

Cytotoxic and Antiproliferation Activity of n-Hexane Fraction of Avocado seed (*Persea americana* Mill.) on MCF7 cell

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Received: 12 Mei 2022; Accepted: 20 Juni 2022; Published: 25 Juni 2022

Abstract

Avocado seeds, which often end up as food waste, have many health benefits, including anticancer activity. This study aimed to determine the potential cytotoxic activity of avocado seeds against breast cancer cells, MCF7. Avocado seeds were macerated with 96% ethanol and fractionated with n-hexane, chloroform, and ethyl acetate as solvents. Cytotoxic activity of the extract and fractions was tested using the MTT method, while antiproliferation activity and apoptosis of the n-hexane fraction were tested using the doubling time and double staining methods, respectively. The selectivity of the n-hexane fraction was also determined. The results showed that the extract and n-hexane fraction of avocado seeds had cytotoxic activity on MCF7 with IC₅₀ values of 211.2±25 and 47.87±1.5 µg/mL, respectively. The cytotoxic activity of the n-hexane fraction was selective (Selectivity Index = 4.73). The fraction can extend the doubling time to 76.54-77.41 hours. The double staining test results showed red fluorescence in the cells, indicating the apoptosis of MCF7 cells. The n-hexane fraction of avocado seed extract has the potential to be developed as an anticancer therapeutic agent.

Keyword: *Persea americana* Mill., MCF7, MTT, cytotoxic, apoptosis

INTRODUCTION

Avocado is a dicotyledonous plant that belongs to the Lauraceae family. Avocado plants are native to Central America and southern Mexico (Yahia, 2012), but the plants can thrive in tropical areas such as Indonesia (Dewi et al., 2014). Avocado plants have many benefits. Avocados are rich in nutrients such as omega fatty acids, tocopherols, phytosterols, and squalene, which are contained in the lipid fraction of avocados (Fulgoni et al., 2013). Avocado is a good source of energy (1.7 kcal/g) because it contains 80% water and dietary fibre (McCormack et al., 2010).

Avocado fruit is mostly used for its flesh, while avocado seeds are discarded. The avocado seed is one of the natural ingredients with many health benefits (Fulgoni et al., 2013). Avocado seeds have certain medicinal properties such as hypoglycemic effect (Edem et al., 2009), hypolipidemic effect (Nwaoguikpe, 2011), cardioprotective activity (Pahua-ramos et al., 2012), antioxidant effect, improvement of lipid

profile (Shehata & Solttan, 2013), and cytotoxic activity on T47D breast cancer cells (Rahmawati & Maryati, 2021; Kristanty et al., 2014) and MCF7 (Nur et al., 2017). Potential cytotoxic activity of natural products is a current concern due to more than two million new cases of breast cancer in women occurring each year worldwide (WCRF, 2018). The purpose of this study was to determine the cytotoxic activity of avocado seed extract and its fraction on MCF7 cells. The most active fraction was tested for its selectivity towards normal cells, tested for anti-proliferative activity, and apoptotic induction activity against MCF7 cells. The results of this study are expected to be the initial information about avocado seed's potency to be developed as an anticancer therapeutic agent for breast cancer.

RESEARCH METHODOLOGY

Equipment

The equipment used in this research were confocal laser scanning microscope (Zeis 900), camera microscope (Optilab), inverted

microscope (Olympus), ELISA reader (Elx800 BioTeK), CO₂ incubator (Binder), cytotoxic safety cabinet (ESCO), micropipette (Socorex), haemocytometer (Neubauer), analytical balance (Precisia, XT 120A), rotary evaporator (IKA RV8), and water bath (Mettler).

Plant Material

The avocado seeds were obtained from avocados that were planted at Nargoyoso Village, Kemuning Tawangmangu, Karanganyar Regency, Central Java, Indonesia. A determination to ensure the correctness of the material was conducted at the Biology Department of Gajah Mada University, Certificate Number 16.12.8/UNI/FFA/BF/PT/2019.

Test Cells

The cells used in this study, breast cancer cells (MCF7) and kidney cells from an African green monkey (Vero), were obtained from the Mammalian Cell Culture Laboratory of the Faculty of Pharmacy, Universitas Muhammadiyah Surakarta.

Chemicals

The chemicals used in the research were 96% ethanol, n-hexane, chloroform, ethyl acetate, distilled water, DMEM (Dulbecco's Modified Eagle Medium) (Gibco), NaHCO₃ (Merck), DMSO (Merck), HCl (Merck), NaOH (Merck), FBS (Fetal Bovine Serum) (Gibco), 3% penicillin-streptomycin (Gibco), 0.025% trypsin (Sigma), PBS (Phosphate Buffered Saline) (Invitrogen), MTT (3-(4,5-dimethylthiazole-2-yl) diphenyl tetrazolium bromide) (Invitrogen), SDS (Sodium Dodecyl Sulphate) (Merck), acridine orange (Merck), propidium iodide (Merck), doxorubicin (Kalbe), 96-well plates (Iwaki), 24-well plates (Iwaki), and plastic round coverslips (Thermanox, Fisher Scientific).

Extraction and Fractionation

Avocado seeds were separated from the flesh, cut into small pieces, and dried. The dried seed was macerated with 96% ethanol and remaceration was done twice. The extract was concentrated using a rotary evaporator and water bath. Viscous extract was then

liquid-liquid fractionated with n-hexane, chloroform, and ethyl acetate to result in 3 fractions. The fractions were concentrated using a rotary evaporator and water bath to obtain thick fractions (Mardiyaningsih & Ismiyati, 2014).

Cytotoxicity Test

A hundred microliters of suspension of MCF7 cells in DMEM, which contains 10% FBS and 3% penicillin-streptomycin (density 10⁴ cells/well) was put into wells in 96-well plates and incubated for 24 hours in an incubator with 5% CO₂. The media were discarded, then 100 µL of samples with concentration of 125, 100, 75, 50, and 25 µg/mL were added to each well. The samples were dissolved in DMSO and diluted in the media. The plates were incubated in an incubator for 24 hours at 37°C with 5% CO₂. After that, 100 µL of 0.01% MTT solution in PBS was added into each well and the plates were incubated for 2 hours at 37°C. Enzymes in the viable cells reduce the MTT to insoluble formazan crystals (purple). Sodium dodecyl sulphate solution in 10% HCl was added as a stopper solution, then the plates were tightly wrapped in aluminium foil and incubated overnight at room temperature. The absorbances of formazan were read by a microplate reader at a wavelength of 550 nm. The percentage of living cells was calculated from the absorbances value. Next, a log concentration versus cell viability curve was made to calculate the IC₅₀ value (CCRC, 2012). Cell viability percentage was calculated with the formula below.

If the absorbance of solvent control is equal to the absorbance of cell control:

$$\% \text{ Cell Viability} = \left(\frac{\text{abs. of sample} - \text{abs. of medium control}}{\text{abs. of cell control} - \text{abs. of medium control}} \right) \times 100\%$$

If the absorbance of solvent control is lower than the absorbance of cell control:

$$\% \text{ Cell Viability} = \left(\frac{\text{abs. of sample} - \text{abs. of medium control}}{\text{abs. of solvent control} - \text{abs. of medium control}} \right) \times 100\%$$

Anti-proliferation Test

Table 1. IC₅₀ value of the Avocado Seed Ethanolic Extract and Fractions

Sample	IC ₅₀ (µg/mL)
Ethanolic extract	211.2±25
Ethyl acetate fraction	>10 ⁶
Chloroform fraction	281.3±110.9
N-Hexane fraction	47.87±1.5
Doxorubicin (positive control)	4.62±3.64

Observation of proliferation inhibition was carried out by the MTT method. The concentration of the n-hexane fraction were 40 µg/mL and 30 µg/mL. The cell viability was measured at 0, 24, 48 and 72 hours of incubation (CCRC, 2009b). The doubling time value was calculated by plotting the incubation time versus the log number of living cells on a graph, yielding the linear regression equation $Y = Bx + A$. Y was the log value of 2 times the initial number of live cells, and X was the value of doubling time (Wati et al., 2016).

Observation of Apoptosis by Double Staining

MCF7 cells with a density of 5×10^4 were seeded in a 24-well plate with coverslips at the bottom of the wells. The cells will attached to the coverslips. After overnight incubation, cells were treated with the n-hexane fraction of avocado seeds at concentrations of 40 µg/mL and 20 µg/mL, then the cells were incubated for 24 hours (CCRC, 2009a). For staining the cells, 0.1% acridine orange was mixed with 0.1% propidium iodide, and then the mixture was diluted to a concentration of 0.05%. The diluted mixture was used to stain the cells. Coverslips were taken from the wells and 10 µL of dye mixture was added to the coverslips, then the cells were observed under a confocal laser scanning microscope.

Selectivity Test

Vero cells with a density of 10^4 were seeded in a 96-well plate and incubated overnight. Then the cells were treated with n-hexane fraction with concentrations of 25, 50, 75, 100, and 125 µg/mL. The cells were incubated at 37°C for 24 hours. Next, 0.01% MTT solution was added to the wells and the

plate was incubated for 2 hours. Then, the SDS in 10% HCl (stopper solution) was added to the wells. The plate was wrapped in aluminium foil and incubated at room temperature overnight. Absorbances from formazan were read by a microplate reader at a wavelength of 550 nm. Next, the IC₅₀ value of the hexane fraction on Vero cells was determined. The selectivity index was calculated using the following formula:

$$SI = \frac{IC_{50} \text{ of normal cell}}{IC_{50} \text{ of cancer cell}}$$

Samples with a SI value less than 3 are considered less selective, while samples with a SI value greater than 3 are considered selective (Sutejo et al., 2016).

Statistical Analysis

Statistical analysis was performed using the SPSS application, version 20. Paired T Test was performed to assess the difference of doubling time values between treatment group and control group.

RESULT AND DISCUSSION

Cytotoxic Test

The cytotoxic test results of avocado seed ethanolic extract and its fractions showed that the n-hexane fraction has the highest activity with an IC₅₀ value of 47.87 ± 1.5 µg/mL (Table 1).

The n-hexane fraction of avocado seeds changes the morphology of MCF7 cells (Figure 1). MCF7 cells treated with the n-hexane fraction with a concentration of 125 µg/mL have dark colour with irregular shapes and some of them aggregating with each other, while untreated MCF7 cells (control cells) were transparent, have solid edges, and

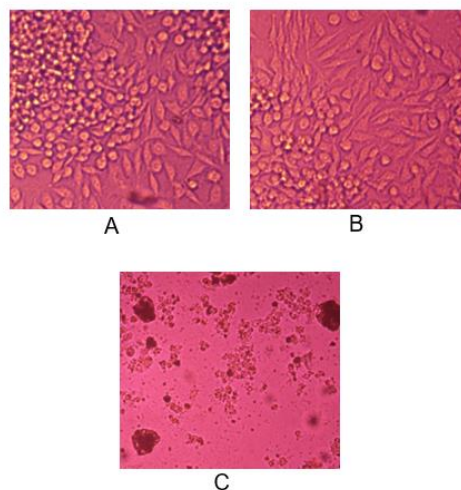


Figure 1. Morphology of control cells (A), MCF7 cells in 0.125% DMSO (B), and treated MCF7 with the n-hexane fraction of 125 µg/mL (C). Control cells and cells in 0.125% DMSO were not significantly different, but there were significant differences between the control cells and treated cells.

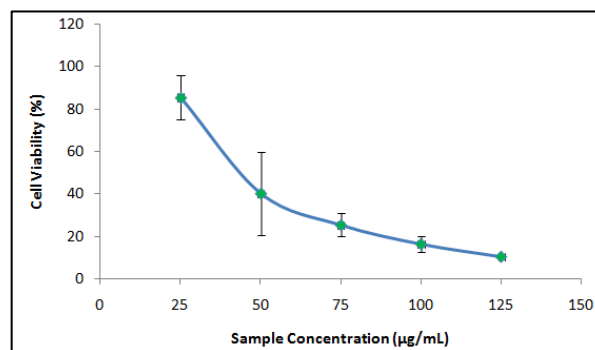


Figure 2. The increase in the concentration of the n-hexane fraction caused a decrease in the viability of MCF7 cells. The MCF7 cells were incubated with various concentrations of the n-hexane fraction for 24 hours. The cell viability was determined by the MTT method.

intact nuclei. The results also showed that there was no difference in the cell morphology between the cells in the control group and cells in the solvent control group (0.125% DMSO). The cytotoxic activity of the n-hexane fraction of avocado seeds is dose dependent. The higher the sample concentration, the greater the inhibition of the cell viability (Maryati et al., 2019) (Figure 2).

N-hexane fraction of avocado seeds contains terpenoids (Grace et al., 2015), long-chain fatty acids, and several fatty acid derivatives (Rivai et al., 2019). These compounds are responsible for the cytotoxic activity of avocado seeds. Nur et al. (2017) reported that triterpenoid isolated from

avocado seed inhibits breast cancer cells (MCF7) and liver cancer cells (Hep-G2) growth with IC₅₀ values of 62 µg/mL and 12 µg/mL, respectively. Unsaturated fatty acids in n-hexane fraction of the seeds have cytotoxic activity against colon cancer cells (Caco-2) with an IC₅₀ value of 28 µg/mL (Lara-Márquez et al., 2020).

Several other studies have also revealed the cytotoxic activity of avocado seeds against various cancer cells. Avocado seed extract has cytotoxic activity against prostate cancer cells (LNCaP), breast cancer cells (MCF7), colon cancer cells (HT29), and lung cancer cells (H1299) with IC₅₀ values of 42.15 µg/mL, 19.1 µg/mL, 67.6 µg/mL, and

Table 2. Inhibitory Effect of the n-Hexane Fraction of Avocado Seeds on MCF7 Cells

Incubation Period (Hours)	% Inhibition		
	0.04% DMSO	30 µg/mL Fraction	40 µg/mL Fraction
24	1.29	9.94	9.24
48	2.7	19.57	20.62
72	1.45	21.78	23.42

132.2 µg/mL, respectively (Dabas et al., 2019). The n-hexane fraction of avocado seeds at a concentration of 200 µg/mL inhibits 81±3% of lung cancer cells (A549) growth, while the dichloromethane fraction at the same concentration could inhibit the growth of gastric cancer cells (BGC823) of with inhibition rate of 75±2% (Vo et al., 2019). Chloroform extract of avocado seed was able to inhibit the growth of MCF7 cells with IC₅₀ value of 94.87 µg/mL (Widiastuti et al., 2018). Alkhalaf et al. (2019) showed that avocado seed extract inhibits the growth of liver cancer (HepG2) and colon cancer (HCT116) cells with lower IC₅₀ values than avocado extract.

The cytotoxic activity of the n-hexane fraction of avocado seeds on MCF7 cells generally depends on its bioactive ability to inhibit the proliferation process and induce apoptosis through various possible mechanisms of action. To determine the possible mechanism of the proliferation inhibition, further testing of cell proliferation kinetics and apoptosis observation was performed using the double staining method with propidium iodide-acridine orange (Maryati et al., 2019).

Anti-proliferative Activity

The purpose of the anti-proliferative test was to investigate the sample's ability to prolong the doubling time of cancer cells. Doubling time is the time required for cells to double in number (Mehrara et al., 2007). Compounds that can delay cell multiplication time are thought to be able to inhibit genes or proteins that regulate cell cycle. MCF7 cells were treated with the n-hexane fraction of avocado seeds with concentrations of 40 and 30 µg/mL, which are lower than the IC₅₀ value

(47.87±1.5 µg/mL). This was done to prevent excessive cell death during the incubation period (Maryati et al., 2019). Observations of the cell death were carried out at incubation times of 24, 48, and 72 hours. The results of the anti-proliferation test showed that the n-hexane fraction of avocado seeds inhibits the proliferation of MCF7 cells (Table 2).

The proliferation inhibition activity was directly proportional to the concentration of the fraction tested. The n-hexane fraction of avocado seeds with concentrations of 40 and 30 µg/mL had doubling time of 77.41 and 76.54 hours, respectively. These results were significantly different (p<0.05) to the doubling time of control MCF7 cells, which had doubling time of 29.01 hours. These findings suggest that administering the n-hexane fraction of avocado seeds at concentrations lower than the IC₅₀ value causes cell cycle arrest.

These results were in line with previous studies which revealed that avocado seeds have anti-proliferative activity on cancer cells. Aliphatic acetogenin compounds isolated from avocado are able to inhibit the proliferation of oral cancer cells through inhibition mechanism of phosphorylation of EGFR (Epidermal Growth Factor Receptors), ERK 1/2 (Extracellular-Signal-regulated-Kinase 1/2), and c-Raf (Ser 338) in cancer cell growth process (D'Ambrosio et al., 2011). Treatment of avocado seed extract with a concentration of 42.5 µg/mL to prostate cancer cells (LNCaP) for 12 hours induces cell cycle arrest in the sub G0/G1 phase. The extract reduces the expression of Cyclin D1 and Cyclin E2 without causing changes in Cyclin A (Dabas et al., 2013). Cyclin D1 and Cyclin E2 are protein kinases that regulate cell

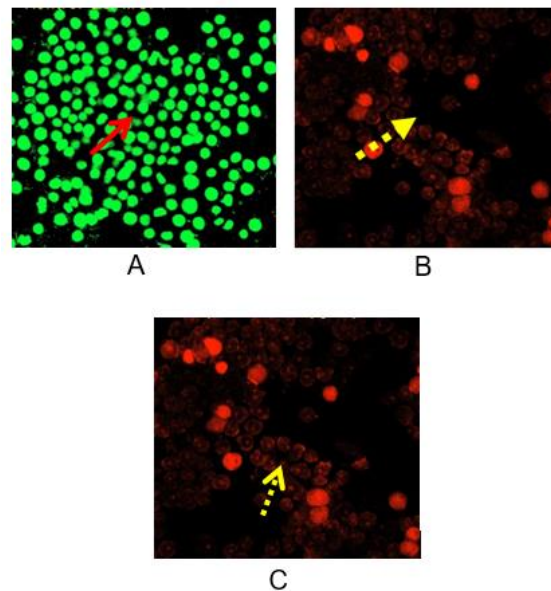


Figure 3. Morphology of MCF7 cells after double staining using propidium iodide-acridine orange on control cells (A), treated cells with the n-hexane fraction (40 µg/ml) (B) and n-hexane fraction (20 µg/ml) (C). In the control cells, the living cells are green. Treated cells with the n-hexane fraction underwent apoptosis (red fluorescence) and DNA fragmentation was seen. (→) normal cell, (→) cells that are likely to be apoptotic.

proliferation processes, which work by sending internal signal transduction at each phase in the cell cycle (Malumbres & Barbacid, 2007).

Apoptosis Observation by Double Staining

The purpose of the double staining test was to investigate the ability of the n-hexane fraction of avocado seeds to induce apoptosis in MCF7 cells through visual observation by the staining method using a mixture of acridine orange and propidium iodide. The mechanism of apoptosis induction is a big concern in cancer therapy research because with this mechanism, cancer cells will initiate their own suicide without causing harm to other normal cells (Wong, 2011). The double staining test results showed that the cells, which were treated with the n-hexane fraction at concentrations of 40 µg/mL and 20 µg/mL, showed red fluorescence whereas untreated cells showed green fluorescence (Figure 3). The red fluorescence in the cells indicated that the cells underwent apoptosis, which could be induced by the n-hexane fraction of avocado seed. The cells might experience membrane

blebbing that results in red fluorescence. These results are consistent with several characteristics of cells undergoing apoptosis, such as the formation of membrane blebbing, cell size shrinkage, organelle fragmentation, and nuclear condensation (Bjelaković et al., 2005).

Acridine orange can be absorbed by living and dead cells. Acridine orange will bind to double-stranded DNA in living cells, producing a green fluorescence, whereas in dead cells, acridine orange will bind to single-stranded DNA, producing an orange fluorescence. Propidium iodide interacts with cell nucleic acids to form a red fluorescence. The dye can only be absorbed by cells undergoing membrane blebbing. The concentration of colouring mixture used was 0.05% (Foglieni et al., 2001).

The ability of avocado seeds to induce apoptosis on cancer cells could occur through various mechanisms of action. The methanol fraction of chloroform extract of avocado seed was known to increase the population of MCF7 cells in the sub G1 phase by 21.9%

compared to control cells (3.7%). These results indicate the induction of apoptosis in MCF7 cells (Widiastuti et al., 2018). Velez-Pardo et al. (2014) reported that avocado peel extract, avocado seed extract, and avocado leaf extract could induce apoptosis in lymphoblastic leukaemia cells. The induction of apoptosis was mediated by mitochondrial membrane depolarization (52-87%) resulting in oxidative stress-dependent, activation of the transcription factor p53 (6.3-25.4%), increased protease caspase-3, and increased (AIF) Apoptosis Inducing Factor (20.6-36 %). Another study reported that avocado lipid extract could increase the expression of caspase 8 and caspase 9 in colon cancer cells (Caco-2) (Lara-Márquez et al., 2020).

Administration of avocado seed extract to prostate cancer cells (LNCaP) was reported to increase the cleavage of caspase-3 and PARP proteins, as well as decrease the translocation of NFkB into cells by more than 50%. A decrease in the expression or translocation of NFkB into cells indicates a decrease in cancer cell defence (Dabas et al., 2019). Various mechanisms of apoptosis induction by avocado plants against cancer cells can be an initial description of the mechanism of apoptosis induction possessed by the n-hexane fraction of avocado seeds. The process of induction of apoptosis by the n-hexane fraction of avocado seeds may occur through inhibition of proliferative signals or through inhibition of cell cycle progression, by inhibiting proto-oncogenes such as CycD or cdk4/6. Apoptosis could also be caused by changing the expression of proteins that control apoptosis, such as p53, Bax, Bcl-2,

NOXA, and so on (Velez-pardo, 2014). The potential of the n-hexane fraction of avocado seeds as an anticancer agent needs to be explored further to ascertain the molecular mechanism of the anticancer activity.

Selectivity Test

The selectivity test aimed to obtain an initial description of the toxicity of the n-hexane fraction of avocado seeds to normal cells. The selectivity test was carried out by comparing the IC₅₀ value of the sample on normal cells to those on cancer cells. The normal cells used were Vero cells, which are normal cells cultured from an African Green Monkey kidney cells (Ammerman et al., 2009). The SI values of n-hexane fraction of avocado seed was calculated from the cytotoxicity of fraction against normal cells (Vero cells) and MCF-7, which were 226.5±21 µg/mL and 47.48±1.5 µg/mL, respectively. Selectivity Index value was 4.73, indicating a highly selective cytotoxic effect of the n-hexane fraction of avocado seed against MCF-7.

The n-hexane fraction of avocado seeds can be a selective anti-cancer agent, which only works on the cancer cells and does not harm the normal cells. In general, agents that are used to treat cancer also interferes with the normal cells, causing adverse side effects such as baldness, digestive disorders, anaemia, and sexual dysfunction (Carvalho et al., 2009).

CONCLUSIONS

N-hexane fraction of avocado seeds has selective cytotoxic effect and could induces apoptosis of MCF7 cells.

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