

Phytochemical Test and Cytotoxic Activity of Macroalgae *Euclima cottonii* against Cervical HeLa Cells

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ABSTRACT

Introduction: Marine resource of macroalgae *Euclima cottonii* from East Lombok, Nusa Tenggara Barat, Indonesia, have potential as anti-cervical cancer agent. **Objectives:** Finding cytotoxic activity of *Euclima cottonii* hexane, ethylacetate, chloroform and ethanol extracts against cervical HeLa cells by MTT cell proliferation assay. **Methods:** The extracts was tested in phytochemical and cytotoxic activity test. Phytochemical test to identify composition of secondary metabolite such as flavonoid, alkaloid, saponins, tannin, triterpenoid, steroid and glycoside. The amount of substances contained in the extract sample was analyzed by Thin Layer Chromatography (TLC). Cytotoxic activity using HeLa cells. **Results:** Phytochemical test of *E. cottonii* extracts showed the positive result for metabolite of flavonoid, whereas the TLC analysis revealed that the extracts containing five chemical compounds. Ethanol, n-hexane, chloroform, and ethyl acetate extracts of *E. cottonii* exhibited a strong cytotoxic activity against cervical HeLa cells with IC₅₀ of 7.54 µg/mL, 5.73 µg/mL, 4.82 µg/mL and 4.34 µg/mL, respectively. **Conclusion:** The results suggest that macroalgae *Euclima cottonii* could be used as a new anti-cervical cancer's candidate.

Key words: Anti-cervical cancer, Cytotoxicity, *Euclima cottonii*, HeLa cell lines, Phytochemical test, seaweed.

INTRODUCTION

Cancer is a second cause of death in the world after cardiovascular disease. Around 7.5 million people die because of cancer; more than 70% were from poor and developing countries. Cervical cancer and breast cancer are kinds of cancer that often suffered by women.¹⁻³ Based on the data of GLOBOCAN project in 2012, 235,000 death cases caused from cervical cancer from a total of 14,067,894 new cancer cases and 8,201,575 death cases were recorded worldwide.⁴ According to *Riset Kesehatan Dasar* (Riskesdas) in 2013, the prevalence of cancer is about 1.4% from which the incidence of cervical cancer in Indonesian women is about 16 over 100.000.¹⁻³ Various treatments have been made to cure cancer, including surgery, chemotherapy, radiotherapy, and targeting therapy. But, all of these procedures have side effects, such as vomiting, malaise, anemia and susceptibility with infection.

Marine organisms, mainly seaweeds, are sources of many natural products with pharmacological, medical and biological activities.⁵⁻⁶ From these seaweeds, the Rhodophyceae *Euclima cottonii* grown in Malaysia and Indonesia beaches showed a broad spectrum of

anticancer activities of different types and cell lines. Shamsabadi *et al.* found that the ethanol extract of *E. cottonii*, collected from Sabah beach-East Malaysia, suppress *in vivo* the mammary gland tumor in rat due to the antioxidant and antiproliferative properties of polyphenols as quercetin, catechin, and rutin. While, Lee *et al.* tested the ethanol extract of *E. cottonii in vitro* against some of cancer cell line such as HeLa cell line, human lung carcinoma cell line (SK-LU-1), and human colon carcinoma cell line (HCT-116) and fibroblast. They confirmed that polyphenols can induct apoptosis, either through intrinsic or extrinsic pathway by increasing the division of poly (adenosine-diphosphate)-ribose polymerase, p53 protein, BCL2-Associated x protein, and activation of caspases 3 and 9.⁷⁻⁹ In 2016, Arsianti and its co-authors reported *in vitro* cytotoxic activity of the n-hexane, ethyl acetate, ethanol and chloroform extract of *E. cottonii*, originated from Salemo island, South Sulawesi, Indonesia, against breast MCF-7 and colon HCT-116 cell lines. They relate those activities to the presence of steroids, glycosides, and flavonoids.¹⁰

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Figure 1: *Euचेuma cottonii*.

Kingdom : Plantae
 Division : Rhodophyta
 Subclass : Floridae
 Class : Rhodopyceae
 Order : Gigartinales
 Family : Soliriaceae
 Genus : Kappaphycus
 Species : *Euचेuma cottonii* or *Kappaphycus alvarezii*

In this investigation, we aimed to test different organic solvents extract of *E. cottonii* for their anti-cervical activity with minimal side effect and higher effectiveness.

MATERIAL

Seaweed

Euचेuma cottonii were collected from East Lombok beach, Nusa Tenggara Barat, Indonesia in March 2017, Figure 1.¹¹

Cervical cancer cell lines

HeLa cervical cancer cells were kindly provided from the Department of Anatomical Pathology, Faculty of Medicine, University of Indonesia.

METHODS

Extraction and Fractionation Samples of Seaweeds

Method of extraction and fractionation of seaweed is adopted from previous research,¹⁰ in which, 1000 g dry powder of macroalgae *E. cottonii* were macerated in ethyl acetate in a sealed glass vessel for a week, with conditional stirring. After maceration process, the filtrates were evaporated to give concentrated extract of *Euचेuma cottonii*. Concentrated extracts of *E. cottonii* were fractionated by column chromatography on silica gel G60 (230-400 mesh ASTM) using chloroform and n-hexane as non-polar solvent, ethyl-acetate as semi-polar solvent, and ethanol as polar solvent, afforded n-hexane, chloroform, ethyl acetate and ethanol fractions, respectively. The fractions then were evaluated using thin layer chromatography to identify chemical components present in every fraction. Subsequently, the fractions were applied for phytochemical test to determine the secondary metabolites, followed by cytotoxicity evaluation of the fractions against HeLa cells by MTT assay.

Phytochemical Tests

Phytochemical tests were used to identify the secondary metabolites contained in the concentrated fractions of macroalgae *E. cottonii*. The procedure was carried out according to Harborne, 1987 as follows:¹²

Saponin screening was done with vertical shuffle of 10 mL of solution in a test tube for 10 sec and then left it still for 10 sec. The presence of stabile foam of around 1-10 cm in height for less than 10 min which stays after the addition of 1 drop of 2N HCl, indicate the existence of saponin in the solution.

Flavonoid screening was done with evaporating 1 mL of solution until it is dry, and the solution left are drenched with acetone. Then, a pinch of smooth powder of boric acid and oxalate acid were added, heated carefully in water bath at 60°C for 15 min. The remaining solution was mixed with 10 mL of ether. The appearance of intensive yellow fluorescence using UV light at 366 nm suggested the presence of flavonoid compound.

Triterpenoid and steroid screening was identified through Liebermann Burchard reaction. 2 mL of solution was evaporated in porcelain cup, the residue was diluted into 0.5 mL of chloroform and then, 0.5 mL of acetic acid anhydrate was added. Finally, 2 mL of concentrated sulfuric acid was put into the wall of the tube. The formation of brownish or violet ring on the border of the solution suggested the presence of triterpenoid while the formation of blue greenish ring suggested the presence of steroid.

Tannin screening was done by reacting 1 mL of solution with 10% ferric chloride solution. The formation of dark blue or black greenish color meant the presence of tannin.

Glycoside screening was done by evaporating 0.1 mL of solution in water bath at 60°C for 15 min. The remaining solution was diluted into 5 mL of acetic acid anhydrate. Then, 10 drops of concentrated sulfuric acid was added. Blue or green product indicated the presence of glycoside.

Alkaloid screening was done through evaporating 2 mL of the solution on a porcelain cup. The residue formed was diluted with 5 mL of 2N HCl. The mixture of the solution was divided into 3 reaction tubes. In the first tube, 2N HCl was added and was considered as the control. The second tube was supplied with 3 drops of Dragendorff reagent (consist of bismuth subnitrate, acetic acid glacial, and potassium iodide) and the third tube was filled with 3 drops of Mayer reagent (consist of mercury (II) chloride and potassium iodide). The presence of orange precipitated in the second tube or yellow precipitated in the third tube suggested the presence of alkaloid.

Thin Layer Chromatography

Thin layer chromatography (TLC) is a procedure to identify the amount of substances contained in the extract sample, which is expressed by Rf values. The extract sample was then applied on the plates (5cm × 20 cm × 0.2 mm) by capillary pipe. Mixture of chloroform (CHCl₃)- methanol(CH₃OH) in ratio 3:1 was used as a mobile phase. After separation, the spots of chemical components were visualized under UV lamp with wavelenght of 254 nm and 366 nm and the Rf value of each spot was recorded. If the Rf value is quite similar or does not differ significantly, so the secondary metabolite in the extract is quite similar.¹³⁻¹⁶

In vitro Cytotoxicity Assay

HeLa cervical cancer cells was cultured into RPMI 1640 (Gibco, USA) and was supplemented with 10% of fetal bovine serum (Gibco, USA). Then the cultured cells were incubated in 5% CO₂ with humidified atmosphere at 37°C. The anti-proliferating effects of n-hexane, ethylacetate, and ethanol extract-fractions towards cervical cancer HeLa cells are determined using MTT assay. Each extract-fraction was diluted until the

concentrations reach 51,2; 25,6; 12,8; 6,4, 3,2; 1,6; 0,8; 0,4 µg/ml, before they was added separately to the target cells. The reaction was incubated for 48 h. Then, 100 µl of the solution MTT phosphate-buffered saline (PBS) 5mg/mL was added into the target cells. The mixture inside the plates was incubated for 4 h. About 100 µl of dimethyl sulfoxide was added into every well forming blue purple sediments. Absorbance was measured at 590 nm on a micro plate reader (Model 550, Bio-Rad, USA). The inhibition rate was calculated using this following formula:

$$\%inhibition = 1 - \left(\frac{\text{Absorbance of treatment group}}{\text{Absorbance of control group}} \right) \times 100\%$$

While the inhibitory concentration that diminished 50% of cancer cells was signed as IC₅₀ value.

RESULTS AND DISCUSSION

Phytochemical Test

Phytochemical test of *Eucheuma cottonii* extracts were recorded in Table 1. All extract of *E. cottonii* showed positive result for flavonoids. Flavonoids as secondary metabolite have anti allergic, anti-inflammatory, anticancer, and antibiotic activities, which are grouped into nine class, i.e. anthocyanin, proanthocyanin, flavonol, flavon, glikoflavon, biflavon, chalcone and aurone, flavanon, and isoflavon. Flavonoids can bind to sugars as glycosides and some are in the form of aglycons. Flavonoid glycosides are polar compounds, so it can be dissolved in the polar solvent.¹⁷

Thin layer chromatography (TLC) of *Eucheuma cottonii*

TLC analysis of *E. cottonii* extracts are showed on Table 2 and Figure 2. The result of TLC analysis showed that the Rf value in each extract of *Eucheuma cottonii* has no significant difference, which means that the secondary metabolite present in all extracts are similar.

Anticancer Activity of Seaweed *Eucheuma cottonii*

Figure 3 illustrated the relationship between concentrations of *E. cottonii* extracts with percentage of inhibition on cervical HeLa cells. The percentage inhibition of *E. cottonii* extracts ranging from 31% to 77% against cervical HeLa cells. The percentage of inhibition has improved by increasing the concentration of extract, it can be observed in ethyl acetate and ethanol extracts of *E. cottonii*. Whereas, percentage of inhibitor of n-hexane and chloroform extracts of *E. cottonii* showed fluctuate pattern by increasing the concentration of extract. Figure 3 revealed that all *Eucheuma cottonii* extracts are concentration-dependents in terms of inhibiting the proliferation of cervical HeLa cells.

IC₅₀ value was calculated using Microsoft Excel 2013 by extrapolating the concentration of *Eucheuma cottonii* extract (in x axis) versus the percentage of inhibition (in y-axis), in which a linear regression was then created. Table 3 summarized IC₅₀ value of *Eucheuma cottonii* extracts against cervical HeLa cells. As reported by Atjanasuppat *et al.* anticancer activity level of the extracts were categorized according to the median inhibitory concentration (IC₅₀) into four groups: ≤20 µg/mL, active; >20–100 µg/mL, moderately active; >100–1000 µg/mL, weakly active; and >1000 µg/mL, inactive.¹⁸ As shown in Table 3, each extract of *Eucheuma cottonii* are less than 20 µg/mL, which assigned to have a good anticancer activity. Ethanol, n-hexane, chloroform, and ethyl acetate extracts of *Eucheuma cottonii* exhibited strong cytotoxic activity against cervical HeLa cells with IC₅₀ of 7.54 µg/mL, 5.73 µg /mL, 4.82 µg /mL and 4.34 µg /mL, respectively.

Similar results with this work have been reported by Lee *et al.* in which, the red algae *Eucheuma cottoinii* from Sabah, East Malaysia that a rich

Table 1: Phytochemical analysis of *Eucheuma cottonii*.

Metabolite	<i>Eucheuma cottonii</i>			
	n-Hexane	Ethyl Acetate	Chloroform	Ethanol
Saponin	-	-	-	-
Flavonoid	+	+	+	+
Triterpenoid	-	-	-	-
Steroid	-	-	-	-
Alkaloid	-	-	-	-
Tannin	-	-	-	-
Glikosida	-	-	-	-

Table 2: TLC analysis and Retention factor (Rf) of *E. cottonii* extracts.

Extract	Rf value				
	1	2	3	4	5
n-Hexane	0.368	0.5	0.735	0.853	0.97
Ethylacetate	0.32	0.5	0.735	0.853	0.97
Chloroform	0.32	0.47	0.735	0.853	0.97
Ethanol	-	-	0.735	0.88	0.97

*Rf = Retention factor

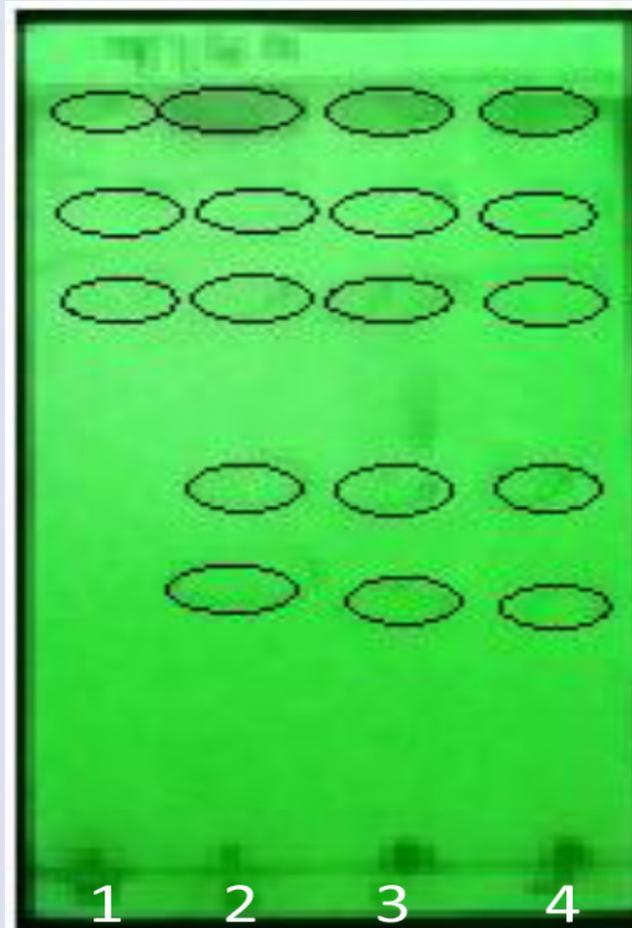


Figure 2: TLC analysis of *Eucheuma cottonii*, 1: ethanol extracts; 2: ethyl acetate extracts; 3: chloroform extract; 4: n-hexane extract.

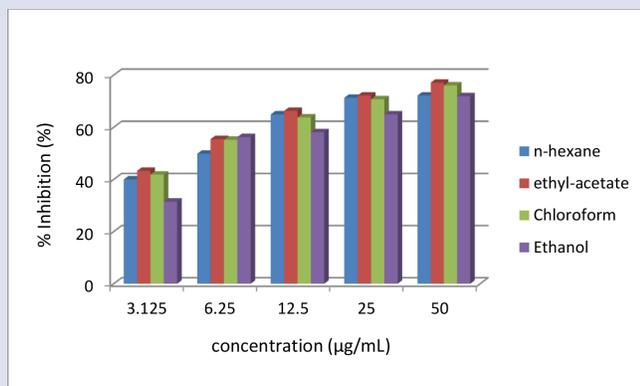


Figure 3: The relationship between concentrations of *Eucheuma cottonii* extracts with percentage of inhibition on cervical HeLa cells.

Table 3: IC₅₀ values of *Eucheuma cottonii* extracts against cervical HeLa cells.

Tested extracts	IC ₅₀ (µg/mL)
n-hexane	5.731
Ethyl acetate	4.344
Chloroform	4.828
Ethanol	7.542

*IC₅₀ is the 50% half maximal inhibitory activity in µg/mL.

source of antioxidants such as flavonoids, showed anticancer effect on Lung carcinoma SK-Lu-1, colorectal HCT-116 cells, and also demonstrated anticancer activity by inducing the apoptosis of cervical HeLa cells dose-dependently.^{8,19} Our previous research on red seaweed *Euchemia cottonii* derived from South Sulawesi, Indonesia that demonstrated a strong cytotoxic activity against breast MCF-7 and colorectal HCT-116 cells, also containing metabolite of flavonoids.¹⁰

Therefore, the mechanism of action of flavonoids as antioxidant and anticancer is a strong point of discussion in this work. Antioxidant systems are proposed to be involved in carcinogenesis because of its capability to damage the reactive oxygen species. Reactive oxygen species cause unrepaired or misrepaired damage in DNA, leads to the mutations. Antioxidant flavonoids such as quercetin and apigenin are stated to be potent inhibitors of cell proliferation. Quercetin and apigenin inhibited the growth of melanoma and influenced the invasive and metastatic potential in mice. Furthermore, it has been reported that flavonoids obstructed the angiogenesis in the human body. Angiogenesis inhibitors showed a strong effect on various steps in angiogenesis, such as the proliferation and migration of endothelial cells and lumen formation. Flavonoids is known as the one of Angiogenesis inhibitors seem to play an important role. However, the mechanism of antiangiogenetic effect of flavonoids is still unclear.²⁰ These facts revealed that the anti-cervical cancer activity of the *Eucheuma cottonii* in this work may be due to the presence of antioxidant flavonoids.

CONCLUSION

Eucheuma cottonii extracts have demonstrated a strong cytotoxic activity on HeLa cells. Thus it could be further developed as a potent new anti-cervical cancer agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

TLC: Thin layer chromatography; MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); IC₅₀: Inhibition Concentration 50%; %: Percentage; µg/mL: microgram/milliliter; HeLa: Henrietta Lacks; SK-LU-1: Human lung cancer cell line; HCT-116: Human Colorectal carcinoma; PARP: Poly (Adenosine-Diphosphate)-Ribose Polymerase; Bax: BCL2-Associated x protein; MCF-7: Michigan Cancer Foundation; ASTM: American Society for Testing and Materials; g: gram; mL: milliliter; N: Normality; UV: Ultra violet, RPMI: Rosewell Park Memorial Institute; h: hour; PBS: Phosphate-Buffered Saline; °C: degree Celsius; µL: microliter; CO₂: Carbon dioxide; Rf: Retention factor; USA: United States of America.

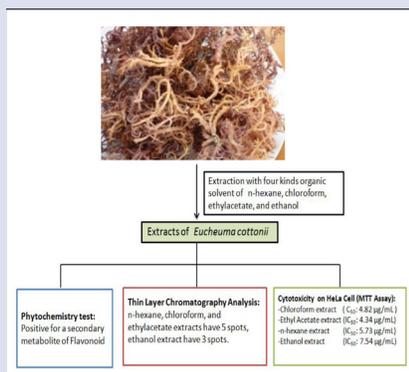
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GRAPHICAL ABSTRACT



SUMMARY

Marine macroalgae of *Euचेuma cottonii* collected from East Lombok beach, Nusa Tenggara Barat, Indonesia, showed potent anti-cervical cancer activity. Phytochemical test of *Euचेuma cottonii* showed the positive result for metabolite of flavonoid. TLC analysis revealed that the extracts of *Euचेuma cottonii* containing five chemical compounds. Ethanol, n-hexane, chloroform, and ethyl acetate extracts of *Euचेuma cottonii* demonstrated strong cytotoxicity against HeLa cells with IC₅₀ of 7.54 µg/mL, 5.73 µg /mL, 4.82 µg /mL and 4.34 µg /mL, respectively.

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