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Metabolites analysis of the marine sponge *Callyspongia affinis* from Kangean Island as a potential source for anticancer candidates

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ABSTRACT

This study has objective to determine the metabolites classes contained in the hexane, EtOAc, BuOH, and aqueous fractions partitioned from the MeOH extract of the *Callyspongia affinis* sponge from Kangean Island, Indonesia. This study used a sponge from Kangean Island collected at 3–10 m depth as a research sample. The collected sample was then identified in the Zoology and Animal Engineering Laboratory, Sepuluh Nopember Institute of Technology, Surabaya, Indonesia. The extracts and fractions were investigated for their ability in terms of free radical scavenging, toxicity, antiproliferation, and apoptotic activity against HeLa and MCF-7 cells. The results showed that all fractions have organic nitrogen metabolites in the form of alkaloids and amide functional groups. The EtOAc and BuOH fractions contain fluorinated organic halides, while the aqueous fraction contains chlorinated organic halides. The EtOAc fraction showed the best ability as free radical 2,2-diphenyl-1-picryl-hydrazyl-hydrate scavenging with an IC_{50} of 27.617 µg/mL. It has the highest toxicity against *Artemia salina* with an LC_{50} of 26.652 µg/mL. The EtOAc fraction showed the best antiproliferative ability against HeLa and MCF-7 cells with IC_{50} values of 94.934 ± 0.700 and 97.804 ± 0.241 µg/mL, respectively. The EtOAc extract also showed the best apoptotic ability to HeLa and MCF-7 cells with IC_{50} values of 143.380 ± 0.010 and 131.646 ± 0.011 µg/mL, followed by MeOH extract and hexane fractions.

INTRODUCTION

Indonesia archipelago is known for its indigenous natural resources, such as marine sponge, which shows high potency of bioactive compounds (Latifah *et al*., 2021). Around 15,000 species of marine sponges are known to exist, and they remain desirable for use in the pharmaceutical industry (Lim *et al*., 2014). Sponges do not have an innate immune system and can only protect themselves by producing metabolites that serve as a kind of self-defence and allow them to adapt to their surroundings (Calcabrini *et al*., 2017; Rocha *et al*., 2001). Kangean Island is part of the Indonesian Coral Triangle area located in East Java Province, with various types of marine sponges. It contains a wide variety of marine natural products with a high benefit value (Lorig-Roach *et al*., 2018; Pawlik and McMurray, 2020). Most benefits are antiinflammatory, antifungal, antibacterial, and anticancer agents. One of the sponge species belonging to the Callyspongiidae family, which is widely distributed in this marine environment, has active chemicals with the potential to develop into future treatments for severe diseases like cancer. *Callyspongia* sp. is a marine sponge that generates secondary metabolites such as steroids, alkaloids, flavonoids, and terpenoids that can be used as antibacterial agents (Wao and Priska, 2021). It is well known that natural products from marine sponges have antioxidant, antiproliferative, and cytotoxic activities since the first discovery of the nucleotides spongouridine and spongotimidine from the marine sponge *Cryptotethia crypta*. Their synthetic derivative compounds are used to treat breast, lung, pancreatic, and bladder cancers (Kumar and Adki, 2018). Secondary metabolites obtained

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from sponges of the Callyspongiidae family in their extract and compound have shown antioxidant (Arunachalam and Amirtham Jacob Appadorai, 2013), cytotoxic, and anticancer activities (Youssef *et al*., 2003).

The hydro-ethanoic extract of sponge *Callyspongia* sp. from East Java, Indonesia, showed the ability to trap 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radicals at IC_{50} 181 µg/mL, toxic to tumor cell line with IC_{50} below 50 µg/mL against HT-29 and T47D cells (Abdillah *et al*., 2013). Aqueous ethanol extract from *Callyspongia crassa* and *Callyspongia siphonella* (*C. siphonella*) showed inhibition against the growth of MCF-7 and Caco-2 cancer cells (Ibrahim *et al*., 2017). Furthermore, the extract of *C. siphonella* extracted with various semi-polar solvents showed antiproliferative and proapoptotic activity against HepG-2, MCF-7, and Caco-2 cells (Rady and Bashar, 2020). Two oxindole brominated alkaloid compounds from the Red Sea sponge *C. siphonella* reveal a strong cytotoxic effect against different human cancer cell lines, presumably through the induction of necrosis (El-Hawary *et al*., 2019). In addition, the methanol extract from *Callyspongia aerizusa* is also active in inhibiting the growth of A549 cells with IC_{50} 9.38 µg/mL (Hadisaputri *et al*., 2021). Callyspongamide, a compound isolated from *Callyspongia fistularis*, showed inhibitory activity against HeLa, MCF-7, and A549 cells (Ki *et al*., 2020; Youssef *et al*., 2003). Polyacetylene diol compounds separation results from EtOAc extract *Callyspongia* sp. showed antiproliferative properties against HL-60 cells with IC_{50} values below 10 µg/mL (Umeyama *et al*., 2010).

Moreover, the organic extracts of marine spongesmay be a viable source of toxic metabolites produced by related bacteria as well as secondary metabolites for the development of novel therapeutics for both human and veterinary illnesses. In light of the enormous potential of marine resources in Indonesia, Kangean Island is one of the islands with various marine, including sponges. The marine sponge *Callyspongia affinis*, a species of sponge that is only found in the Kangean archipelago of Indonesia, so there are no research reports related to its metabolites and potential benefits. This article aims to learn more about the biological activity of the extract, which includes antioxidant, toxic, and anticancer properties linked to the presence of secondary metabolites that make up the extract.

MATERIALS AND METHODS

Sponge material

The sponges were collected by scuba diving (3–10 m depth) from Kangean Island in the Province of East Java, Indonesia. The samples were identified at the Sepuluh Nopember Institute of Technology's Zoology and Animal Engineering Laboratory, Surabaya, Indonesia, using scanning and optical microscope studies on skeletal slides and fragmented spicule mounts by the keys of John N. A. Hooper's sponge guide (Erpenbeck *et al*., 2020; Umeyama *et al*., 2010) and identified as *C. affinis*. The samples were put in plastic bags containing seawater, transferred to the laboratory on ice, and then stored at −10°C.

Preparation and partition of methanol extract

The frozen sponge was thawed, carefully cleansed with 70% alcohol, dried, cut into small pieces, and ground to create a finely powdered sample for 1.91 kg. The powdered sample was dissolved in MeOH under sonication and evaporated using a rotary vacuum evaporator to obtain 75.15 g of brownish-green concentrated extracts. The MeOH extract was partitioned with n-hexane:water and the n-hexane fraction (3.46 g) was obtained. The water fraction was partitioned with EtOAc and BuOH, respectively, in order to obtain EtOAc (19.06 g), BuOH (3.05 g), and water (29.97 g) fractions.

Metabolites analysis

Metabolites analysis was analyzed on the *C. affinis* crude extract on a high-performance liquid chromatography Thermo Scientific Dionex Ultimate 3000 RSLCnano with a micro flow meter system coupled to an HRMS Thermo Scientific Q Exactive, full scan at 70,000 resolutions and 17,500 resolutions of data-dependent MS2. Chromatographic separation was carried out on a Hypersil GOLD aQ (50 × 1 mm × 1.9 u particle size). A linear binary solvent gradient of 0.1% formic acid in water (v/v) as solvent A and acetonitrile as solvent B was performed over 30 minutes at a flow rate of 40 µl per minute. The column temperature was 30°C, and the injection volume was 2 µl. The raw data were transformed into positive and negative ionization files using MS Converter and subjected using mzCloud MS/MS Library Compound Discoverer software.

DPPH assay

DPPH radicals have an absorption maximum of 515 nm. When an antioxidant reduces DPPH radicals, the color of the solution changes, and the reaction may be easily tracked using a spectrophotometer (UV–Vis Shimadzu). The previously mentioned approach was modified to test the extract's capacity to eradicate free radicals (Oogarah *et al*., 2020). Determination procedures were as follows: 0.1 mM DPPH solution was prepared by dissolving 3.94 mg DPPH powder in 100 ml MeOH. A methanolic solution of the sample was prepared by dissolving 2.5 mg samples in 10 ml MeOH. After mixing 0.5 ml of DPPH solution with 20 µl of test extract at various concentrations, the mixture's absorbance was measured at 515 nm after 20 minutes at 37°C (A). Daily measurements were taken at the same wavelength on a blank sample that contained 20 µl of the aforementioned DPPH solution. Three duplicates of the experiment were performed. The amount of radical scavenging activity was determined using the following formula: Inhibition (%) is calculated using the formula $[(A_b - A_s)/A_b] \times 100$, where A_b and A_s represent the absorbance of the reaction solutions containing the blank and the samples, respectively.

Brine shrimp lethality test

This test was used to predict the cytotoxic activity of MeOH extract and fraction of n-hexane, EtOAc, BuOH, and water. *Artemia salina* L. nauplii (Artemiidae) were employed in brine shrimp lethality test. For 48 hours at 28°C, brine shrimp eggs were incubated in 500 ml of filtered seawater with continual aeration. The active egg cells nauplii were gathered and used for testing. After that, the active nauplii of the egg cell were collected and used for testing. One mg of each extract dissolved into 1 mL of Dimethyl Sulfoxide (DMSO) and solutions of varying concentrations of 150, 100, 75, 50, 25, 10, and 5 µg/mL in triplicate were obtained by the serial dilution using seawater. Fifteen nauplii were then inserted into each vial and kept at room temperature for 24 hours.The vials were inspected using a magnifying glass to count the number of

survived nauplii. This data calculated the percent of the lethality of the brine shrimp nauplii for each concentration. As negative controls, filtered seawater and DMSO were utilized. The LC_{50} value was calculated using the curve method based on the probit analysis (Chakraborty and Francis, 2021; Meyer *et al*., 1982).

MTT assay

MTT is a colorimetric assay that can evaluate the antiproliferative effect of *C. affinis* extracts. Two cell lines, MCF-7 as a human breast cancer cell line and HeLa as a cervix cell line, have been produced for cytotoxic testing using MTT. All cell lines were acquired from the Brawijaya University Life Science Central Laboratory. HeLa and MCF-7 were cultured in RPMI-1650 (Gibco, USA) medium. After mild trypsinization with 0.25% trypsin-EDTA/Ethylenediaminetetraacetic acid (Gibco, USA), all cells were subcultured, and the vitality and quantity of cells were assessed. The cells were incubated overnight and seeded in 96-well plates at a density of 5×10^3 cells/well in a 100 µl medium. All media contained 10% fetal bovine serum (FBS) (Gibco, USA) and 1% streptomycin-penicillin (Gibco, USA). The cell line was kept at 37°C, with a 5% CO_2 atmosphere. The samples were dissolved to create a stock solution in 1% DMSO to give a 2,000 mg/l concentration. Cells incubated for 24 hours were then divided into cell control and media control (blank) groups. The medium is removed and washed using phosphate-buffered saline (PBS) sterile, then each well is added 100 µL PBS. Each extract was added to each well to produce various concentrations of 50, 100, 200, 400, 600, and 800 µg/mL, and then it was incubated for 24 hours at 37°C, with 5% CO_2. Using sterile PBS, cells that had been cultured and incubated for 24 hours were disseminated throughout the medium. Then in each well was added 100 µl of MTT (5 mg/ml) in a culture medium followed by 4 hours of incubation at 37°C, with 5% CO_2. To dissolve the formazan crystals, 100 l of DMSO was added to each well and incubated at room temperature for 30 minutes. Determine the optical density (OD) of each well using an ELISA reader at 595 nm.

The cell viability percentage was then calculated using the following formula:

$$\frac{\text{OD of treatment} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}} \times 100\%$$

Flow cytometry assay

HeLa and MCF-7cells were cultured in RPMI-1640 (Gibco, USA) with 10% of FBS in the presence of different concentrations (50, 100, 200, 400, 600, and 800 µg/mL) to determine the effect of the sponge extracts on apoptosis. After 24 hours, the cells were taken out, washed in PBS, and stained with 50 µl Annexin V–PI solution in PBS (50 µg/mL) according to the manufacturer's instruction (Invitrogen, Life Technologies, IN, USA). For 40 minutes, the cells were incubated at room temperature. The cells were re-suspended in 500 µl PBS for running flow cytometry using BD FACS Calibur Flow Cytometer. The data were analyzed using the Cell Quest Program.

Statistical methods

The mean and standard deviation were used to express all experimental results. Using one-way analysis of variance and Dunnett's post-test with GraphPad Prism version 8.00, GraphPad Prism Software, statistically significant differences between means were evaluated (San Diego, CA). Data with a *p*-value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Metabolites analysis

The MeOH extract was partitioned into hexane, EtOAc, BuOH, and aqueous fractions to obtain non-polar, semi-polar, and polar metabolites, giving different molecular structures. Thus, the toxicity, antioxidative, and apoptotic properties of each fraction could be studied and compared with the MeOH extracts.

Table 1 shows data from the chromatogram peaks of each fraction, including retention time, molecular weight, and chemical formulas of compounds. The chemical formula was obtained from the molecular ion analysis of each compound's mass spectra based on the output of the Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) In general, the metabolites in each fraction are organic molecules containing heteroatoms such as N, O, and a small number of halides such as F, Br, or Cl. The O and N atoms can form functional groups such as lactam amide, phenolic, alcohol, acid, ester, or amines bonded in aliphatic or aromatic chains. The presence of organo halides compounds was also shown in semi-polar and polar fractions. The semi-polar fractions, EtOAc and BuOH, and the polar aqueous fraction contained organochlorine compounds. The sulfide groups are also found in the compounds that make up the semi-polar and polar fractions. The presence of O atoms in the molecular structure of the fraction may form hydroxyl groups of alcohol or phenolic hydroxyl OH−groups, ether, or ester as found in the Callypyrone A and B structures (Chakraborty and Francis, 2021). The presence of nitrogen could be derived from alkaloids which can be presented in the form of primary, secondary, or tertiary amines. Most combinations of O and N form an amide group, as found in the structures of Callyspongiamide (Kapojos *et al*., 2018), Callyptide (Shaala *et al*., 2016), and Callyaerins (Ibrahim *et al*., 2010).

Semi-polar fractions such as EtOAc and BuOH contain fluorinated organic compounds. The existence of this flour is a new thing in reporting the content of secondary metabolites in marine sponges, especially *Callyspongia* species. In the EtOAc fraction, there were three fluorinated organic compounds with the formulas of $C_{23}H_{43}N_7O_3F$, $C_{27}H_{50}N_2FS$, and $C_{28}H_{57}NO_3FS$, while in the BuOH fraction there are $C_{16}H_{18}O_2F$, $C_{16}H_{18}OF$, and $C_{19}H_{27}NFS$.

DPPH assay

The ability to scavenge DPPH radicals from each fraction is shown in Table 2. The ability to scavenge radicals is directly related to the antioxidant properties of each fraction. The stronger the ability to scavenge radicals is (marked by a decrease in the absorbance of DPPH), the stronger the antioxidant properties are. The EtOAc fraction showed the highest radical scavenging ability with an IC_{50} value of 27.617 µg/mL compared to the MeOH extract and other fractions. The hexane fraction with IC_{50} of 153.854 µg/mL showed the lowest radical scavenging ability. This radical scavenging ability describes the ability of the fractions to act as antioxidants. From the data in Table 2, it can be seen that although all fractions showed oxidative properties under ascorbic acid as a positive control (IC50 11.529 µg/mL), the EtOAc fraction was a potent antioxidant (IC50< 30 µg/mL), while MeOH extract, aqueous fraction, and BuOH fraction are included as moderate antioxidant (IC50< 100 µg/mL).

Table 1. Analysis of LC-HRMS from each fraction of *C. affinis* (*).

Fractions	RT (minute)	$[M + H]^+$	Calculated mass	Chemical formula
Hexane (31 peaks)	7.98	216.1966	216.1964	$C_{12}H_{26}NO_2$
	8.18	482.3268	482.3270	$C_{30}H_{44}NO_4$
	9.25	244.2280	244.2277	$C_{14}H_{30}NO_2$
	9.76	258.2437	258.2433	$C_{15}H_{32}NO_2$
	11.38	296.2953	296.2953	$C_{19}H_{38}NO$
	12.51	506.4052	506.4043	$C_{24}H_{48}N_{11}O$
	13.01	480.3451	480.3437	$C_{26}H_{46}N_3O_5$
	4.73	227.1398	227.1396	$C_{11}H_{19}N_2O_3$
EtOAc (35 peaks)	5.06	261.1239	261.1239	$C_{14}H_{17}N_2O_3$
	5.37	211.1447	211.1447	$C_{11}H_{19}N_2O_2$
	6.11	245.1290	245.1290	$C_{14}H_{17}N_2O_2$
	8.20	482.3269	482.3270	$C_{30}H_{44}NO_4$
	8.83	484.3415	484.3411	$C_{23}H_{43}N_7O_3F$
	9.27	258.2435	258.2433	$C_{15}H_{32}NO_2$
	10.33	272.2593	272.2590	$C_{16}H_{34}NO_2$
	10.81	286.2747	286.2746	$C_{17}H_{36}NO_2$
	12.24	453.3690	453.3679	$C_{27}H_{50}N_2FS$
	12.51	506.4055	506.4043	$C_{28}H_{57}NO_3FS$
BuOH (29 peaks)	3.87	213.1237	213.1239	$C_{10}H_{17}N_2O_3$
	5.06	261.1244	261.1291	$C_{16}H_{18}O_2F$
	5.37	211.1448	211.1447	$C_{11}H_{19}N_2O_2$
	6.13	245.1292	245.1342	$C_{16}H_{18}OF$
	7.32	320.1860	320.1848	$C_{19}H_{27}NFS$
	7.61	352.2020	352.2025	$C_{21}H_{26}N_3O_2$
	8.00	216.1966	216.1964	$C_{12}H_{26}NO_2$
	9.78	258.2431	258.2433	$C_{15}H_{32}NO_2$
	11.52	296.2952	296.2953	$C_{19}H_{38}NO$
Aqueous (24 peaks)	13.01	480.3455	480.3437	$C_{26}H_{46}N_3O_5$
	0.95	151.0360	151.0387	$C_3H_8N_4OCl$
	3.98	164.1438	164.1473	$C_8H_{22}NS$
	4.75	227.1397	227.1396	$C_{11}H_{19}N_2O_3$
	5.01	261.1238	261.1239	$C_{14}H_{17}N_2O_3$
	5.32	346.1217	346.1225	$C_{17}H_{20}N_3O_3S$
	5.91	305.0992	305.0994	$C_{12}H_{21}N_2O_3S_2$
	9.39	144.0449	144.0449	C_9H_6NO

*Only major compounds are included in this table.

Table 2. Antioxidative activity and IC_{50} value of extracts and fractions of sponge *C. affinis*

Samples	IC_{50} value (µg/mL)	R^2	Antioxidative activity categories
MeOH extract	38.694 ± 0.073	0.9978	moderate
Hexane fraction	153.854 ± 0.160	0.8731	weak
EtOAc fraction	27.617 ± 0.013	0.9920	strong
BuOH fraction	64.692 ± 0.192	0.9854	moderate
Aqueous fraction	43.309 ± 0.100	0.9930	moderate
Ascorbic acid	11.529 ± 0.004	0.9895	strong

Antioxidative properties are closely related to preventing the growth of harmful tissues triggered by free radical reaction mechanisms. The formation of free radical substances in the body triggers the growth and development of cancer cells. Therefore, the antioxidant ability of the *C. affinis* fractions also illustrates its ability to inhibit the formation of cancer cells. Constituent compounds support this activity in each fraction, acting as free radical scavenging. In this case, the compounds that make up the EtOAc fraction are more effective than the MeOH extract as the parent solution as an antioxidant and can inhibit the formation of cancer cells.

Brine shrimp lethality test result

Brine shrimp LC_{50} values for the MeOH extract and four fractions of sponge *C. affinis* evaluated in this study are reported in Table 3. All tested fractions and extracts were dose-dependent and highly toxic to *A. salina* with LC_{50} values below 100 µg/mL, according to Clarkson *et al.*'s (2004) toxicity criterion. The EtOAc fraction showed the strongest and highest toxicity among all samples, with an LC_{50} value of 26.652 µg/mL. MeOH extract with an LC_{50} value of 27.451 µg/mL was relatively more toxic than other fractions except for the EtOAc fraction. Although the

LC_{50} values of all samples did not show much difference, the MeOH extract and aqueous fraction, which were polar, tended to show a higher level of toxicity than the non-polar and semi-polar hexane and BuOH fractions. These results show a direct relationship with the fractional extraction performed. MeOH was the first solvent used for total extraction, while other solvents were used for fractionation, which showed different toxicity according to the distribution of polar and non-polar toxicants.

The EtOAc fraction had a higher level of toxicity than the extract and other fractions. This high toxicity can be attributed to the presence of fluorinated organic halides with non-polar properties (bound to long hydrocarbon chains) in the EtOAc fraction, which is thought to contribute to the cytotoxicity effect *on A. salina*.

MTT assay

The toxicity effects of the extract and sponge fractions on HeLa and MCF-7 cells were determined using the MTT assay. The toxicity effect can also be interpreted as the antiproliferative ability of the extract and fractions against HeLa and MCF-7 cells. The toxicity level of the extract and all sponge fractions can be seen in Figure 1, indicated by the decrease in the number of living cells as the concentration of the extract/fractions increases. The EtOAc and hexane fractions showed higher antiproliferative properties than the MeOH extract and other fractions. The EtOAc fraction was more toxic to HeLa and MCF-7 cells than extracts and other fractions with IC_{50} values of 94.934 ± 0.700 and 97.804 ± 0.24 µg/mL, which are presented in Table 4. Non-polar fractions such as hexane also showed high toxicity even though it was still below the EtOAc fraction. In general, the compound constituents in the fraction with lower polarity showed better antiproliferative activity than the more polar fraction.

The increase in antiproliferative ability is partly due to electronic interactions such as electron-withdrawing groups attached to the aromatic ring, electron density in the double bond structure in both alkene and aromatic structures, and the presence of heterocyclic rings (Shu *et al*., 2021). Table 1 shows that the EtOAc fraction contains compounds with molecular formulas indicating the presence of aromatic and heterocyclic rings. Most alkaloids with heterocyclic rings can inhibit CDK-Cyclin Complex activity (Zhang *et al*., 2021).

Flow cytometry assay

The ability of extract and fractions of *C. affinis* to exert apoptotic effects on HeLa and MCF-7 cells was determined by flow cytometry assay. Apoptosis is a form of planned necrosis that is defined by morphological and biochemical changes. It is crucial for the development and maintenance of a healthy body since it eliminates old, unneeded, and diseased cells (Raja *et al*., 2020). Apoptosis can be triggered by factors external to the cell. In this experiment, the EtOAc fraction showed a higher apoptotic effect than the MeOH extract and other fractions with IC_{50} values

Table 3. LC_{50} values based on brine shrimp activity of MeOH extract and fractions from *C. affinis.*

Samples	LC_{50} value (µg/ml)	R^2	Toxicity category
MeOH extract	27.451 ± 1.911	0.9738	highly toxic
n-Hexane fraction	30.425 ± 2.237	0.9178	highly toxic
EtOAc fraction	26.652 ± 2.962	0.9544	highly toxic
BuOH fraction	42.167 ± 3.531	0.9287	highly toxic
Aqueous fraction	29.123 ± 1.330	0.9842	highly toxic

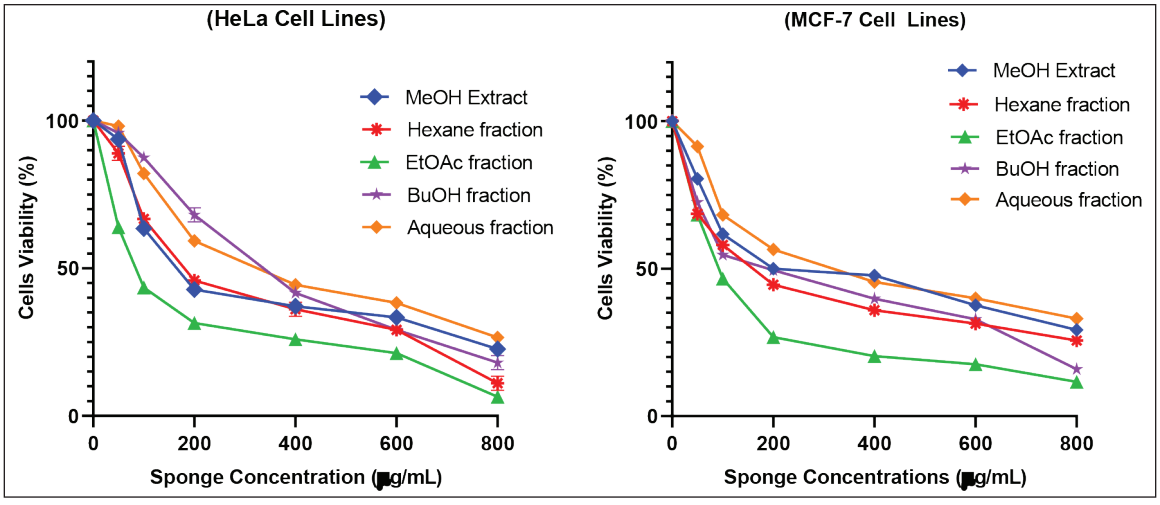

Figure 1. Cytotoxicity of various concentrations of *C. affinis* extract and fractions on HeLa and MCF-7 cells. The experiment was done three times, and the results were presented as the mean standard deviation (SD) with $p < 0.05$.

of 143.380 ± 0.010 µg/mL for HeLa cells and 131.646 ± 0.011 µg/mL for MCF-7 cells, as shown in Table 5. The proapoptotic ability of the fractions other than EtOAc was still lower than the MeOH extract.

Figure 2 shows the level of apoptosis of MeOH extract cells and various fractions of *C. affinis* at a concentration of 200 µg/mL. Some samples had entered 50% of cell apoptosis. The process of apoptosis in the two types of cells showed different conditions in terms of cells undergoing early apoptosis (AnnexinV/PI +/−) and late apoptosis (AnnexinV/PI +/+). In HeLa cells, the EtOAc fraction was more potent in inducing early apoptosis. In contrast, MCF-7 cells exhibited late apoptotic cells. Meanwhile, the fraction other than EtOAc showed that most of the apoptotic cells were in late apoptosis. Early apoptotic cells attract phagocytes by releasing specific signals without enhancing inflammation, while late apoptotic cells release additional pro-inflammatory danger signals (Khazaei *et al*., 2017). In general, extracts and fractions can trigger cells to repair themselves through the process of apoptosis against unwanted cells, such as cancer cells.

Table 4. The level of toxicity of extracts and fractions of *C. affinis* was determined by the IC_{50}value.

Samples	IC_{50} value (µg/ml)	
	HeLa	MCF-7
MeOH extract	167.619 ± 2.224	184.034 ± 0.209
Hexane fraction	180.315 ± 3.821	139.720 ± 0.613
EtOAc fraction	94.934 ± 0.700	97.804 ± 0.241
BuOH fraction	340.189 ± 3.785	147.333 ± 0.313
Aqueous fraction	328.568 ± 3.924	259.767 ± 1.129

Table 5. IC_{50} values of apoptosis of extracts and fractions of *C. affinis*.

Samples	IC_{50} value (µg/mL)	
	HeLa	MCF-7
MeOH extract	151.938 ± 0.047	192.547 ± 0.035
Hexane fraction	218.160 ± 0.055	203.394 ± 0.027
EtOAc fraction	143.380 ± 0.010	131.646 ± 0.011
BuOH fraction	436.584 ± 0.103	244.283 ± 0.036
Aqueous fraction	302.896 ± 0.093	349.153 ± 0.085

CONCLUSION

Callyspongia affinis is one of the marine natural products in Kangean Island, which is abundant and has good potential and opportunity to be developed as a new medicinal ingredient which was first investigated and reported in this study. This study presents the content of compounds in *C. affinis* as a new candidate for cancer treatment of selected marine sponge species that act on different targets in different cancer cells. The chemical content of *C. affinis* extract and fractions can be developed as a promising anticancer agent. The EtOAc fraction of sponge *C. affinis* showed the most promising anticancer potential among the investigated fractions and had potential antioxidant, toxic, antiproliferative effects, and apoptotic inducers. Nevertheless, isolating the pure compound from the EtOAc fraction from the *C. affinis* sponge remains challenging. Thus, it can also be explored as an anticancer agent in various types of cancer.

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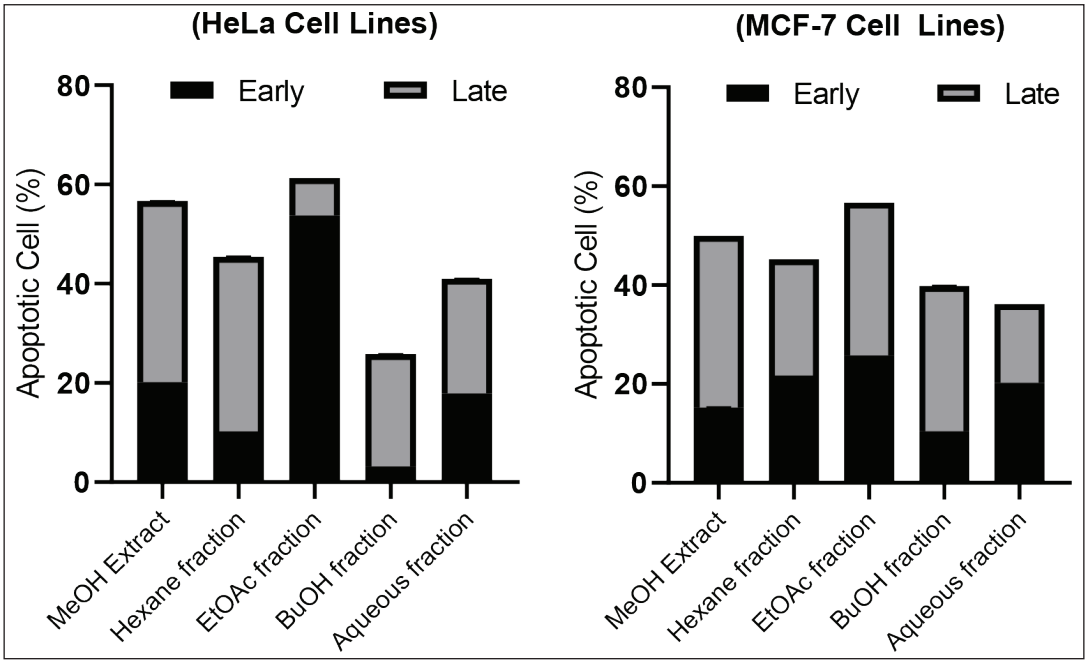

Figure 2. Effects of *C. affinis* extract and various fractions on the apoptotic of HeLa and MCF-7 cells at 200 mg/mL.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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