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Cytotoxic Effects and Anticancer Activity Assay of *Sonneratia alba* Fruit on Cancer Cell Cultures CaCo-2 and MCF-7

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ABSTRACT

Background and Objective: *Sonneratia alba* has a variety of bioactivities due to its secondary metabolites, one of its potential is as an anti-cancer agent. There are some previous studies on the toxicity of this plant. However, there is no information about the cytotoxic activity of the fruit part against colon and breast cancer cell cultures. Therefore, the study investigated the ethanol extract of *Sonneratia alba*, thus adding evidence of its potential as an anti-cancer agent. **Materials and Methods:** *Sonneratia alba* fruit extract was obtained using 70% ethanol through the maceration technique. Initial screening was done using the Brine Shrimp Lethality Test (BSLT) method and continued with the WST-1 assay on colon cancer cell line (CaCo-2) and breast cancer cell line (MCF-7). The Cytotoxicity of *Sonneratia alba* fruit was determined by the LC_{50} value derived from probit analysis and the IC_{50} value obtained through regression analysis. **Results:** The study found the ethanol extract of *Sonneratia alba* fruit was toxic to shrimp larvae, causing significant mortality. In breast cancer cell line MCF-7, the extract exhibited moderate cytotoxicity, indicating a potential for therapeutic application. Conversely, the extract showed only mild cytotoxic effects on CaCo-2 colon cancer cells, suggesting a lower efficacy against this cell type. **Conclusion:** Further research on specific bioactive compounds and their mechanisms of action can provide valuable insights into the therapeutic potential of *Sonneratia alba* in cancer treatment.

KEYWORDS

CaCo-2, cytotoxic, MCF-7, *Sonneratia alba*, WST-1 assay

INTRODUCTION

Cancer is a condition in which cells change and reproduce uncontrollably[1]. Cancer is currently a global health problem, including in Indonesia. According to data from the Global Burden of Cancer Society (Globocan) regarding the incidence of cancer in the world in 2022, there were 19.9 million new cancer cases recorded, with a death rate of 9.7 million. The total number of cancer cases in Indonesia in 2020 reached 396,914 new cases, with a total of 234,511 deaths[2]. Breast cancer and colon cancer are the most common types of cancer suffered by the Indonesian population. Breast cancer is in first place, cervical cancer is in second place, lung cancer is in third place, followed by colorectal cancer in fourth place[2].

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Breast cancer is the most common type of cancer detected in women worldwide, with more than 2 million new cases recorded in 2020[3]. In 2020, there were 65,8581 new cases of breast cancer in Indonesia, accounting for around 16.6% of the total of 396,914 new cancer cases. Meanwhile, the number of deaths due to breast cancer reached more than 22 thousand people[2]. The number of new cases of colon cancer in both men and women is 1.9 million and the death rate is 10%, reported 935,000 per year[4]. The GLOBOCAN recorded the cancer situation in Indonesia in 2020: There were 33,427 new cases of colon cancer, with a death rate of 8.6%[2].

There are several therapy options for cancer, such as chemotherapy, radiotherapy and biological therapy. However, this therapy is not selective and can damage healthy cells and tissues, causing side effects in the body[5]. Therefore, it is necessary to have alternative anticancer therapy treatments that have no or minimal side effects but have the same level of effectiveness. Natural products from plants cause fewer undesirable side effects due to their similarity to chemicals found in human food that can increase tolerance capabilities[6]. Indonesia is a country with the richest types of mangroves in the world[7]. The use of mangrove as a source of food and traditional medicine has been carried out by coastal communities for a long time. *Sonneratia alba* is one of the species in the genus *Sonneratia*, which is a group of mangrove plants from the *Lythraceae* family that widely grows in areas between sea and land, both tropical and subtropical[8].

The phytochemical test found that the ethanol extract of *Sonneratia alba* fruits contains flavonoids, alkaloids, phenolics, steroids and triterpenoids[9]. Based on these contents, *Sonneratia alba* fruit has the potential to be anti-tumor and inhibit the proliferation of cancer cells[7]. The benefits of *Sonneratia alba* fruit as an anticancer need to be strengthened by research on the toxicity of *S. alba* fruit using the initial Brine Shrimp Lethality Test (BSLT) method. According to previous BSLT test using various *Sonneratia alba* extracts after 24 hrs, the results showed that *S. alba* had promising cytotoxicity values[10]. Another cytotoxic test that can be used is a viability test targeted at cancer cells. This test is basically used to screen cell responses to drugs or chemicals. There are various types of viability tests, one of which is the WST-1 assay[11]. The study reported by Suryaningrum[12], an IC_{50} value of 478,630 µg/mL was obtained from *Sonneratia alba* extract and included in the category of being quite active against cervical cancer cells. This study aims to explore the cytotoxic effects and anticancer activity of *S. alba* fruit. This can provide important information regarding the potential of *Sonneratia alba* fruit as an *in vitro* anticancer in Colon Cancer Cells (CaCo-2) and Breast Cancer Cells (MCF-7).

MATERIALS AND METHODS

The study was carried out from January, 2024 to June, 2024 at Abdurrab University's Natural Materials Chemistry Laboratory and Institute of Medical Molecular Biotechnology, Universiti Teknologi MARA (UiTM) Laboratory, Malaysia.

Plant extraction: *Sonneratia alba* fruits are taken from the mangrove forest in Concong Luar Village Indragiri Hilir Riau Indonesia. A total of 4 kg of collected fruit was identified at the Biology Department Laboratory, Faculty of Mathematics and Natural Sciences, Riau University with letter number: 564/UN19.5.1.1.3-4/EP/2022. The fruits were wet sorted to separate them from dirt and unnecessary parts. Afterward the dried fruit was mashed with a blender to obtain dry simplicia (400 g). The extraction process for *Sonneratia alba* fruit used 70% ethanol solvent using the maceration method. The maceration results are then filtered and concentrated using a rotary evaporator (EYELA N-1300, Tokyo, Japan) to obtain a thick extract (29,50 g).

Brine shrimp lethality test protocol: *Artemia salina* shrimp eggs (100 mg) are hatched in a clear container containing seawater which is divided into two parts with a perforated partition, creating a dark and light area. Shrimp eggs are placed in the dark. To maintain the hatching temperature (25-30°C), light

is provided in the bright part of the container. Shrimp eggs are left for 48 hrs until they hatch into active larvae[13]. To obtain sample stock (1000 µg/mL), before carrying out the BSLT test, 0.50 g of sample was dissolved in 50 mL seawater. To obtain a 10 µg/mL solution, 0.5 µL was taken from the stock (1000 µg/mL) and pipetted into a 5 mL calibrated vial, then seawater was added to the calibration limit. Next, a series solution was made with a concentration of 20; 30; 40; 60; 80 and 100 ppm. The test solution for each concentration was pipetted as much as the calibration limit into all vials except the control group which only contained seawater, then 10 *Artemia salina* shrimp larvae were put into them[14,15]. The tube was left in the open for 24 hrs and the level of toxicity was assessed by counting the number of dead larvae. The standard criterion for determining the death of shrimp larvae is when no movement of the shrimp larvae is visible during several seconds of observation. This experiment was replicated 3 times for each group[13,16].

WST-1 protocol: The CaCo-2 and MCF-7 cells used were from the collection of the Institute of Medical Molecular Biotechnology, Universiti Teknologi MARA (UiTM) Laboratory. The CaCo-2 cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 media, while MCF-7 cells were cultured in DMEM (Dubelco's Modified Eagle's Medium) media. To obtain sample stock (100 mg/mL), before treating the cells, 100 mg of sample was dissolved in 1 mL DMSO. Furthermore, 10 µL was taken from the stock (100 mg/mL) and 9990 µL of medium was added to obtain a substock solution of 100 µg/mL. Afterwards, a series solution was made with a concentration of 10; 20; 30; 40; 60; 80 and 100 ppm was pipetted into a 96-well plate. Colon Cancer (CaCo-2) and Breast Cancer (MCF-7) cells that had been cultured were tested in 96 well plates with a total volume of 100 µl/well[17]. Then the wells were incubated in a 5% CO2 incubator at 37°C for 24 hrs. *Sonneratia alba* fruit extract which had been dissolved in DMSO co-solvent was added to the wells with 7 concentration series, namely: 10; 20; 30; 40; 60; 80; 100 ppm and incubated for 48 hrs. After incubation, the media and extract were discarded and then the cells were washed with PBS (phosphate-buffered saline)[18]. Then, 10 µL of WST-1 reagent solution was added to each well and the plate was incubated at 37°C for 4 hrs. After incubation, absorbance was measured at 450 nm with a microplate reader (Multiskan™ FC, Waltham, Massachusetts).

Data analysis: After data on the number of larval deaths has been collected, a probit analysis is carried out in Microsoft Excel 2016 using the Windows 10 system. The following probit calculation formula is used[19]:

$$\text{Larval death (\%)} = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

Probit analysis is performed by running a linear regression on the presentation of the larvae's death to generate LC_{50} values.

Regarding the WST-1 test, the results of absorbance readings with a microplate reader are converted into % viability cells in the following way[20]:

$$\text{Viability cells (\%)} = \frac{\text{Absorbance test well}}{\text{Absorbance control well}} \times 100$$

Based on % viability cell data, the IC_{50} value can be calculated using a linear regression equation which is the relationship between % viability cells vs concentration of *Sonneratia alba* fruit extract. The smaller the IC_{50} value, the more potential the extract has in inhibiting the growth of cancer cells.

RESULTS

Cytotoxic test using BSLT method: Cytotoxic test of ethanol extract of *Sonneratia alba* fruit using the BSLT (Brine Shrimp Lethality Test) method using 48 hrs old *Artemia salina* Leach larvae, with concentrations of 10, 20, 30, 40, 60, 80, 100 µg/m. Total mortality was obtained by adding up the larval

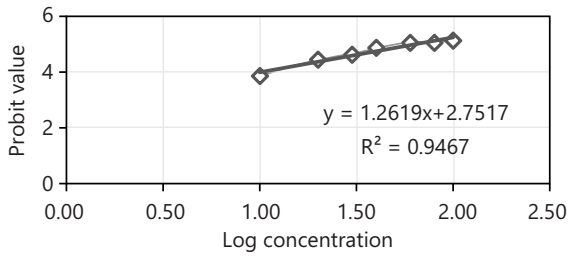

Fig. 1: A Linear regression graph of probit values for larval death and log concentration for *Sonneratia alba* fruit extract

*Calculating the LC_{50}, the straight line equation y = 1.2619x+2.7517 is obtained, entering 5 in the y value to obtain an LC_{50} of 60.48 µg/mL

Table 1: BSLT results show *Artemina salina* Leach mortality due to *Sonneratia alba* fruit extract

Concentration (µg/mL)	Total mortality	Average of mortality	Precentage of mortality (%)	LC_{50} (µg/mL)
10	4	0.13	13.3	60.48
20	9	0.30	30.0	
30	11	0.36	36.6	
40	14	0.46	46.6	
60	16	0.53	53.3	
80	16	0.53	53.3	
100	17	0.56	56.6	
Control	0	0	0	

deaths at each concentration, while the average was obtained by dividing the total deaths by the total larvae used at each concentration. The percentage of larval deaths was obtained by multiplying the average death by 100. Based on Table 1, the number of deaths of shrimp larvae increased along with increasing extract concentration and the negative control group did not show any death of shrimp larvae. Figure 1 shows the relationship between the concentration of *S. alba* fruit extract and the mortality precentage of the *Artemia salina* Leach larvae. These results indicate that an extract concentration of about 60.48 µg/mL is required to achieve 50% mortality, demonstrating significant cytotoxic potential of *S. alba* fruit extract against shrimp larvae, suggesting potential anticancer activity.

Cytotoxic test using WST-1 assay

Test on Colon Cancer (CaCo-2): Cytotoxicity test of ethanol extract of *Sonneratia alba* fruit using the WST-1 method on CaCo-2 cells using concentrations of 10, 20, 40, 60, 80, 100 µg/mL. The experiment was carried out 2 times at each concentration. Apart from that, a negative control was also made containing media and cancer cells without the addition of extract. The absorbance results in Table 2 show that the WST-1 test carried out on CaCo-2 cell cultures with the addition of various doses of ethanol extract of *S. alba* fruit, resulted in cell viability that was not much different from the control group. The cytotoxic activity of *S. alba* fruit extract against the CaCo-2 cell line was evaluated by measuring cell viability. As displayed in Figure 2, the IC_{50} value was calculated from the dose-response curves obtained by plotting precentage of viability cells.

Test on Breast Cancer (MCF-7): Cytotoxicity test of ethanol extract of *Sonneratia alba* fruit using the WST-1 method on MCF-7 cells using concentrations of 10, 20, 30, 40, 60, 80, 100 µg/mL. The experiment was carried out 3 times in each concentration. Apart from that, a negative control was also made containing media and cancer cells without the addition of extract. The results of the cytotoxic test of ethanol extract of *S. alba* fruit against MCF-7 breast cancer cells are shown in Table 3. The absorbance presented in Table 3 shows the results of the WST-1 test carried out on MCF-7 cells which had been cultured with additional doses of ethanol extract of *S. alba* fruit with a concentration of different, resulting in less MCF-7 cell viability when compared to controls. Cytotoxic data shows that each *S. alba* fruit extract

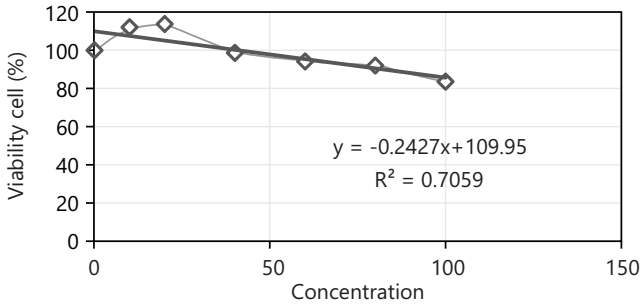

Fig. 2: A linear regression graph of the relationship between *Sonneratia alba* fruit extract concentration and precentage of CaCo-2 cell viability

*Resulting in the equation y =-0.2427x+109.95. and setting y to 50%, the IC_{50} was calculated as 247.012 µg/mL

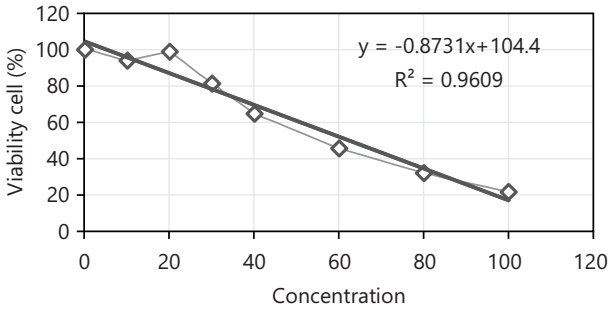

Fig. 3: A linear regression graph of the relationship between *Sonneratia alba* fruit extract concentration and precentage of MCF-7 cell viability

*Resulting in the equation y =-0.8731x+104.4, by setting y to 50%, the IC_{50} was calculated as 62.30 µg/mL

Table 2: WST-1 test results on CaCo-2 cells for 48 hrs

Concentration (µg/mL)	Absorbance average	Viability cell (%)	IC_{50} (µg/mL)
0	1.5482	100	247.012
10	1.7357	112.11	
20	1.7613	113.76	
40	1.5276	98.67	
60	1.4583	94.19	
80	1.4248	92.03	
100	1.2948	83.63	

Table 3: WST-1 test results on MCF-7 cells for 48 hrs

Concentration (µg/mL)	Absorbance average	Viability cell (%)	IC_{50} (µg/mL)
0	1.9596	100	62.30
10	1.8425	94.02	
20	1.9380	98.89	
30	1.5942	81.35	
40	1.2691	64.76	
60	0.8943	45.63	
80	0.6278	32.03	
100	0.4243	21.65	

at various concentrations has the ability to inhibit MCF-7 cells differently which can be seen in the average cell viability. Cytotoxic activity of *S. alba* fruit extract against the MCF-7 cell line was evaluated by measuring cell viability. The IC_{50} value was calculated from the dose-response curves obtained by plotting precentage of viability cells (Fig. 3).

From the results of calculating the percentage of cell viability, it was found that the average percentage of cell viability decreased at each dose tested for WST-1, meaning that fewer cells were alive. This is because the higher the test concentration dose, the lower the viability of MCF-7 cells as seen at the lowest

dose of 10 µg/mL, a high % of cell viability is obtained, in contrast to the viability at the highest dose of 100 µg/mL which has a low % cell viability.

DISCUSSION

In the present study, the ethanol extract of *S. alba* fruit demonstrated significant cytotoxic activity particularly against *Artemia salina* in the Brine Shrimp Lethality Test (BSLT), with an LC_{50} value of 60.48 µg/mL, categorizing it as toxic within the range of 30-1000 µg/mL[16]. This finding suggests the extract's potential as an anticancer agent. The cytotoxic effect was further observed in MCF-7 breast cancer cells with an IC_{50} value of 62.30 µg/mL, indicating moderate cytotoxic activity and in CaCo-2 colon cancer cells with an IC_{50} of 247.012 µg/mL, indicating weak cytotoxic activity.

Compared to previous research, this study aligns with findings by Latief *et al.*[10], regarding the toxicity of *S. alba* root extract, which reported an LC_{50} value of 23.98 µg/mL. On the other hand, this result contrast with the research conducted by Hendri *et al.*[21], which found that the methanol extract of *S. alba* exhibited a high LC_{50} value, indicating a low potential to kill *Artemia salina* larvae. The same as previous research conducted by Suryaningrum 12, that *S. alba* extract was cytotoxic to the viability of HeLa cells with an IC_{50} value of 478,630 µg/mL.

The cytotoxic test using BSLT method is a preliminary test in identifying anticancer potential by determining the LC_{50} value after 24 hrs of exposure to the extract solution[22]. The LC_{50} represents the concentration of a substance that causes 50% mortality in test organisms[23]. *Artemia salina,* used in the BSLT must be 48 hrs old, to be most actively dividing by mitosis, akin to cancer cells[16] and sensitive to test substances[24]. The control group, containing only seawater, showed no shrimp larvae mortality, indicating that the deaths in the test group were due to the extract and not seawater[14]. Differences in LC_{50} results can be influenced by several factors, including the quality and concentration of the extract, the extraction method and testing conditions such as temperature and pH. Aditionally, the withdrawal of active compounds from natural ingredients is affected by internal factors such as genetics and enzyme activity, as well as external factors, namely the environment including light, temperature, temperature, water, sampling location and sample type[25]. Apart from that, the type and amount of extraction solvent also affects the amount of active compounds contained in the extract. In this study, the type of solvent used was ethanol because it is relatively non-toxic and has a very high extraction rate[26]. The ethanol concentration used is no more than 70% because it can reduce the extraction rate of the target component, due to protein denaturation which increases diffusion resistance at higher ethanol concentrations[27].

The mechanism of death in larvae is caused by the secondary metabolite content of *S. alba* fruit extract[9]. Alkaloids and triterpenoids act as stomach poisoning which can cause larvae to experience digestive tract disorders[13]. Other than that, this compound can also inhibit taste receptors on the surface of the larva's mouth, making it unable to detect food and ultimately dying of starvation. Flavonoids can kill larvae by entering the larva's body through the respiratory system which will then cause weakness in the nerves and damage to the respiratory system so that the larvae cannot breathe and eventually die[28].

The WST-1 *(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)* is a colorimetric test to determine cellular viability. In principle, WST-1 reacts with mitochondrial succinate tetrazolium reductase to form yellow-orange water-soluble formazan[29]. An indicator that the cells being tested are able to reduce this salt is a color change in the media. The more living cells, the higher the color intensity that will be produced[30]. In this study, the cytotoxic test of MCF-7 breast cancer cells treated with *S. alba* fruit extract, at concentrations of 60, 80, 100 µg/mL there was no change in WST-1 (dark yellow), however at a lowest concentration of 10 µg/mL and the control group there was a significant color change, initially

the yellow color becomes more intense. Tests carried out on CaCo-2 colon cancer cells showed that the smaller the concentration of *S. alba* fruit extract, the more intense the color change that occurred. The occurrence of a color change in the test indicates cell life. This is due to the formation of formazan crystals as a product of the WST-1 reduction process by the oxidoreductase enzyme produced by the mitochondria of cancer cells. However, if there is no color change, it indicates that there are no living cells, so they are unable to metabolically produce the oxidoreductase enzyme which reduces WST-1 to formazan crystals[30].

The cytotoxic effect of a substance is assessed by the amount of half maximum inhibition (IC_{50}). The IC_{50} indicates the concentration required to kill 50% of cancer cells. The lower the IC_{50} value, the higher the cytotoxic activity of the substance. Based on the U.S. National Cancer Institute (NCI) and the Geran protocol, IC_{50} values <20 µg/mL indicate high cytotoxic activity, IC_{50} between 21-200 µg/mL has moderate cytotoxic activity, 201-500 µg/mL less toxic activity and IC_{50} >500 µg/mL has no cytotoxic activity[31]. The cytotoxic test on MCF-7 and CaCo-2 cells with ethanol extract of *S. alba* fruit revealed varyingIC_{50} values (62.30 and 247.012 µg/mL). Differences in cytotoxic activity are influenced by the response or sensitivity of each cancer cell which has different characteristics[32]. Based on the results of this research, *S. alba* fruit has the potential to be used as a co-prevention agent. Materials containing cytotoxic compounds with moderate activity can function as chemoprevention agents that play a role in inhibiting and preventing the development of cancer cells[32].

There is no literature that discusses the mechanism of action of *S. alba* induces cancer cell death. However, several previous studies explained that the cytotoxic mechanism for cancer cell death is related to the content of secondary metabolite compounds[12]. This compound has a regulatory effect on metabolic and signaling pathways, thereby controlling angiogenesis and inhibition of the formation of microtubule assembly in cells and their apoptosis[33]. Steroids have inhibitory enzymes such as aromatase and sulfatase inhibitors for breast cancer, these compounds can also damage the permeability of mitochondrial membranes in cells or cause cells to experience necrosis and death[34]. Alkaloids exert a restraining effect on the topoisomerase enzyme, thereby stopping DNA replication and cell death[35]. Flavonoids induce cancer cell death by inhibiting autophagy or vice versa can trigger cancer cell death through autophagy[36]. Overexpression of Bcl-2 can inhibit apoptotic signals, which means that the mechanism of apoptosis can be seen from Bcl-2 expression[37]. Flavonoids have been proven to activate the apoptotic pathway in CaCo-2 cells and are able to induce the intrinsic apoptotic pathway through upregulation of Bax, caspase-3 and downregulation of Bcl-2 in MCF-7 cells[38]. Various studies explain that triterpenoids taken from plants can have a positive impact on various types of breast cancer cells[39]. It efficiently prevented cell cycle progression and cell survival by lowering mitochondrial membrane potential. This was accomplished by lowering Bcl-2 expression while also changing the expression of Bax, caspases, HDAC-2 and H_3K27Ac. Furthermore, it caused programmed cell death in breast cancer cells by epigenetic modifications, upregulating Beclin 1 and LC_3B while downregulating p62 and mTOR protein expression[40].

This study highlights the potential of *S. alba* fruit extract as a source of bioactive compounds with cytotoxic activity. The significant toxicity to shrimp larvae, MCF-7 breast cancer cells and CaCo-2 colon cancer cells suggest that the extract could be further explored as a natural product with anticancer properties. These findings suggest further research including isolating and identifying the spesific secondary metabolites responsible for the cytotoxic effects and conducting mechanistic studies to elucidate their action on cancer cells. However, limitations such as the study's focus on a limited number of cancer cell lines and the lack of assessment of the extract's selectivity between cancer cells and normal cells must be addressed to gain a comprehensive understanding of *S. alba*'s anticancer potential.

CONCLUSION

The ethanol extract of *Sonneratia alba* fruit demonstrates significant cytotoxic activity against shrimp larvae, classifying them as toxic. In studies involving MCF-7 breast cancer cells, the extract exhibits moderate cytotoxic effects while it shows weaker cytotoxicity against CaCo-2 colon cancer cells. For further research on the anticancer mechanisms of *Sonneratia alba,* the isolation of secondary metabolite compounds is necessary.

SIGNIFICANCE STATEMENT

This study investigates the cytotoxic effects and anticancer activity of *Sonneratia alba* fruit on CaCo-2 and MCF-7 cancer cell cultures. The purpose is to explore natural anticancer agents that could serve as alternative or complementary treatments to current therapies. The study reveal that the fruit extract significantly reduces cell viability in both CaCo-2 and MCF-7 cells. These results highlight the potential of *Sonneratia alba* as a source of bioactive compounds with anticancer properties. To confirm the therapeutic potential and safety of these compounds in cancer treatment, further research is required, including the isolation and characterization of the active compounds, *in vivo* studies and clinical trials. This study creates opportunities for the development of novel, efficient natural cancer treatments.

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