. For nanoparticle preparations in the category of very active against MCF-7 cancer cells with an IC_{50} value of 12.35 ± 2.8 µg/mL.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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