Indonesian Journal of Cancer Chemoprevention, February 2023
ISSN: 2088–0197
e-ISSN: 2355-8989

Cytotoxic and Anti-proliferative Effects of Ethanol Extract of Marine Sponge *Stylissa carteri* on Colon Cancer HCT-116 Cell Line

Resti Gradia Dwiwina[1], Sarah Sabrina[2], Yuni Susanti Pratiwi[2], Putri Halleyana Adrikni Rahman[2], Muhammad Hasan Bashari[2*]

[1]Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran
[2]Faculty of Medicine, Universitas Padjadjaran

Abstract

Colorectal cancer is one of the most diagnosed cancers in the world. KRAS mutations in colon cancer are being responsible for the progressiveness and resistance of the standard therapeutic available. Marine sponge is one the sources for chemotherapy. Stylissa carteri is a marine sponge that lives in Indonesia and its anti-cancer effects are starting to be explored nowadays. The purpose of this study was to determine the cytotoxicity and anti-proliferative effect of ethanol extract of *Stylissa carteri* against colon cancer cells with KRAS mutations HCT-116 cells. This was an *in vitro* study using colon cancer cell line HCT-116 which was tested by 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue assay. The trypan blue assay was performed on control and treated cells at 72 h. The MTT assay was performed on cells which were previously incubated with Stylissa carteri extract. Sponge *Stylissa carteri* was taken from Pramuka island, Taman Nasional Kepulauan Seribu, Jakarta. The IC_{50} of ethanol extract of *Stylissa carteri* is 5 µg/mL (R square 0.9550) in HCT-116 cells. The trypan blue assay showed doubling time of incubated cell for 48 h was 26.10 h for control and 39.69 h for treated cells, respectively. Ethanol extract of *Stylissa carteri* has cytotoxic and anti-proliferative effects in HCT-116 colon cancer cells with KRAS mutation.

Keywords: *colon cancer, HCT-116, KRAS mutation, marine sponge, Stylissa carteri.*

INTRODUCTION

Colorectal cancer is one of the most common cancers diagnosed worldwide (Ardekani, *et al*., 2012). Colorectal cancer is the third most common cancer in men, and the second most common cancer in women. In 2020, there were 1.1 million new cases of colorectal cancer and 576,858 deaths (Ardekani, *et al*., 2012).

Mutations in the KRAS gene is one of the causes of the current high incidence of colorectal cancer deaths. In more than 30% of colorectal cancer cases, the KRAS gene is mutated (Eder, *et al*., 1999). KRAS is a form of RAS and is an oncogene. The KRAS gene codes the KRAS protein, which acts as a molecular "switch" that regulates a variety of signaling pathways (Fouad, *et al.*, 2012).

Submitted: December 24, 2022
Revised: March 08, 2023
Accepted: March 10, 2023
Published online: April 03, 2023

*Corresponding author: bashari.hasan@unpad.ac.id

Mutations in the RAS protein gene would cause a disruption in signaling function, causing the RAS protein to remain in an active GTP-bound state all of the time. As a result, aberrant cell proliferation, differentiation, and growth occur (Eder, *et al*., 1999). These mutations will also have an impact on current cancer treatments, such as reduced drug effectiveness, decrease patient response rates, and therapeutic resistance or failure. Resistance to the drug cetuximab in colorectal cancer patients with the KRAS mutation is one of them (Hardani, *et al*., 2018).

The currently recommended colon cancer therapy is surgical removal of the tumor followed by adjuvant therapy in the form of chemotherapy at stages II and III (Jannah, *et al*., 2019 and Kimman, *et al*., 2012). However, some patients do not respond well to chemotherapy (Jannah, *et al*., 2012; Labianca, *et al*., 2010). Therefore, to reduce the number of colorectal cancer deaths, especially colorectal cancer with KRAS mutations, it is necessary to develop treatments, one of which is the development of anticancer drugs (Labianca, *et al*., 2010).

Chemotherapy is still the major treatment for colon cancer, Marine Sponge has anti cancer effect derive from genus Stylissa as part of the Scopalinidae family (Little, *et al*., 2011). One of the *Stylissa* species is *Stylissa carteri* which can be found in Indonesia, specifically in Sulawesi and Ambon (Morrow, 2015). The genus *Stylissa* has a variety of secondary metabolites, some of which are brominated fatty acids, sterols, alkaloids, peptides, and sphingoglycolipids. These biological compounds act as cytotoxic, anticancer, antimicrobial, antibacterial, antifungal, anti-inflammatory, and antiplasmodial agents (Mioso, *et al*., 2017).

Previous study showed that the ethanol extract, the ethyl acetate fraction as well as the n-hexane fraction of *Stylissa carteri* induce cell death in various breast cancer cells including luminal, HER2+ as well as the triple negative cells. Moreover, they also cytotoxic in cervical cancer cells (Bashari, *et al*., 2022; Bashari *et al*., 2019; Dhillon, *et al*., 2007). However, there has been no specific research on the cytotoxic effects of *Stylissa carteri* on colon cancer.

The purpose of this study was to determine the anti-cancer effect, the cytotoxicity and antiproliferative of ethanolic extract of the marine sponge *Stylissa carteri* on the HCT-116 colon cancer cell line with KRAS mutation.

MATERIALS AND METHODS

This was an *in vitro* study which conducted at the Cell Culture and Cytogenic Laboratory, Faculty of Medicine, Universitas Padjadjaran.

Chemical and Reagents

In this study these following materials were used, RPMI medium (Gibco, Invitrogen, USA), fetal calf serum (FBS; Gibco) dan Penisilin/ Strepromisin (Gibco), dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA) 100%, tripsin-EDTA (Sigma-Aldrich), medium that contain MTT reactor (Sigma-Aldrich), trypan blue (Sigma-Aldrich).

Cell Culture and Conditions

The study was conducted on the human colon cancer cell line, HCT-116, which was obtained from Prof. Henning Schulze-Bergkamen (NCT, Heidelberg, Germany). Cell lines were cultured using RPMI medium (Gibco,) with supplementation of 10% fetal calf serum (FBS; Gibco) and 1% Penicillin/Streptomycin (Gibco) in a cell culture incubator at 37°C and 5% CO_2 content. The age of the HCT-116 cell line used was no more than 20 passages.

Extraction of *Stylissa carteri*

Stylissa carteri sponge samples were obtained from Pramuka Island, Thousand Islands National Park, Jakarta. The extraction process of *Stylissa carteri* was cut into small pieces then macerated for 2-3 days using ethanol. The solution

obtained then filtered using filter paper and the solvent was evaporated to produce a dry powder of ethanol extract. The ethanol extract diluted using 100% dimethylsulfoxide (DMSO; Sigma-Aldrich) and mixed with the medium.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) Assay

Colon cancer cell line HCT-116 was harvested from culture containers using trypsin-EDTA (Sigma-Aldrich). In a culture plate of 96 wells, 10,000 cells were planted for each then incubated for 24 h before being treated. Culture media containing 1% DMSO was used as a control. Next, cells were incubated for 72 h and tested for 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). The MTT test was carried out on cells by replacing the medium containing *Stylissa carteri* solution with medium containing MTT reagent (Sigma-Aldrich) which had been incubated for 4 h. The MTT reaction will then be stopped using DMSO. Absorbance measurements were carried out using a plate reader (Multiskan Ex, Thermofisher, USA) with a wavelength of 450 nm. The obtained results would be interpreted in non viable cell percentage with following formulation:

"% non-viable cell=100%-("sample absorbance-blank absorbance"/"control absorbance-blank absorbance"x100%)"

Furthermore, the IC_{50} value analysis was carried out using the sigmaplot software version 12, and this indicate the cytotoxic of medicine.

Trypan Blue Exclusion assay

In the serial trypan blue exclusion test, cell cultivation was carried out with a total of 100,000 cells for each well on a duplicate culture plate of 6 wells and cell counts were carried out at 0 and 72 h after administration of *Stylissa carteri* solution using trypan blue dye (Sigma-Aldrich). The cell doubling time is the time it takes a cell culture to double. Each cell type has its particular doubling time that depends on cell culture conditions. Factors that affect cell growth rate are air and nutrient accessibility, temperature, and surrounding pressure.

Statistical Analysis

The results of the MTT test were analyzed using Sigmaplot 12 version to determine the IC_{50} of the *Stylissa carteri* extract using the four parametric logistic regression methods. The Trypan Blue exclusion test is presented in graphical form.

RESULTS

Ethanol Extract of *Stylissa carteri* Induces HCT-116 Cell Death

From MTT assay, the IC_{50} value was 5 µg/mL (R square 0.9550). This value is the concentration of *Stylissa carteri* as potent inhibitor that is able to reduce the viability of HCT-116 cell line as much as 50%. (See Figure 1).

From the graph it is indicating that the ethanolic extract of *Stylissa carteri* has the ability to cause cell death in the HCT-116 colon cancer line, lower of IC_{50} means the greater its cytotoxic effect (Bashari, *et al.*, 2019).

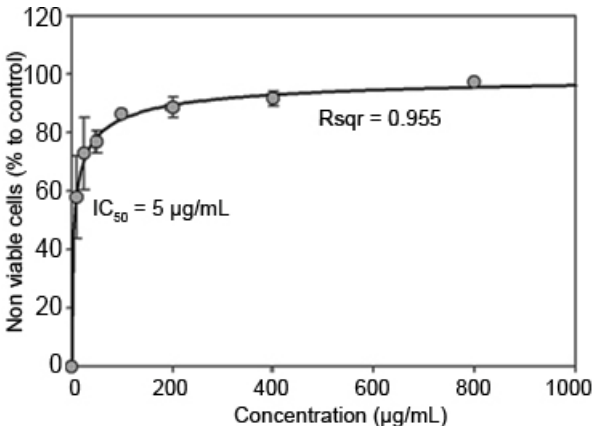


Figure 1. *Stylissa carteri* poses cytotoxic effect towards HCT-116 cells.

Ethanol Extract of *Stylissa carteri* as Antiproliferative Properties

Furthermore, the trypan blue exclusion serial test was used to calculate the mean cell doubling time to evaluate the cell growth inhibition ability of the ethanolic extract of *Stylissa carteri* on HCT-116 colon cancer cells.

From the graph above, the results showed that at 72 h after cell implantation, the mean number of viable cells was $67.7x10^4$ in the control group and $35.2x10^4$ in the treatment group (Figure 2), while the mean doubling time for control cells was 26.10 h and 39.69 h for treated cells.

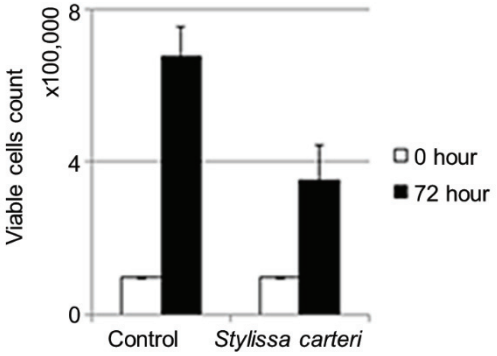


Figure 2. Ethanol extract of *Stylissa carteri* have anti-proliferative properties.

DISCUSSION

The cytotoxicity of *Stylissa carteri* extract against cancer cells has been studied in breast cancer cells (Bashari, *et al.*, 2022; Dhillon, *et al*., 2007; Eder, *et al*., 1999). Previous study was performed on human monocytic leukemia cells (MONO-MAC-6), and the resulting alkaloids actively killed these cells (Morrow, 2015). The methanol extract of *Stylissa carteri* has been shown to be cytotoxic against these leukemia cells, with an IC_{50} value of 2.4 µg/mL for the (Z)-debromohymenialdisine alkaloids and 0.2 µg/mL for the (Z)-hymenialdisine alkaloids (Morrow, 2015). This cytotoxicity test, however, has not been used specifically on colon cancer cell lines HCT-116.

The alkaloid aldisine was discovered to be a potent inhibitor of mitogen-activated protein kinase kinase-1 in another species, Stylissa massa (MEK-1) (Quasar Collaborative Group, 2007). The Raf/MEK/MAPK (mitogen activated MAP kinase) pathway is important in cell signaling processes, which eventually lead to cell proliferation and differentiation (Quasar Collaborative Group, 2007). Stylissa extract fractionation yields two aldisine alkaloids, 10E-hymenialdisine and 10Z-hymenialdisine, which selectively inhibit MAPK phosphorylation by MEK-1 and thus block the Raf/MEK/MAPK pathway (Quasar Collaborative Group, 2007). The hymenialdisine alkaloid was also discovered in *Stylissa carteri*, and based on the preceding research, it is possible that *Stylissa carteri* has a similar effect (Lee, *et al*., 2019).22

Four new brominated alkaloids, 12-N-methyl stevensine, 12-N-methyl-2-debromostevensine, 3-debromolatonduine B methyl ester, and 3-debromolatonduine A, as well as eight other alkaloids, were discovered in a study using a marine sponge of the genus Stylissa with an unspecified species. Only four of the twelve alkaloids found to have high cytotoxicity *in vitro* in the mouse lymphoma cell line L5187Y (Misale, *et al*., 2012). Those alkaloids were 12-N-methyl stevensin, (Z)-hymenialdisine, (Z)-debromohymenialdisine, and Lantoduine. The four produce EC_{50} values of 3.5, 1.8, 2.1, and 9.0 µg/mL in that order (Misale, *et al.*, 2012). The four alkaloids found are similar to those found in *Stylissa carteri* species.

The cytotoxicity of *Stylissa* on cancer cell lines is thought to be due to the sponge's diverse alkaloids. Some alkaloids in the genus *Stylissa* have been tested for cytotoxicity based on data from the genus. Inhibiting MEK-1, which is involved in the MAPK cell signaling pathway, is one of the mechanisms described in *Stylissa* massa. In cancer, inhibiting MEK-1 results in the cessation of proliferation and differentiation, increased apoptosis, and inhibition of abnormal cell migration (Fouad, *et al*., 2012; Quasar Collaborative Group, 2007).

Based on our research into the cytotoxic effect of the ethanolic extract of *Stylissa carteri*, we discovered that this sponge had a cytotoxic effect and was potent. On the HCT-116 colon cancer cell line with the KRAS mutation, the ethanolic extract of *Stylissa carteri* had an IC_{50} value of 5 µg/mL. This suggests that *Stylissa carteri* is cytotoxic to the HCT-116 colon cancer cell line. A natural material is considered cytotoxic if its IC_{50} value is less than 90 µg/mL, and non-cytotoxic if it has an IC_{50} value of 90 µg/mL or higher (Schmoll, *et al*., 2012). The differences of IC_{50} values that have been found and mentioned among *Stylissa carteri* extract may be due to differences in the group of compounds present in the extracts used and the characteristics of the cancer cell lines tested.

The ethanolic extract of *Stylissa carteri* has antiproliferative activity. This was shown by a doubling time that is greater than the control after 72 h of incubation, indicating that the HCT-116 colon cancer cell line that was treated has been inhibited in its proliferation (Figure 2).

CONCLUSION

Based on the findings, *Stylissa carteri* has cytotoxic and anti-proliferative properties, particularly against the HCT-116 colon cancer cell line with KRAS mutation. Furthermore, the findings of this study are expected to be considered when expanding knowledge about marine sponges, particularly those found in Indonesia.

ACKNOWLEDGEMENT

We would like to express our gratitude to Balai Taman Nasional Kepulauan Seribu for allowing to collect samples, Mr. Beginer Subhan from Bogor Agricultural University for collecting samples, Harold Atmaja, M.Sc., who extract the Stylissa carteri. Also to Tenny Putri and Nurul Qomarilla for their assistance with research in the Laboratorium Culture and Cytogenetics at Faculty of Medical, Universitas Padjadjaran. This study was funded by Ministry of Research and Technology/National Research and Innovation for MHB. This study was carried out with the support of the infrastructure provided by Universitas Padjadjaran. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This study did not use any primary cells and has no ethical issues.

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