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*Original Article*

# Cytotoxicity and apoptosis activities of passion fruit (*Passiflora edulis* Sims) seed extract on HSC-2, HSC-3, MCF-7, and HaCaT cell lines

[Citotoxicidad y actividades apoptóticas del extracto de semilla de fruta de la pasión (*Passiflora edulis* Sims) en las líneas celulares HSC-2, HSC-3, MCF-7 y HaCaT]

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## Abstract

*Context*: Passion fruit (*Passiflora edulis* Sims) is a well-known plant in the tropical islands, especially in North Sumatra, Indonesia, because of its sweet and refreshing taste. Although the fruit is often used as a syrup and sweetener, the efficacy of *P. edulis* fruit seed extract for anticancer activity has not been widely studied.

*Aims*: To evaluate the ability of *P. edulis* fruit seed extract to induce cytotoxicity and apoptosis in HSC-2, HSC-3, MCF-7, and HaCaT cell lines.

*Methods*: *P. edulis* fruit seed extract was extracted by using 96% ethanol. The cytotoxicity activity was measured by using an MTT assay for 48 hours. Apoptosis was examined using a TUNEL assay, and the morphology of the apoptosis bodies was analyzed using electron microscopy.

*Results*: The $IC_{50}$ values of *P. edulis* fruit seed extract were 572.79 µg/mL and 439.54 µg/mL for HSC-3 and MCF-7, respectively. The seed did not show cytotoxic activity on the HSC-2 cell line, but it induced proliferation of the HaCaT cell line in the maximum dose. The *P. edulis* fruit seed extract induced apoptosis activity in HSC-3, MCF-7, and HaCaT cell lines. Most DNA fragments were found in the cytosol of HSC-3 cells and the nucleus of MCF-7 cells.

*Conclusions*: The *P. edulis* fruit seed extract has cytotoxicity and apoptosis activities in oral cancer (HSC-3 cells) and breast cancer (MCF-7 cells), as well as being safe in normal human keratinocytes (HaCaT cell line). The *P. edulis* fruit seed extract can be considered an adjuvant agent in cancer treatment.

*Keywords*: apoptosis; breast cancer; cytotoxicity; oral cancer; passion fruit seed.

## Resumen

*Contexto*: La fruta de la pasión (*Passiflora edulis* Sims) es una planta muy conocida en las islas tropicales, especialmente en el norte de Sumatra, Indonesia, por su sabor dulce y refrescante. Aunque el fruto se utiliza a menudo como jarabe y edulcorante, la eficacia del extracto de semilla del fruto de *P. edulis* para la actividad anticancerígena no se ha estudiado ampliamente.

*Objetivos*: Evaluar la capacidad del extracto de semilla de *P. edulis* para inducir citotoxicidad y apoptosis en las líneas celulares HSC-2, HSC-3, MCF-7 y HaCaT.

*Métodos:* El extracto de semilla de *P. edulis* se extrajo utilizando etanol al 96%. La actividad citotóxica se midió mediante un ensayo MTT durante 48 horas. La apoptosis se examinó mediante un ensayo TUNEL, y la morfología de los cuerpos de apoptosis se analizó mediante microscopía electrónica.

*Resultados*: Los valores $IC_{50}$ del extracto de semilla del fruto de *P. edulis* fueron 572,79 µg/mL y 439,54 µg/mL para HSC-3 y MCF-7, respectivamente. La semilla no mostró actividad citotóxica en la línea celular HSC-2, pero indujo la proliferación de la línea celular HaCaT en la dosis máxima. El extracto de semilla del fruto de *P. edulis* indujo la actividad de apoptosis en las líneas celulares HSC-3, MCF-7 y HaCaT. La mayoría de los fragmentos de ADN se encontraron en el citosol de las células HSC-3 y en el núcleo de las células MCF-7.

*Conclusiones*: El extracto de semilla de fruto de *P. edulis* tiene actividades de citotoxicidad y apoptosis en cáncer oral (células HSC-3) y cáncer de mama (células MCF-7), además de ser seguro en queratinocitos humanos normales (línea celular HaCaT). El extracto de semilla del fruto de *P. edulis* puede considerarse un agente adyuvante en el tratamiento del cáncer.

*Palabras Clave*: apoptosis; cáncer de mama; citotoxicidad; cáncer oral; semilla de fruta de la pasión.

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## INTRODUCTION

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 (Ferlay et al., 2020). As the second major cause of mortality in the US, cancer is a serious global public health issue. Oral squamous cell carcinoma (OSCC), which ranks 16th globally, is the most frequent type of cancer in head and neck cancer. It is listed as one of the most aggressive malignant tumors because of its propensity to spread and high recidivism rate (Chamoli et al., 2021). It has been demonstrated that surgical resection, radiotherapy and/or chemotherapy, and nanotechnologies provide a major clinical benefit, particularly in cases with locally advanced OSCC (Imbesi Bellantoni et al., 2023). The goals of these treatments reveal beneficial anticancer activity in terms of angiogenesis activity inhibition, recurrence risk, and adverse effect reduction. However, many side effects caused by these treatments are unavoidable including mucositis, xerostomia, trismus, and osteoradionecrosis which may cause disturbances for patients' survival and quality of life (Rezazadeh et al., 2023). Apart from OSCC, breast cancer is also known as the fifth leading cause of cancer mortality worldwide, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases (Sung et al., 2021). The permanent side effects of breast cancer treatment, such as lymphedema and neuropathy, are also increased in morbidity rates (Nordin et al., 2018; Tommasi et al., 2022). Many drugs depend on bioactive compounds, some of which have anticancer properties. This has prompted many studies to explore alternative therapies, especially those derived from herbal ingredients, to overcome this problem (Ali et al., 2023). All this research aims to increase the survival rate and reduce the side effects of cancer treatments. Currently, the pharmaceutical industry is targeting the autophagy-apoptosis signaling cascade by using plant phytochemical compounds to modify modern cancer treatments with natural phytocompound-based anticancer agents (Yin et al., 2013).

Many plants that grow in Indonesia have potential as medicines. Currently, the development of anticancer herbal medicines is aimed at inducing apoptosis in cancer cells optimally without damaging normal cells, so as to minimize the side effects of drugs. One of the popular plants is passion fruit (*Passiflora edulis* Sims; *Passifloraceae).* It is known as a sweet fruit that grows in tropical and subtropical areas, especially in Brazil, the Caribbean, Central and South America, Mexico, South Florida, South Africa, and many countries in Asia (Barbosa Santos et al., 2021; Gadioli et al., 2018). In Indonesia, the most common distribution of *P. edulis* fruit is found in the North Sumatra area, and it has become one of the most popular products, such as fresh juice, syrup, and sweetener. The fruit contains phytochemical content such as phenols, carotenoids, flavonoids, tannins, and saponins (Ramos et al., 2018). This fruit contains vitamins A and C, potassium, magnesium, calcium, iron, fibers, and organic acids (Ramaiya et al., 2019; Viera et al., 2022). *P. edulis* fruit is divided into three parts, namely rind, pulp, and seed. The seed is a part of the *P. edulis* fruit, which has good antioxidant potential because it contains piceatannol (trans-3,4,30,50-tetrahydroxy-stilbene) which can inhibit melanogenesis and promote collagen synthesis (Matsui et al., 2010). The phytochemicals in *P. edulis* fruit are natural analogs of resveratrol, which can also be found in red wine, grapes, and sugar cane. Several biological actions have been demonstrated for piceatannol, including immunomodulatory, anti-inflammatory, antiproliferative, and anti-cancer properties (He et al., 2020). Currently, previous studies have reported the effects of *P. edulis* fruit seed extract in inhibiting the proliferation of lungs and colorectal cancer cells and also act as an antiaging agent because it can stimulate skin collagenase and tyrosinase (Matsui et al., 2010; Yamamoto et al., 2019). Previous research using leaves and juices of *P. edulis* fruit was also carried out on liver cancer (HepG2 cell) and colon adenocarcinoma and showed satisfactory results (Aguillón et al., 2018; Ramos et al., 2018). The use of the seeds has not been widely explored, and this has prompted researchers to investigate the effects of *P. edulis* fruit seed extract further. *P. edulis* fruit seed extract has been shown to be an excellent source of antioxidants as it contains phenolic compounds, vit C, total carotenoids, flavonoids, tannins, saponins, piceatannol, and organic acids, which makes it a potential seed for cancer treatment.

Therefore, research of innovation in *P. edulis* fruit seed extract needs to be developed with various possible mechanisms and cell targets. Although several studies have investigated the effect of *P. edulis* fruit as an immunomodulator, antiproliferative, anti-inflammatory, antiangiogenic, anticancer, and antiaging activities, the effect of *P. edulis* fruit seed extract from North Sumatra, Indonesia, on anticancer activity has not been explored. This study aimed to determine the cytotoxicity and apoptosis activities of *P. edulis* fruit seed extract on oral carcinoma, breast cancer, and normal keratinocyte cell lines.

## MATERIAL AND METHODS

### Plant material

Sample material was taken from the plantation in Brastagi, North Sumatra, Indonesia ( GPS coordinates 3.1853 °N latitude and 98.5049 °E longitude). The stems, leaves, roots, flowers, and seeds were determined by Dr. Sunaryo in the Botanical Division of Biological Research Center LIPI Cibinong, Bogor, Indonesia, in 2015. Authentication number 440/IPH.1.01/lf.07/IV/2015.

### Extraction

Two kilograms of ripe *P. edulis* fruit, which had a yellow color, were picked from the Brastagi farm, and then the seeds were taken and separated from the rind. The seeds were then cleaned from the juicy layer that covered them under running water and dried in the open air. Afterward, it was dried in a 50°C oven. The dried material was then crushed with a blender to become powder and soaked in 96% ethanol. This ethanol solution was evaporated at a temperature of 20-25°C using a rotary evaporator for three days. Dry extract was stored and protected from direct exposure to light at 5°C until use.

### Cisplatin preparation

This study used 10 µg/mL cisplatin injection (Pfizer, Ltd.) as the positive or reference control.

### Cell line culture

The experiments were carried out with HSC-2 and HSC-3 (human oral squamous carcinoma cell lines), MCF-7 (human breast cancer cell lines), and HaCaT (immortalized human keratinocyte cell lines). HSC-2 (JCRB0622), HSC-3 (JCRB0623), and HaCaT cell lines used in this study were given from the Section of Molecular Embryology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University. The MCF-7 cell lines were provided at the Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo General Hospital Integrated Laboratory. Cell concentrations in the culture were adjusted to allow for exponential growth. A 10 mg/mL cisplatin stock solution was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, D2650) just before the experimental assay. The concentration of the *P. edulis* fruit seed extract solution used in this study was 200, 250, 300, 500, 600, and 800 µg/mL. This study used 0.01 g of *P. edulis* fruit seed extract dry powder and was dissolved in Dulbecco's Eagle medium (DMEM, Gibco, No. 11965-092, Life Technologies, California, USA) to produce 10 000 µg/mL. The supplementation given to DMEM consisted of 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (10 000 U/mL, and 25 mg/mL Fungizone® antimycotic (Gibco®). The solution was used for stock with 1 mL *P. edulis* fruit seed extract which was derived from 10 000 µg/mL, then 9 mL of complete DMEM was added to produce a stock solution of 1000 µg/mL. The solution was then filtered using a filter (Minisart® NML Syringe Filter) and put into a 15 mL tube. This stock solution was made before the assay was carried out. The cells were exposed to different concentrations (200-800 µg/mL) for 48 h.

### Viability assay

Cell viability was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction (MTT) assay, which aimed to assess the mitochondrial activity of the cells, which involves reaction with NADH or similar reducing molecules that transfer electrons to MTT. The MTT reagent was converted into a purple-colored formazan product with a maximum absorbance near 570 nm by viable cells with active metabolism. Cell cultures were reached and counted with a hemocytometer to obtain a concentration of $2 \times 10^4$ cells/100 µL and put into 96-well plates and later into the incubator for two hours. The MTT reagent (CellTiter 96® Aqueous One Solution Reagent) was thawed for 90 minutes at room temperature and then mixed with the electron coupling reagent phenazine methosulfate (PMS) in a ratio of 20:1. The solution was covered with aluminum foil and left for 2 hours. The 20 µL MTT reagent solution was put into each well, which was filled with $2 \times 10^4$ cells/100 µL in a culture medium. A $CO_2$ 5% incubator incubated the plates at 37°C for four hours to complete the solubilization of formazan crystals and added 100 µL DMSO into each well after incubation. The absorbance was measured at a wavelength of 490 nm using a microplate reader (Multiskan Go, USA). These assays were carried out with three replicates of cell and media control. By using the absorbance data obtained from measurements, the percentage of cell viability was calculated in relation to nontreated cells (assumed as 100% value). The relationship between the concentration of the extract test solution and the inhibition of the cells was displayed in graphical form to determine the $IC_{50}$ value of the extract solution to see the living cells. The $IC_{50}$ values were calculated as the concentration of extract that inhibited 50% of cell viability after exposure time. After calculating the $IC_{50}$ value against cancer cells, the selectivity index against non-malignant (HaCaT) cells was calculated. The selectivity index (SI) was defined as the ratio of the $IC_{50}$ value observed in tumor cell lines to the $IC_{50}$ value observed in the non-malignant cells. The SI value indicates the sample's selectivity to the cell lines

tested. Samples with a SI>3 were considered highly selective for cancerous cells (Yahuafai et al., 2023).

### TUNEL assay

The TUNEL assay used a commercially available kit (In Situ Cell Death Detection Kit, fluorescein, TMR Red, 12156792910 Roche, Mannheim, Germany) to quantify the number of apoptosis cells and DNA fragmentation. A suspension containing 5 × $10^4$ cells/well was inoculated into a slide chamber (Nunc Lab-Tek Chamber Slide System 171080, Thermo Fisher Scientific) and incubated for 48 hours. Data was obtained from two independent culture batches. The cell groups used were HSC-3, MCF-7, HaCaT, positive control with cisplatin, and TUNEL positive control using DNAse I recombinant (30 U/mL in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mg/mL BSA) to induce DNA strand breaks. The cell incubation was carried out for 48 hours. The TUNEL assay test protocol followed the instructions for the *in situ* cell detection kit. The manufacture of cell suspension began with incubating cells before fixation and permeabilization of the cell. The sample test was washed 3 times in PBS and adjusted to 5 × $10^4$ cells/mL. A 100 µL/well cell suspension was transferred into a 96-well microplate. Added 100 µL/well of a freshly prepared fixation solution to the cell suspension, resuspend completely and incubate for 60 minutes at 15 to 25°C. To avoid extensive clumping of cells, the microplate on a shaker during fixation was incubated. The microplate was centrifuged at 300 × *g* for 10 minutes, and the fixative was removed by flicking off or suctioning. Cells were washed once with 200 µL/well PBS and the microplate at 300 × *g* for 10 minutes, centrifuged and removed PBS by flicking off or suction. Cells in 100 µL/well with permeabilization solution were resuspended for 2 minutes on ice at 2 to 8°C. For the labeling protocol cell suspensions, cells were washed twice with 200 µL/well PBS and resuspended in a 50 µL/well TUNEL reaction mixture. For the negative control, 50 µL Label Solution each was added. The lid was also added and incubated for 60 minutes at 37°C in a humidified atmosphere in the dark. Then, samples were washed twice in PBS, and the cells were transferred into a tube to a final volume of 250 to 500 µL in PBS. The samples were analyzed directly under a fluorescence microscope with an excitation wavelength of 450 to 500 nm and a detection wavelength of 515 to 565 nm. Cells that undergo apoptosis were fluoresced red (TMR red), which means a positive signal of fragmentation of genetic material, while cells that do not undergo apoptosis were fluoresced blue or 4′,6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA. The proportion of the genetic material that was broken during apoptosis in each cell line was divided into three locations, specifically in the nucleus, cytosol, and both.

### Statistical analysis

Descriptive analysis data was presented as mean and standard deviation. The determination of $IC_{50}$ concentration was performed using a linear regression equation from log concentration, and the final results formed a cytotoxicity curve. To confirm the effects of PFSE on the apoptotic activity of the cancer cell lines, data were analyzed with one-way ANOVA followed by Post Hoc test LSD for HSC-3 cell lines, Kruskal Wallis for MCF-7, and HaCaT cell lines. Significance was determined at $p<0.05$.

## RESULTS

### The effect of *P. edulis* fruit seed extract in inhibiting the proliferation of HSC-2, HSC-3, MCF-7, and HaCaT cell lines

The cytotoxicity assay aims to calculate the number of cell viability based on the mitochondrial activity of cell cultures. The study examined the effect of *P. edulis* fruit seed extract in inhibiting the proliferation of HSC-3, HSC-2, MCF-7, and HaCaT cell lines. As seen in Fig. 1, the $IC_{50}$ value of *P. edulis* fruit seed extract on HSC-2 cells could not be determined because the extract was unable to kill cells by up to 50%. The HSC-2 cells were still viable up to 71% with the highest dose of *P. edulis* fruit seed extract (800 µg/mL). The results also demonstrated that HSC-3 and MCF-7 cells decreased by 50% of the number of viable cells so that the $IC_{50}$ of HSC-3 and MCF-7 are 572.79 ± 17.29 and 439.59 ± 17.81 µg/mL, respectively (Table 1, Fig. 1). As the non-malignant cells were almost unaffected by the extract, to calculate the selectivity index, we assumed that for these cells, the $IC_{50}$ was reached at the maximum concentration evaluated (800 µg/mL). From this assumption, the selectivity index for HSC-2 and MCF-7 cell lines must be values above 1.3 and 1.8, respectively.

Cisplatin induced a significant decrease in viability in all cell line groups. The percentage of the viability of all cell lines was below 20% of total cells after exposure to cisplatin. These results showed that cisplatin was not only capable of inducing cellular death in cancer cells but also toxic to keratinocyte cells. This study used HaCaT cells to compare the effects of the extract on human keratinocyte cells. The study reported an increase in HaCaT cell viability up to 0.74% of the total number of living cells. This research aimed to observe whether the extract was cytotoxic to normal cells. The result showed that the extract did not cause cytotoxicity in HaCaT cells (Fig. 1).

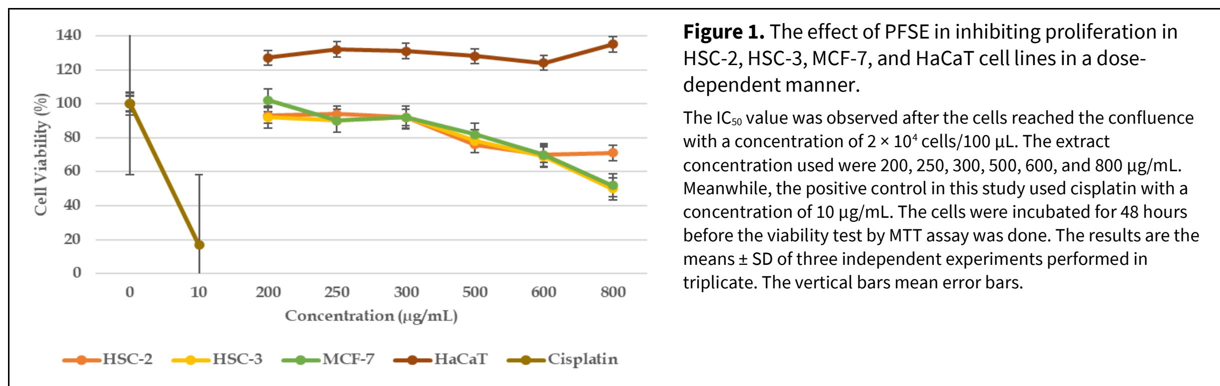


**Figure 1.** The effect of PFSE in inhibiting proliferation in HSC-2, HSC-3, MCF-7, and HaCaT cell lines in a dose-dependent manner.

The $IC_{50}$ value was observed after the cells reached the confluence with a concentration of $2 \times 10^4$ cells/100 µL. The extract concentration used were 200, 250, 300, 500, 600, and 800 µg/mL. Meanwhile, the positive control in this study used cisplatin with a concentration of 10 µg/mL. The cells were incubated for 48 hours before the viability test by MTT assay was done. The results are the means ± SD of three independent experiments performed in triplicate. The vertical bars mean error bars.

**Table 1.** Cytotoxicity evaluation of *P. edulis* fruit seed extract in different cells.

| Cell line | Origin | MTT assay $IC_{50}$ (µg/mL) (SI) |
|---|---|---|
| HaCaT | Human keratinocyte | >800 |
| HSC-2 | Human oral squamous cell carcinoma | >800 |
| HSC-3 | Human tongue squamous cell carcinoma | 572.79 (>1.3) |
| MCF-7 | Breast carcinoma | 439.59 (>1.8) |

Selectivity index (SI) was calculated by dividing the $IC_{50}$ value into normal cells by the $IC_{50}$ value on cancer cells. The SI value indicates the sample selectivity of the cell lines tested.

This study could not determine the $IC_{50}$ value because there was no cell death up to 50% of the total number of living cells. The viability assay on HaCaT cells proved that the *P. edulis* fruit seed extract was relatively safe when used on normal, non-cancerous cells. The cell cytotoxicity caused changes in the shape and structure of the cells after 48 hours. The extract administration to HSC-2, HSC-3, MCF-7, and HaCaT cells showed the morphological features of the early apoptosis process, such as rounded and big, swelling, granular changes, and separation of attachments between cells followed by a decrease in cell numbers. Some cells appeared to detach or float from the growth medium.

## The effect of *P. edulis* fruit seed extract in apoptotic activities in HSC-3, MCF-7, and HaCaT cell lines

The TUNEL assay was conducted to investigate the ability of PFSE to induce apoptosis through the assessment of the cleavage of genomic DNA or DNA damage in a population of cells. The treatment given used a TUNEL *in vitro* positive control, cisplatin as a positive control, the group without treatment as a negative control, and *P. edulis* fruit seed extract. The HSC-3, MCF-7, and HaCaT cell lines were carried out in the TUNEL assay. Meanwhile, this TUNEL assay did not continue using HSC-2 cells because the cell condition was fragile and the ability to replicate was decreased, making it difficult to achieve 100% confluency when the TUNEL assay was ready to be carried out. The doses used were the $IC_{50}$ for HSC-3 and MCF-7 (572.79 ± 17.29 and 439.59 ± 17.81 µg/mL, respectively), whereas, for HaCaT cells, the maximum dose was used (800 µg/mL).

The result of the TUNEL assay on HSC-3 cells showed a significant difference between the TUNEL positive control with cisplatin, *P. edulis* fruit seed extract treatment, and the negative control groups ($p<0.05$), as seen in Fig. 2. The seed extract caused cell apoptosis up to 68.65 ± 23.67%, and this result was 13.96% different from treatment with cisplatin. In the MCF-7 cell line, the exposure of the extract also induced an apoptotic effect in 29.66 ± 35.58%. This result explained the ability of *P. edulis* fruit seed extract to work optimally in various types of cancer cells. In the HaCaT cell lines, the extract caused 11.15 ± 6.54% apoptosis. Although the number of cells undergoing apoptosis was small, this assay was not in line with the results of the MTT assay, where the extract could not induce cellular death, but it turns out that the *P. edulis* fruit seed extract was able to increase the HaCaT cells proliferation above 100% of total viable cells. In the treatment group with cisplatin, it was seen that this drug caused apoptosis in HaCaT cells.

## The proportion of genetic material fragments in HSC-3, MCF-7, and HaCaT cell lines after being exposed to *P. edulis* fruit seed extract

The result of observing apoptotic activity was done through immunofluorescence microscopy, and

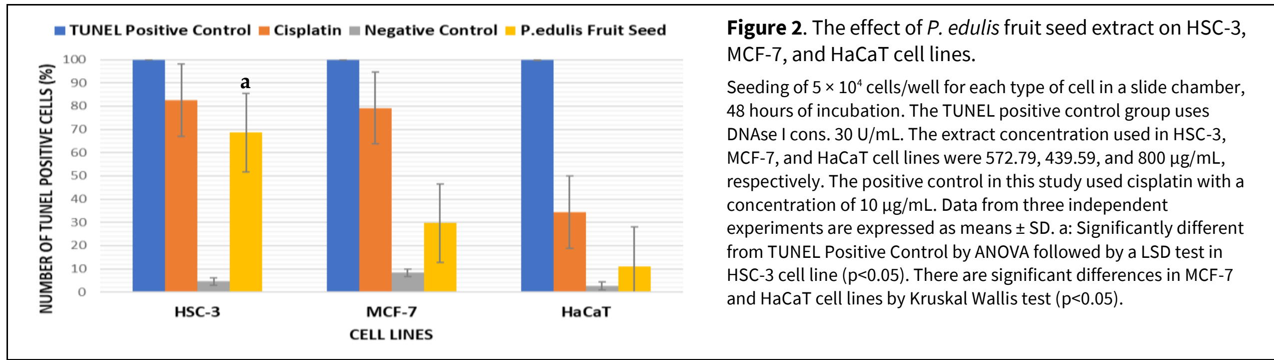


**Figure 2**. The effect of *P. edulis* fruit seed extract on HSC-3, MCF-7, and HaCaT cell lines.

Seeding of 5 × $10^4$ cells/well for each type of cell in a slide chamber, 48 hours of incubation. The TUNEL positive control group uses DNAse I cons. 30 U/mL. The extract concentration used in HSC-3, MCF-7, and HaCaT cell lines were 572.79, 439.59, and 800 µg/mL, respectively. The positive control in this study used cisplatin with a concentration of 10 µg/mL. Data from three independent experiments are expressed as means ± SD. a: Significantly different from TUNEL Positive Control by ANOVA followed by a LSD test in HSC-3 cell line ($p<0.05$). There are significant differences in MCF-7 and HaCaT cell lines by Kruskal Wallis test ($p<0.05$).

**Table 2**. Descriptive analysis of the number of cells with fragments of genetic material based on TMR red luminescence.

| Cell lines | Group treatment | TUNEL positive proportion mean (%) | | |
|---|---|---|---|---|
| | | Nucleus | Cytosol | Nucleus and Cytosol |
| HSC-3 | TUNEL positive control | 100.00 ± 46.50 | 0 | 0 |
| | Negative control | 6.97 ± 8.72 | 19.92 ± 26.47 | 0.20 ± 0.45 |
| | Cisplatin | 1.67 ± 2.24 | 65.00 ± 20.70 | 25.71 ± 15.52 |
| | *P. edulis* fruit seed extract | 13.78 ± 12.82 | 34.44 ± 21.04 | 9.98 ± 7.32 |
| MCF-7 | TUNEL positive control | 100 ± 27.45 | 0 | 0 |
| | Negative control | 0 | 8.31 ± 19.05 | 0 |
| | Cisplatin | 10.20 ± 8.96 | 67.45 ± 25.02 | 1.57 ± 2.00 |
| | *P. edulis* fruit seed extract | 17.91 ± 35.88 | 11.19 ± 20.54 | 0.56 ± 0.89 |
| HaCaT | TUNEL positive control | 99.83 ± 55.48 | 0 | 0 |
| | Negative control | 2.66 ± 6.02 | 0 | 0 |
| | Cisplatin | 5.06 ± 6.54 | 11.67±17.26 | 17.7 ± 17.26 |
| | *P. edulis* fruit seed extract | 11.15 ± 6.54 | 0 | 0 |

Data are expressed as mean ± SD (n = 12).

positive fragmentation of genetic material was found with a TMR red stain and in the nucleus of cells stained with DAPI (blue). This study reported that in the group treated with TUNEL positive control, the fragments of material genetic of cellular DNA occurred in the nucleus. The signal color became purple-magenta when the two objects were located in the same coordinate of the field of view. In HSC-3 cells induced by *P. edulis* fruit seed extract, most of the cell fragmentation was found in the cell cytosol. In cisplatin treatment, all HSC-3 staining results showed a pattern similar to the treatment with *P. edulis* fruit seed extract, which was consistent in each repetition. In this pattern, it can be seen that both of them showed DNA fragmentation that occurred more in the cytosol, not in the cell nucleus. The average number of cells that experience genetic material breakdown during apoptosis can be seen in Table 2. The morphology of the HSC-3 apoptotic bodies is shown in Fig. 3.

The apoptotic cell observations were also carried out in the MCF-7 cells. In most of the cells induced by cisplatin, the fragmentation also occurred in the cytosol cell, as much as 67.45 ± 25.02%. In contrast, in the treatment with *P. edulis* fruit seed extract, the dominant apoptosis occurred in the nucleus (Table 2, Fig. 4). In the HaCaT cells group, the dominant genetic fragmentation occurred in the nucleus after being exposed to *P. edulis* fruit seed extract. A similar description was seen in the HaCaT cells with cisplatin treatment (Table 2, Fig. 5).

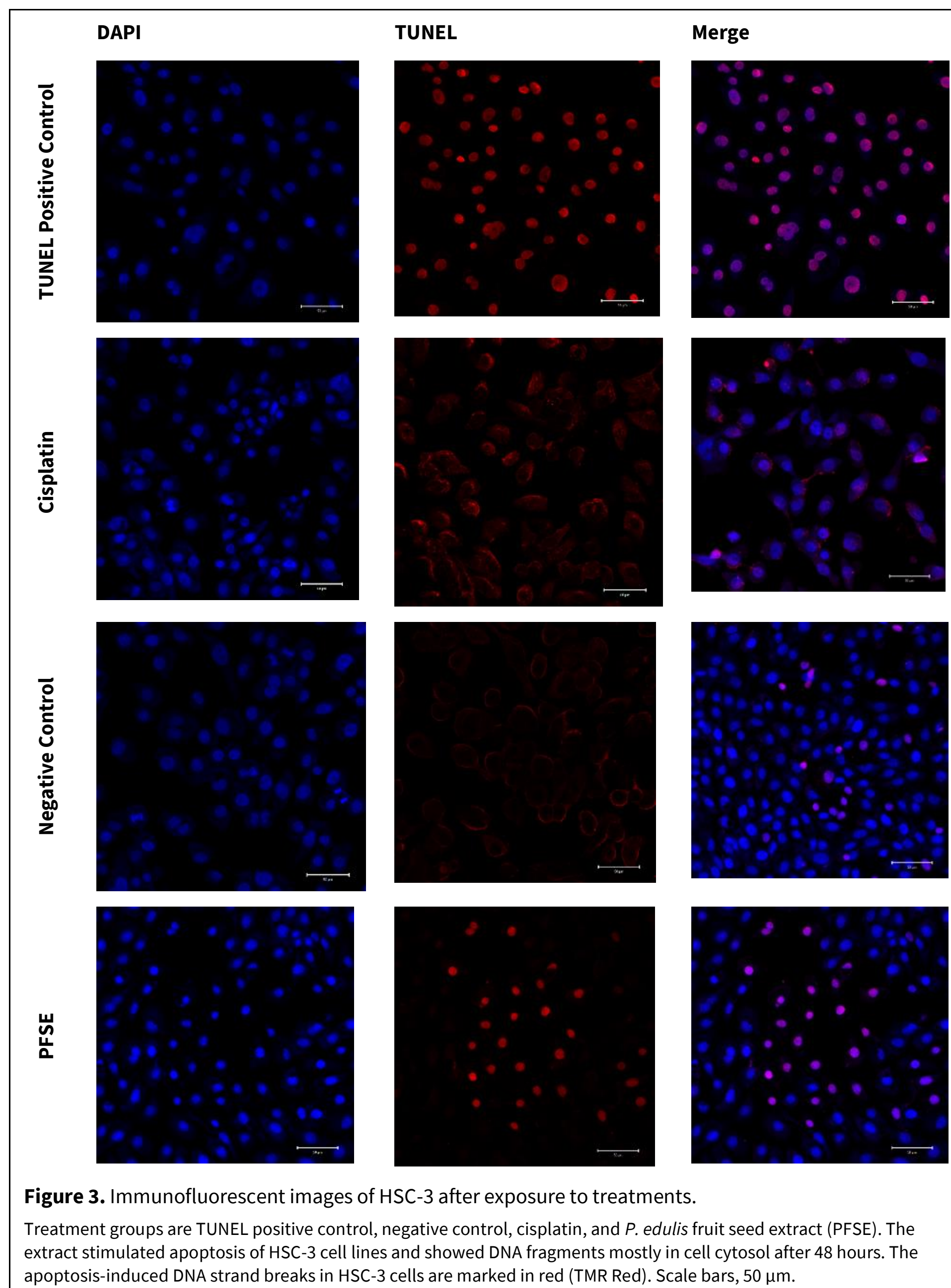


**Figure 3.** Immunofluorescent images of HSC-3 after exposure to treatments.

Treatment groups are TUNEL positive control, negative control, cisplatin, and *P. edulis* fruit seed extract (PFSE). The extract stimulated apoptosis of HSC-3 cell lines and showed DNA fragments mostly in cell cytosol after 48 hours. The apoptosis-induced DNA strand breaks in HSC-3 cells are marked in red (TMR Red). Scale bars, 50 µm.

## DISCUSSION

This study demonstrated that *P. edulis* fruit seed extract exhibited cytotoxic effects on HSC-3 and MCF-7 cell lines, but not on HSC-2 and HaCaT cell lines. The results are in line with several previous studies which showed that *P. edulis* fruit seed extract that contains piceatannol and scirpusin B was able to inhibit the development of cancer cells, including lung and colorectal epithelial cancer cells by inhibiting human glyoxalase I (GLO I) activity (Yamamoto et al., 2019). Piceatannol has anticancer and antiangiogenic activity by blocking vascular endothelial growth factor binding to its receptor (Matsui et al., 2010). Several other anticancer studies have shown that the leaves, seeds, and juice of *P. edulis* fruit can cause cytotoxicity in HepG2, MCF-7, SW480, SW620, Caco-2, CCRF-CEM, CEM/ ADR5000, and HCT116 cell lines (Aguillón et al., 2018; Mota et al., 2018; Ramirez et al., 2017). The anti-cancer property is due to *P. edulis* fruit being able to inhibit the matrix-metalloprotease MMP-2 and MMP-9 activities, which play a role in angiogenesis and inflammatory process (Puricelli et al., 2003). Another study also showed the same result as ours using methanol extract of *P. edulis* fruit. Kuete et al. (2016) reported that *P. edulis* fruit has cytotoxicity activity to drug-sensitive leukemia CCRF–CEM ($IC_{50}$ value below 1 µg/mL) and multidrug-resistant P-glycoprotein-over-expressing subline CEM/ADR 5000 cells ($IC_{50}$ value below 10 µg/mL). The antioxidant activity of flavonoids in plant extracts has an important role in cell cytotoxicity assay. Flavonoids

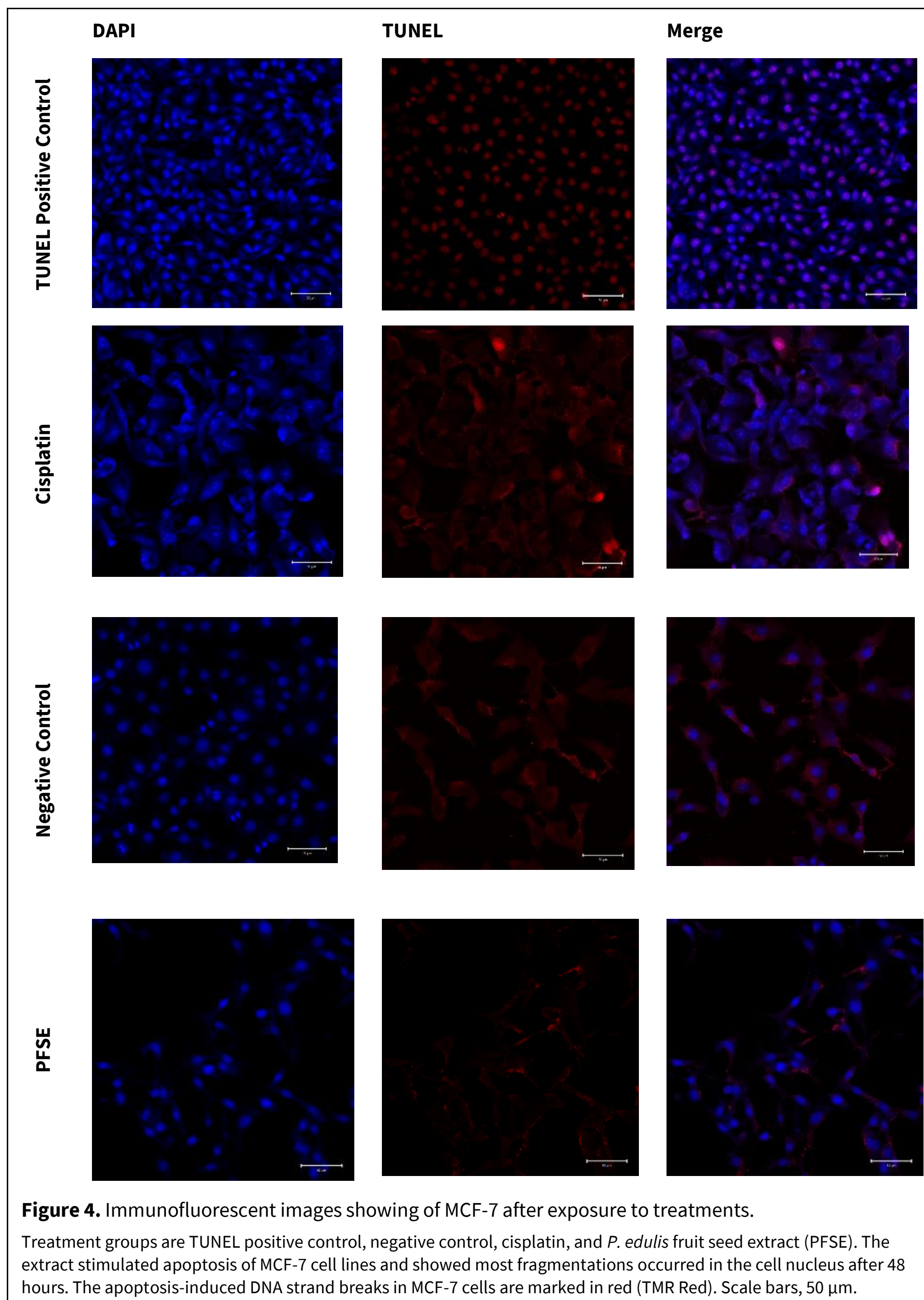


**Figure 4.** Immunofluorescent images showing of MCF-7 after exposure to treatments.

Treatment groups are TUNEL positive control, negative control, cisplatin, and *P. edulis* fruit seed extract (PFSE). The extract stimulated apoptosis of MCF-7 cell lines and showed most fragmentations occurred in the cell nucleus after 48 hours. The apoptosis-induced DNA strand breaks in MCF-7 cells are marked in red (TMR Red). Scale bars, 50 µm.

have a direct stimulatory effect on mitochondria, making them more efficient at producing energy and scavenging free radicals (Brusselmans et al., 2005).

The viability assay resulted in two different $IC_{50}$ values in the two types of cancer cells. These differences indicate that there is selective toxicity and different types of cell death roles in several cancer cell lines when exposed to natural or synthetic agents. Flavonoid compounds show weak cytotoxicity activity against HSC-2 cells, so the $IC_{50}$ values could not be determined in the study (Sakagami et al., 2007). The factors involved in this condition might be from the extract plant and the cells. Factors from compounds in plants that affect the ability of cytotoxicity are whether there are hydrophilic and hydrophobic in the same molecule, the presence or absence of isoprenyl groups, polycyclic and/or halogen structures, highly condensed (lower molecular weights are more cytotoxic), and lipophilicity. Factors within the cell that may influence this cytotoxicity are differences in the expression of proteins resistant to several anticancer agents and the expression of enzymes metabolizing drugs or herbal extracts (Sakagami et al., 2007). Environmental factors include serum type, presence of metal ions, oxygen concentration, and external pressure (Sakagami et al., 2007). The success of the cytotoxicity assay is highly dependent on the ability of cell

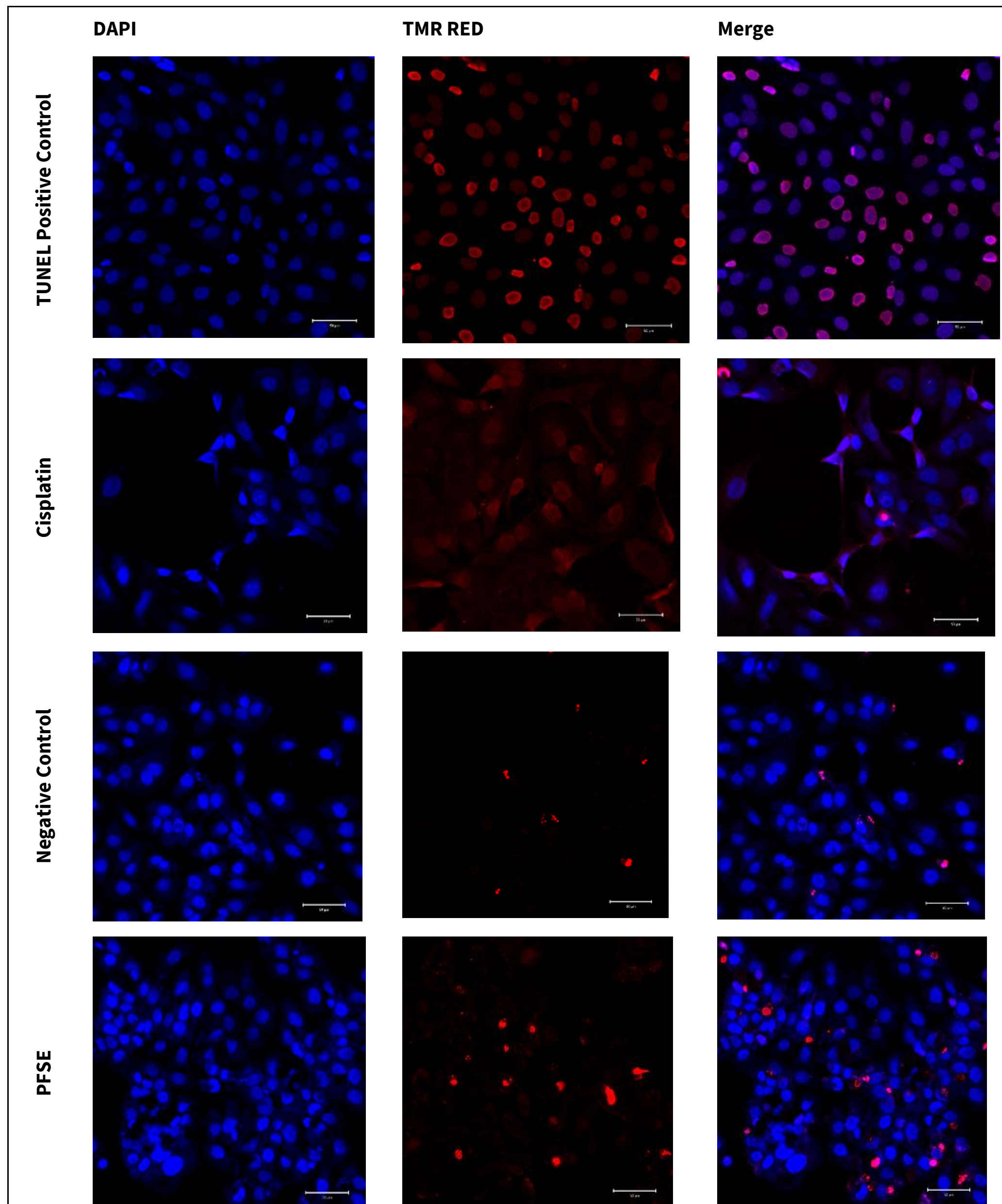


**Figure 5.** Immunofluorescent images of HaCaT cell line after exposure to treatments.

Treatment groups are TUNEL positive control, negative control, cisplatin, and *P. edulis* fruit seed extract (PFSE). The extract stimulated apoptosis of HaCaT cell lines after 48 hours with DNA fragment distribution in the cell nucleus. The apoptosis-induced DNA strand breaks in HaCaT cells are marked in red (TMR Red). Scale bars, 50 µm.

metabolism and respiration. Several previous studies have also found that growth inhibitory capacity in many plant extracts on several types of cancer cells generally also varies (Campoccia et al., 2021; Esmaeilbeig et al., 2015). This study also shows the morphological features of HSC-2 cells, which have a larger shape, more extensive cytoplasm, and tighter attachment between cells than HSC-3 cells. This might cause the extract's cytotoxicity to be weaker on HSC-2 cells, resulting in a higher viable cell number, so a larger dose of the extract might be needed to cause cellular toxicity. Chemotherapy is a primary therapeutic approach for treating cancer, although its selectivity against cancer cells is restricted. The SI represents an experimental chemical compound's (plant extract) ability to effectively cause apoptosis in a certain type of cell cancer while posing the least amount of harm to non-cancerous cells (Céspedes et al., 2023). The study noted that SI values above 1 were found after exposure to *P. edulis* fruit seed extract for HSC-3 and MCF-7. SI values higher than 1.0 indicate compounds of considerable anticancer specificity, and SI much greater than 1.0 indicates highly selective ones (Bartmańska et al., 2018).

For the TUNEL assay, the study used the maximum dose for HaCaT cells. This dose is still considered safe for further tests because it did not cause cell death. The dose (800 µg/mL) is lower than the previous studies using *P. edulis* fruit leaf extract at a dose of 1100 µg/mL, which is still safe to use in the experimental rats to cure colitis by decreasing the level of pro-inflammatory, especially by reducing IL-1β five-fold and TNF-α (Cazarin et al., 2015). The acute toxicity study for seven days in rats using the *P. edulis* fruit extract can also reach a maximum dose of 2000 mg/kg body weight (BW) and did not cause side effects on the bone marrow function, neither hepatotoxic nor nephrotoxic (Devaki et al., 2012). Another study reported that acute and sub-chronic toxicity studies using the *P. edulis* fruit seed extract itself are still relatively safe with a maximum dose of 5000 mg/kg BW. However, it was reported that the *P. edulis* fruit seed extract can cause an increase in ALT, ALP, AST, and GGT levels accompanied by distortion of hepatic architecture at a dose of 3000 mg/kg BW (Amedu et al., 2016).

In the viability assay, the HaCaT cells experienced an increase in the number of cells. The *P. edulis* fruit seed extract can induce normal human keratinocyte cell proliferation. This result is in line with previous research, which demonstrated that seed oil and nanoemulsion can induce the proliferation of dermal keratinocyte cells (de Souza et al., 2022). The oil from the seed has a high level of linoleic (omega 6) and oleic acids (omega 9), and the high one is 60% of its composition (Rana and Blazquez, 2008). Among polyunsaturated fatty acids, omega-6 and 9 are essential to maintain active hormone function and to improve cell function. Moreover, previous research has reported that these fatty acids have dermal regeneration activity on the skin (McCusker and Grant-Kels, 2010). Both of these acids work through a proliferative effect on cells and as precursors of inflammatory mediators responsible for tissue repair (Lin and Khnykin, 2014). This can benefit *P. edulis* fruit in treating cancer cells because it is not toxic to normal keratinocyte cells. However, apart from the possibility that *P. edulis* fruit seed extract can stimulate cell proliferation, this condition can also be caused by the high intensity of extracellular formazan resulting from the reduction of tetrazolium by dehydrogenase succinate enzyme in cells that carry out respiration and metabolism, so that the more energy and results of cellular respiration, the more formazan is formed.

The TUNEL assay aims to determine the effect of *P. edulis* fruit seed extract on apoptosis activity in cancer cells. The study used HSC-3, MCF-7, and HaCaT cell lines, and the optimum number of apoptosis cells was in HSC-3, followed by MCF-7 and HaCaT cells. This study is the first to report the effect of *P. edulis* fruit seed extract on OSCC and breast cancer. Although the studies of *P. edulis* fruit against oral and breast cancer have not been much reported; however, several previous studies using leaves and juices of *P. edulis* fruit have been used on liver, colon, and breast cancer, and colitis (Aguillón et al., 2018; Fotsing et al., 2023). These effects might be due to the antioxidant content derived from polyphenolic and polysaccharide content.

This study is the first report showing the effect of *P. edulis* fruit seed extract on OSCC cell lines. However, research on breast cancer cells has been done before and has given similar results that *P. edulis* fruit seed extract can induce apoptosis in MCF-7 cell lines and Ehrlich ascites carcinoma using crude and supercritical fluid *P. edulis* fruit seed extract (Mota et al., 2018). Another study reported that *P. edulis* fruit seed extract had a protective effect against beta-amyloid-induced neuronal cell death in differentiated human neuroblastoma, so it can be used as a treatment for neurodegenerative diseases such as Alzheimer's disease (Sato et al., 2022). The phytochemical content in seed, especially piceatannol and quercetin, likely contributes to the anticancer effect. The previous research reported that flavonoids such as quercetin, luteolin, kaempferol, apigenin, and taxifolin induced apoptosis in cancer cells, and it was proven strongly associated with their fatty acid synthase (FAS) inhibitory properties (Brusselmans et al., 2005). Quercetin can induce apoptosis and cell cycle arrest via upregulation of Bax expression and caspase-3, downregulation of Bcl-2 protein, and caspase cascade induced by a mitochondrial apoptotic pathway in human breast cancer (Abotaleb et al., 2019; Chou et al., 2010; Duo et al., 2012). Besides quercetin, piceatannol also has a major role in the anticancer activity of PFSE. The previous research reported that piceatannol can inhibit WM266-4 and A2058 cell growth and induce apoptosis by increasing the expression level of miR-181a to suppress melanoma growth (Du et al., 2017). Piceatannol can also induce apoptosis of osteosarcoma cells by regulating PI3K/AKT/mTOR pathway and bladder cancer cells through regulation of the PTEN/AKT signal pathway (Wang and Li, 2020).

Apoptosis is a programmed cell death mechanism that is expected to occur in chemotherapy treatment because it does not cause an inflammatory reaction, so the risk of side effects that are detrimental to the patient's body could be minimal. The anticancer drugs trigger apoptosis by inducing the expression of death receptor ligands or the release of cytochrome c from mitochondria. Cancer cells undergoing apoptosis show DNA breakage/ nuclear fragmentation both at an early and late stage of the apoptosis stage (Sa-

marghandian and Shabestari, 2013). TUNEL assay is used to calculate the cells that undergo extensive DNA degradation during the late stages of apoptosis. The method is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template (Mirzayans and Murray, 2020). The TUNEL assay is a gold standard for detecting cell death through apoptosis. However, the TUNEL staining is not always associated with apoptosis, and apoptosis is not always associated with cell demise. This study examined that *P. edulis* fruit seed extract induced more apoptosis in HSC-3 than MCF-7 cell lines. Most of the DNA fragments were found in the cytosol of HSC-3, whereas in MCF-7, most of the fragments were found in the cell nucleus.

Most DNA is located in the cell nucleus (nuclear DNA), but a small amount of DNA can also be found in the mitochondria, which is called mitochondrial DNA or mtDNA (Yasukawa and Kang, 2018). Although it was suspected that there was a possibility that the mtDNA detected by the TUNEL assay was involved so that many DNA fragment was found in the cytosol, this study could not prove whether the DNA fragments were from the nucleus or indeed came from the cell's mtDNA. Another possibility is that most DNA fragments detected in HSC-3 cells are in the final stage of the apoptotic process, which includes membrane blebbing, ultrastructural modification of cytoplasmic organelles, and loss of membrane integrity so that many of the fragments were found in the cytosol (Kroemer et al., 2009). Drug or herbal treatment strategies targeted to restore the apoptotic signaling pathways toward normality have the potential activity to eliminate the cancer cells. These findings suggest new sources of extract plants that are capable of inducing apoptosis *in vitro*. However, these results are from preliminary cytotoxicity and apoptosis analysis studies. An in-depth apoptosis analysis such as caspase level, antiproliferative, and cell cycle analysis are necessary to guarantee the therapeutic effect of *P. edulis* fruit seed extract against cancer cells. This study used two positive and negative controls. The use of these controls aims to reduce the risk of negative results. In the TUNEL assay, the DNA strand break labeling procedure can cause undetected cell apoptosis due to methodological problems, such as TdT activity or degradation of BrdUTP, so it is very important to use positive and negative controls (Gorczyca et al., 1993). This study used cisplatin as a positive control. This drug was chosen because it can induce DNA damage through the formation of cisplatin-DNA adducts, resulting in cell cycle arrest and apoptosis (Cheng et al., 2021). Cisplatin-based chemotherapy is the first line of treatment in cancer especially in the late stage of oral cancer.

The HaCaT cells also underwent apoptosis, although, in the viability assay, the seed extract stimulated cell proliferation in a dose-dependent manner. Loo et al. (2011) reported that not only detecting the apoptotic cells, the TUNEL staining could also detect non-apoptotic cells, including necrotic degenerating cells, cells undergoing DNA repair, cells damaged by mechanical forces, and even cells undergoing active gene transcription. So, in this case, there is the possibility of events other than apoptosis that trigger the appearance of the staining; however, this must be proven by other, more in-depth assays.

## CONCLUSION

The present study described that *P. edulis* fruit seed ethanolic extract can induce cytotoxicity in HSC-3 and MCF-7, but not in HSC-2 cells. The extract can also induce the proliferation of normal human keratinocyte cells at maximum dose. The *P. edulis* fruit seed extract exerted anticancer activity through the induction of apoptosis or an increase in TUNEL-positive cells in HSC-3 and MCF-7. The *P. edulis* fruit seed extract could be developed as a potential anticancer agent for OSCC and breast cancer.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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| Contribution | Sari LM | Sari DK | Bustami A | Gazali AD | Auerkari EI |
|---|---|---|---|---|---|
| Concepts or ideas | x | | | | |
| Design | x | | | | |
| Definition of intellectual content | x | x | | | |
| Literature search | | x | | | |
| Experimental studies | x | x | x | x | |
| Data acquisition | | | x | x | |
| Data analysis | x | | x | x | |
| Statistical analysis | x | x | x | | |
| Manuscript preparation | x | | | | |
| Manuscript editing | x | | | | x |
| Manuscript review | x | x | x | x | x |

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