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Green algae *Caulerpa racemosa* inhibit proliferation and promote apoptosis in human HT-29 colon cancer by suppressing PI3K/AKT pathway

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ABSTRACT

Introduction: Colon cancer is a malignancy of the gastrointestinal which is one of the most common causes of death worldwide. Tumorigenesis involved oncologic pathway such as PI3K/AKT as the common pathway with the challenges is therapy resistance. Therefore, an alternative colon cancer treatments is needed that comes from biological resources such as sea grapes.

Objective: This study aims to determine anticancer potency of hexane extract from sea grapes (*Caulerpa racemosa*) on HT-29 cell line colon cancer based on suppressing PI3K/AKT pathway.

Method: The study was conducted in vitro using the extract of *Caulerpa racemosa* at concentrations of (0, 400, 800, and 1.200) µg/mL on the HT-29 cell line based on the level expression of p-akt related to PI3K/AKT pathway, cleaved caspase 3 related to apoptosis, and viable cells related to proliferation.

Results: The research results was found that the hexane extract of *Caulerpa racemosa* decreased the expression level of p-akt (Kruskal wallis, p=0,027), increased the cleaved caspase 3 (Kruskal wallis, p=0,016), and decreased the viable cells (Kruskal wallis, 24 and 48 hours: p=0,024 and 0,034).

Conclusion: *Caulerpa racemosa* hexane extract can inhibit colon cancer cells growth by suppressing PI3K/AKT pathway based on inhibition proliferation and induce apoptosis.

KEYWORDS

Caulerpa racemosa, sea grapes, HT-29, colon cancer, PI3K/AKT, proliferation, apoptosis.

ABBREVIATION LIST

Caspase: Cysteinyl Aspartate Specific Proteinase-3.

CCND1: Cyclin D1.

GSK: Glycogen Synthase Kinase-3.

IARC: *International Agency for Research on Cancer.*

P-Akt: Phosporylated Serine/threonine kinase.

PI3K/AKT: Phosphatydil-Inositol-3-Kinase/Serine-Threonine Kinase.

PIP_2: *Phosphatidylinositol 4,5-biphosphate.*

PIP_3: *Phosphatidylinositol 3,4,5-biphosphate.*

PTEN: *Phosphatase and Tensin homolog.*

RTK: *Receptor Tyrosine Kinase.*

TNF-α: Tumor Necrosis Factor-α.

WHO: World Health Organization.

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INTRODUCTION

Colon cancer is a malignancy of the gastrointestinal system which is one of the most common causes of death worldwide. According to WHO, colon cancer is in third position as the most common malignancy affecting men (1.03 million new cases/year) after lung cancer and prostate cancer while women are in second place (0.82 million new cases/year). years) after breast cancer[1]. Based on statistical data from the International Agency for Research on Cancer (IARC), colon cancer occupies the third position as the most common malignancy that attacks the world's population, namely 1.85 million new cases/year and includes 10.2% of the total other malignancies[2]. In the next few years, colon cancer is expected to continue to increase by 2.15 times the number of new cases this year[3].

Pathophysiologically, the incidence of colon cancer is strongly influenced by the path of oncology. One of the oncological pathways that often induce cancer is the Phosphatidyl-Inositol-3-Kinase/Serine-threonine kinase (PI3K/AKT) pathway. The majority of the PI3K/AKT pathways contribute to colon cancer[4]. Dysregulation of the PI3K/AKT pathway induces continuous cell proliferation and inhibits apoptosis so that cell growth becomes uncontrollable and turns into malignancy. Obstacles in cancer treatment are the emergence of side effects that attack healthy cells due to drugs that are not on target, resistant, or do not target the appropriate oncological pathways[5].

Utilizing the potential of natural ingredients is an alternative to colon cancer therapy. In fact, many cancer patients avoid treatments such as chemotherapy and radiotherapy because they are afraid of failure, fear side effects, are expensive, and last a long[6]. Based on these conditions, utilizing natural materials as an alternative to cancer treatment, one of which is sea grapes *(Caulerpa racemosa)* is very realizable. In Indonesia, sea grapes are often used by the community as a food source and are quite easy to find in several Indonesian waters[7]. In previous research by Dissanayake, *et al.,* (2018) *Caulerpa racemosa* has been shown to have anticancer potential in breast cancer[8].

OBJECTIVES

This study aims to determine anticancer potency of hexane extract from sea grapes (*Caulerpa racemosa*) on HT-29 cell line colon cancer based on suppressing PI3K/AKT pathway. This is interesting to study based on the involvement of the PI3K/AKT pathway in influencing colon cancer tumorigenesis. In addition, utilizing biological resources as an alternative therapy for colon cancer is believed to be appropriate and reliable because there has been no previous research examining the potential of *Caulerpa racemosa* extract against colon cancer via the PI3K/AKT pathway.

MATERIAL AND METHODS

Sample preparation

Sea grapes were obtained from Jepara sea, Central Java, Indonesia at more or less 10-20 m above sea level. The sea grapes were air-dried at 24 °C without direct sunlight, then dried in an oven at 40 °C for three days to reduce the water content. After drying, the sea grapes were crushed using an electric mill, and 400 grams of dry sea grape powder was obtained from 1.75 kg of wet sea grapes.

Sea grapes coarse powder is macerated in an n-hexane solution with the ratio of sea grape powder: solvent is 1:10 (100 gr powder: 1 L solvent). Stirring was carried out using a stirrer for 15 minutes at a speed of 400 rpm and then tightly closed using aluminum foil. The initial maceration mixture was allowed to stand at room temperature and stored for 1 x 24 hours, then filtered. The maceration procedure was repeated three times. The resulting macerate was concentrated using a vacuum rotary evaporator with a speed of 50-70 rpm at 40°C until the macerate was in the form of a paste. The thick extract was dried in an oven at 40°C for 24 hours. The *Caulerpa racemosa* extraction produced a thick extract of 0.6 g with a yield of 0.15%.

Cell line

The colon cancer cell line (HT-29) was randomly obtained from the Biomedical Central Laboratorium, Faculty of Medicine, Brawijaya University. The cell line was purchased from American Type Culture Collection (USA). HT-29 cells were cultured in Mc Coy's medium with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Trypan blue test

The harvested HT-29 cells were centrifuged at 1,000 rpm for 5 minutes, removing the supernatant. Take 20 µl centrifuged pellet cells to eppendorf. Add 20 µl of trypan blue solution to the pellet in eppendorf, and homogenize. Take 10 µl of mixed cells and trypan blue solution into the hemocytometer. Observe using a microscope and count cells that do not absorb the blue color (viable cells). Cell counts were performed at 24 and 48 hours to assess cell proliferation.

(Viable cell) = a Total number of viable cells x 2 x 10.000

Immunofluorescence assay

Cells grown on coverslips were washed three times with PBS, washed with PBS Triton-X 100 0,2%, and then washed with PBS again three times. Incubated with 1% bovine serum albumin (BSA) at room temperature, and washed with PBS three times. Incubated with primary antibody overnight (p-Akt S473 rabbit was purchased from Cell Signaling Technology-USA; *cleaved caspase 3* D175 rabbit was purchased from Cell Signaling Technology-USA). After that incubated with a sec-

ondary antibody (goat anti-rabbit IgG ab6718-red) at room temperature, cells washed with PBS three times. Finally, cells were incubated with DAPI, washed with PBS again three times, and covered with an antifade mounting medium. Observed under the microscope fluorescence.

Statistical analysis

Each experiment was performed at least in triplicate, and the results are expressed as the mean. The normality test saphiro-wilk was performed to check the data normality. Between two variables, compared test using one-way anova or kruskal-wallis test. Whereas the correlation of two variables using pearson or spearman. $p<0.05$ was considered statistically significant.

RESULTS

The hexane extract of Caulerpa racemosa yielded significant results ($p<0.05$) in inhibiting the growth of the HT-29 cell line through several mechanisms: 1) inhibition of HT-29 cell line proliferation; 2) inhibition of the PI3K/AKT pathway; and 3) induction of apoptosis.

Caulerpa racemosa inhibits proliferation HT-29

Proliferation test on the HT-29 cell line was carried out using the trypan blue test method which was measured at the 24th and 48th hours. This test aims to see the effect of the hexane extract of *Caulerpa racemosa* in inhibiting HT-29 cell proliferation. The results showed that *Caulerpa racemosa* extract significantly inhibited HT-29 cell proliferation. The statistical test results are shown in **(Figure 1)**.

Based on the results of the cell proliferation statistical test at the 24th hour, the data distribution was not normal ($p<0.05$). The results of the statistical test between the dose and the number of cell proliferation at 24 hours with kruskal wallis showed a significant relationship, $p=0.024$ ($p<0.05$) whereas with the spearman correlation test, it was significant $p=0.000$ ($p<0.05$) with a correlation value coefficient ($r=-0.907$), a very strong negative correlation (>0.8).

Based on the results of the cell proliferation statistical test at the 48th hour, the data distribution was not normal ($p<0.05$). The statistical test results between the dose and the number of cell proliferation at 48 hours with kruskal wallis showed a significant relationship, $p=0.034$ ($p<0.05$) whereas with the spearman correlation test, it was significant $p=0.000$ ($p<0.05$) with a correlation value coefficient ($r=-0.848$), a very strong negative correlation (>0.8).

Caulerpa racemosa suppressing PI3K/AKT pathway

The effect of *Caulerpa racemosa* inhibits PI3K/AKT pathway was carried out using the immunofluorescence assay method which measured the expression level of p-Akt. The results showed that *Caulerpa racemosa* extract significantly decreased the expression level of p-Akt. The immunofluorescence result is shown in **(Figure 2)** and the statistical test are shown in **(Figure 1)**.

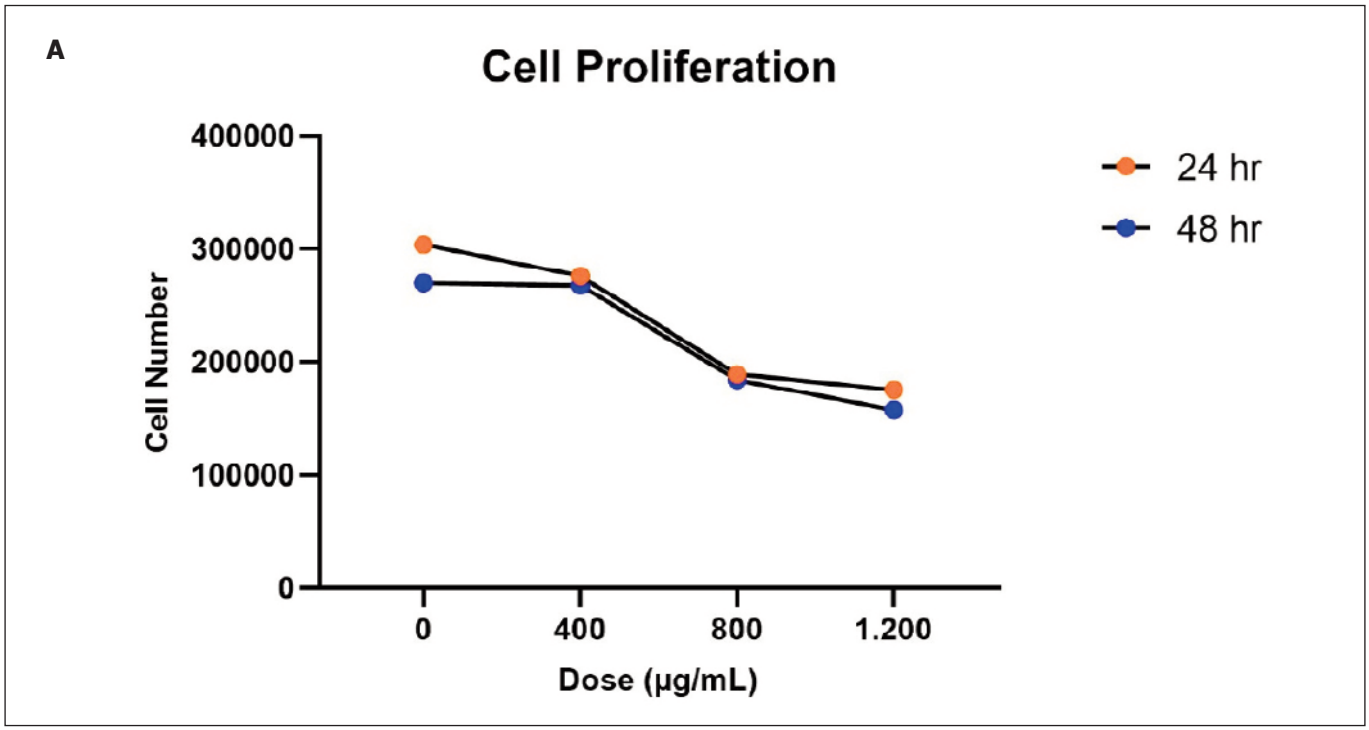

Figure 1. Statistical Analysis. a) summary of cell proliferation for 24 and 48 hours

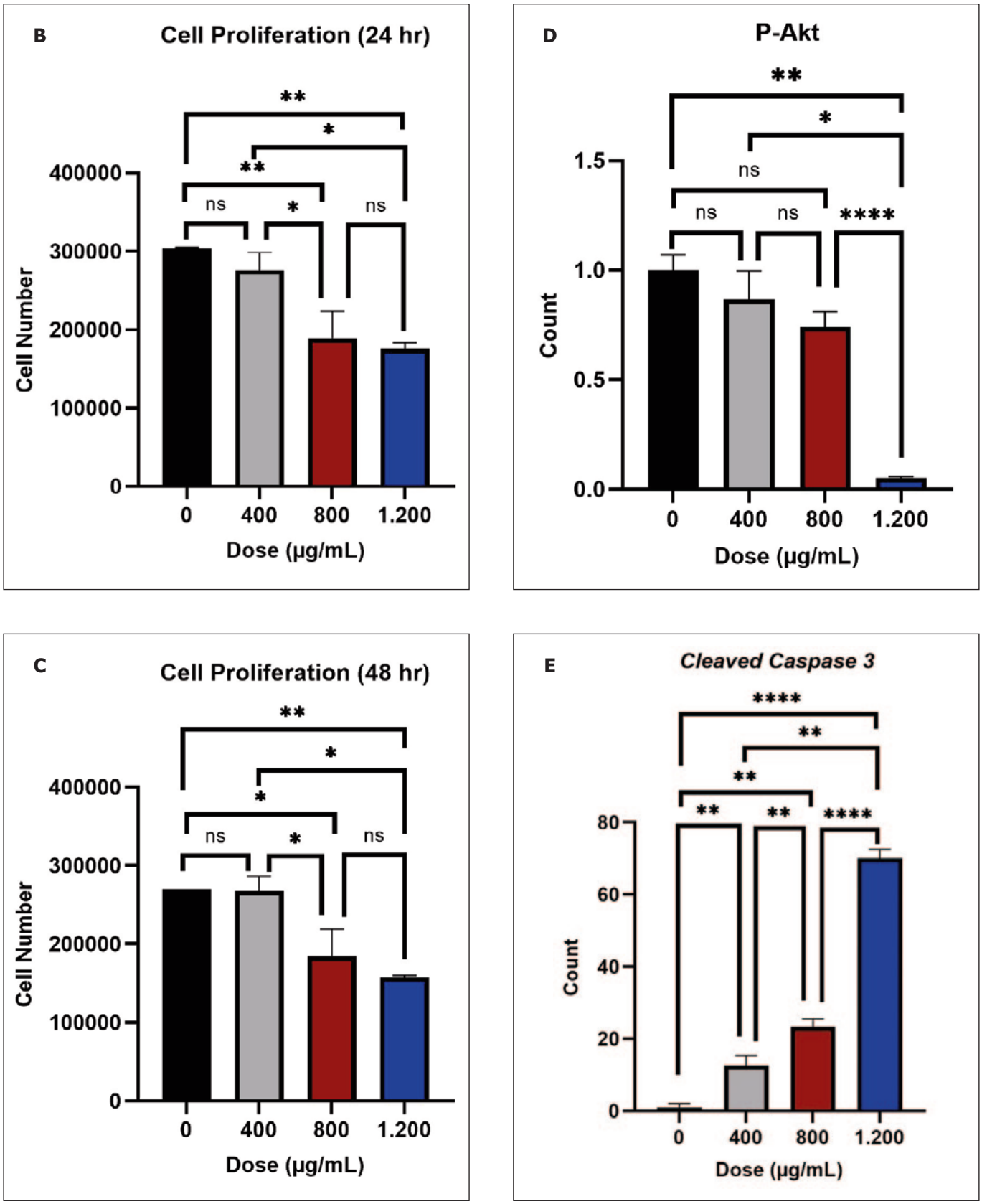

Figure 1 continuation. Statistical Analysis. b) cell proliferation for 24 hour; c) cell proliferation for 48 hour; d) expression intensity of p-akt; e) expression intensity of cleaved caspase 3; *significant (p<0,05)

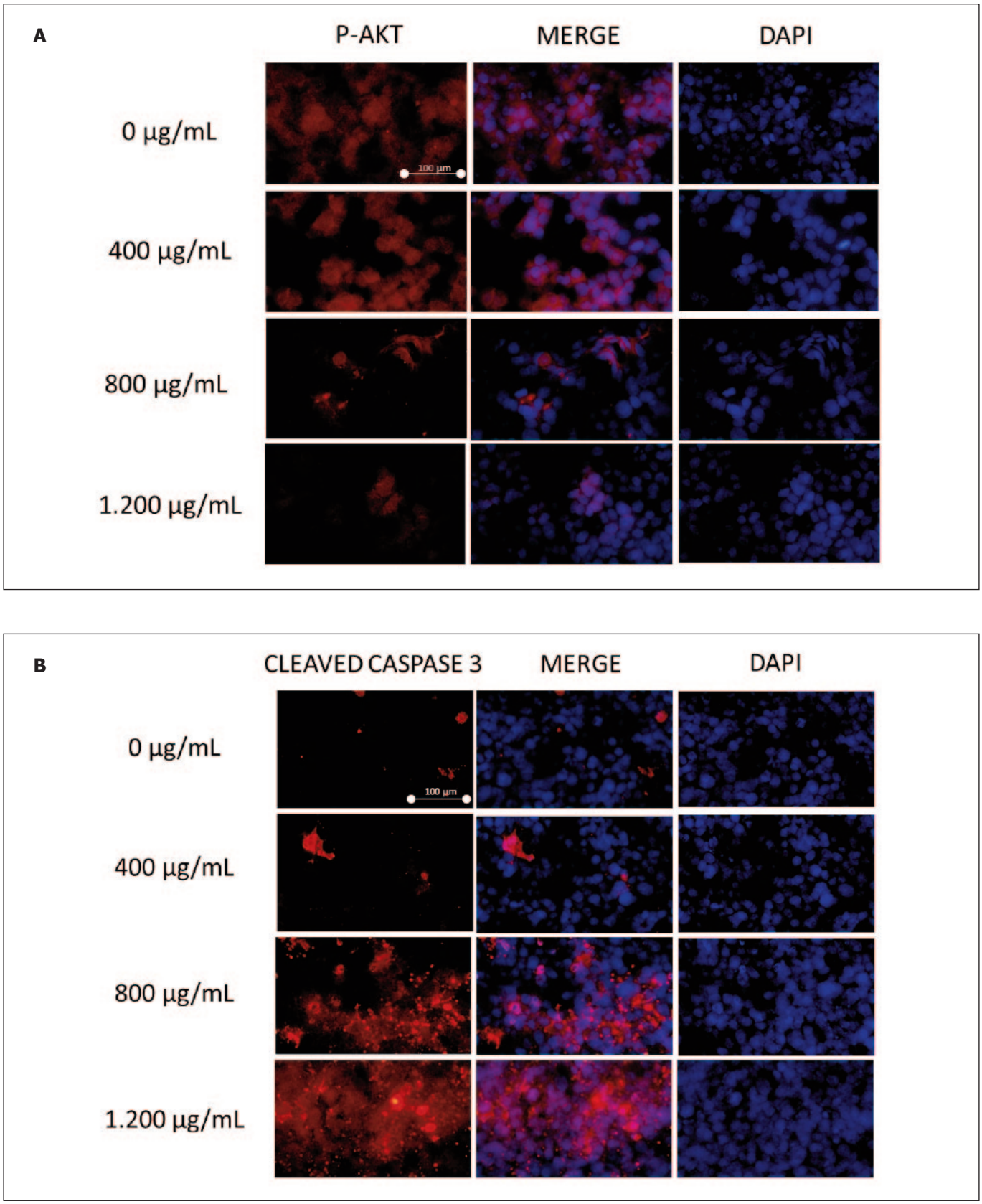

Figure 2. Immunofluorescence. a) increasing doses can decreased expression of p-akt; b) increasing doses can increased expression of cleaved caspase 3

Based on the results of the expression level of p-Akt, the data quantification using ImageJ software and the distribution was not normal ($p<0.05$). The statistical test results between the dose and p-Akt level with kruskal wallis showed a significant relationship, $p=0.027$ ($p<0.05$) whereas with the spearman correlation test, it was significant $p=0.000$ ($p<0.05$) with a correlation value coefficient ($r=-0.907$), a very strong negative correlation (>0.8).

Caulerpa racemosa induces apoptosis HT-29

The effect of *Caulerpa racemosa* induces apoptosis was carried out using the immunofluorescence assay method which measured the expression level of cleaved caspase 3. The results showed that *Caulerpa racemosa* extract significantly increased the expression level of cleaved caspase 3. The immunofluorescence result is shown in **(Figure 3)** and the statistical test are shown in **(Figure 1)**.

Based on the results of the expression level of cleaved caspase 3, the data quantification using ImageJ software and the distribution was not normal ($p<0.05$). The statistical test results between the dose and cleaved caspase 3 level with kruskal wallis showed a significant relationship, $p=0.016$ ($p<0.05$) whereas with the spearman correlation test, it was significant $p=0.000$ ($p<0.05$) with a correlation value coefficient ($r=0.972$), a very strong positive correlation (>0.8).

DISCUSSION

The proliferation test was carried out using the trypan blue test method for 24 and 48 hours. As the dose increased, the number of HT-29 cell proliferation at the 24th and 48th hours was effectively inhibited by *Caulerpa racemosa* hexane extract. This is based on a decrease in HT-29 cells 24 and 48 hours after intervention with *Caulerpa racemosa* hexane extract. Based on previous research conducted by Manikandakrishnan *et al.,* (2019) regarding the proliferation test using the MTT assay method on HT-29 cells, it was found that *Caulerpa racemosa* significantly inhibited the viability of HT-29 cells at 24 hours[9]. Based on another study conducted by Mert-Ozupek *et al.,* (2022) regarding the HT-29 cell proliferation test, it was found that Caulerpa extract significantly inhibited HT-29 cell proliferation at 48 hours[10].

In this study, dead cells will absorb the trypan blue color because the cell membranes are not intact, so when observed under a microscope, dead cells will turn blue. In contrast, cells that are still viable will not absorb the blue color because the cell membranes remain intact[11]. The decrease in the number of proliferating HT-29 cells can be through mechanisms that inhibit the expression of proteins that play a role in cell proliferation and agents that induce cells to apoptosis. The antiproliferative effect on HT-29 cells can be generated by the induction of apoptosis in the PI3K/AKT pathway. Induction of apoptosis involves the role of caspase both as initiator and executor, which causes an increase in the number of dead cells resulting in a decrease in the number of cells[12].

The antiproliferative effect of *Caulerpa racemosa* hexane extract is related to its association with the PI3K/AKT pathway, which inhibits the expression of myc and CCND1, which play a role in cell proliferation. If there is a decrease in the expression of myc and CCND1, then the proliferation process will be hampered and have an impact on reducing the number of HT-29 cells. Myc and CCND1 are regulators in the cell cycle's G1/S phase, which will encourage cells to enter the S phase of G1. If there is a decrease in the expression of myc and CCND1, the cell cycle will stop and cannot continue to the next stage, reducing the number of cells (suppresses proliferation)[13]. In this study, *Caulerpa racemosa* hexane extract significantly inhibited HT-29 cell proliferation for 24 hours.

Observation of the PI3K/AKT pathway of HT-29 cells was investigated based on the amount of p-Akt expression through the immunofluorescence method. Administration of *Caulerpa racemosa* hexane extract aims to inhibit the PI3K/AKT pathway in the hope that there will be a decrease in p-Akt expression along with increasing doses of *Caulerpa racemosa* hexane extract. In this study, significant results were obtained that there was a decrease in p-Akt expression after HT-29 cells were intervened with *Caulerpa racemosa* hexane extract. Based on previous research conducted by Manandhar *et al.* (2020) found that Caulerpa significantly inhibited the PI3K/AKT pathway based on p-akt expression[14].

The PI3K/AKT pathway plays a role in regulating proliferation and apoptosis. In cancer cells, this pathway is dysregulated, leading to increased proliferation and inhibition of the apoptotic process. In this study, the effect of inhibition on the PI3K/AKT pathway was assessed directly by looking at the amount of p-akt expression. The expression of p-Akt is stimulated by PI3K when the growth factor attaches to the tyrosine kinase receptor on the cell membrane. P-Akt is the primary regulators of the PI3K/AKT pathway; when its expression decreases, there is a decrease in proliferation and an increase in apoptosis of cancer cells. This effect is the goal of this research so that the tumorigenesis of colon cancer cells can be stopped. This happened because the decrease in p-Akt expression resulted in the absence of p-Akt, which inhibited GSK-3β and caspase expression. GSK-3β plays a role in inhibiting myc and CCND1 to benefit the cell cycle. If the amount of GSK-3β increases due to the absence of p-Akt, which inhibits it, then myc and CCND1 will be hampered by GSK-3β, resulting in a decrease in cell proliferation. In addition, the decreased expression of p-Akt causes caspase to work to induce apoptosis[15].

The apoptotic test in this study was investigated using the amount of cleaved caspase 3 expression using the immunofluorescence method. The expected result is an increase in the amount of caspase 3 expression along with an increase

in the dose of *Caulerpa racemosa* hexane extract. In this study, significant results were obtained that there was an increase in the expression of caspase 3, which induces apoptosis in HT-29 cells after intervention with *Caulerpa racemosa* hexane extract. Based on previous research conducted by Ferdous, *et al.,* (2021), the caulerpin content found in *Caulerpa racemosa* significantly induces apoptosis in cancer cells by increasing caspase expression, which plays a role in the apoptotic process[16].

Cleaved caspase 3 is one of the primary executor proteins in the process of apoptosis that acts directly on target cells. In this study, the effect of inducing apoptosis of *Caulerpa racemosa* hexane extract was observed by looking at the increased expression of cleaved caspase 3. Endogenous and exogenous pathways can stimulate the high expression of cleaved caspase 3. In the endogenous pathway, mitochondrial damage occurs, causing cytochrome c to be released into the cytoplasm from the mitochondria. Cytochrome c converts caspase 9 into the active form (cleaved caspase 9), then cleaved caspase 9 activates and increases cleaved caspase 3 expression. Meanwhile, cleaved caspase 3 expression increases in the exogenous pathway through stimulation of Tumor Necrosis Factor-α (TNF-α). Cleaved caspase 3 is the last executor protein among other types of caspase in the apoptotic process, so it acts directly on target cells and gives the effect of the induced apoptotic process. Induction of apoptosis by cleaved caspase 3 occurs through the mechanism of nuclear fragmentation of cancer cells (chromatin condensation and DNA cleavage), causing swelling of the cell membrane, continuing to rupture until the cell finally dies[17].

In addition, the effect of *Caulerpa racemosa* hexane extract on increasing cleaved caspase 3 expression was also induced by the influence of *C. racemosa* in inhibiting the PI3K/AKT pathway. It is known that the primary regulator of the PI3K/AKT pathway is p-Akt. P-Akt plays a role in inhibiting caspase expression, so p-Akt can be said to be upstream of caspase expression. Suppose there is a decrease in p-Akt expression due to being inhibited by C. racemosa. In that case, the expression of cleaved caspase 3 will not deter it, thus triggering an increase in cleaved caspase 3 expression and encouraging cancer cells to apoptosis[18,19]. Therefore, the effects of antiproliferation and induction of apoptosis that we wish to observe in this study indirectly illustrate the results of inhibition of the PI3K/AKT pathway by *Caulerpa racemosa*.

The limitations of this study did not explore the bioactive compound of *Caulerpa racemosa* that have an anticancer effect and did not explore the anticancer effect on the other hallmarks of cancer.

CONCLUSION

Caulerpa racemosa has potential as an anticancer agent in colon cancer because it inhibits cancer cell growth by suppressing the PI3K/AKT pathway so that the effects are antiproliferation and induction of apoptosis. Based on the antiproliferation effect, *C.racemosa* decresed the number of viable count cells since 24-48 hours. Whereas on inducing apoptosis effect, *C. racemosa* increased the level expression of cleaved caspase 3 as the executioner of apoptosis process.

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