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Effectiveness of Ethanol Extract of *Aegle Marmelos* Fruit Flesh on Hela Cell Apoptosis

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Abstract

Background: Maja plant (*Aegle marmelos*) or plant commonly known as mojo, is one of the plants that contains many secondary metabolites, one of which is flavonoids. Flavonoids can be used to induce apoptosis mechanisms in cancer cells, so that specific apoptosis mechanisms in cancer cells become more effective and toxicity to normal cells is minimized.
Aim : This study aims to evaluate the effectiveness of ethanol extract of maja fruit flesh (*Aegle marmelos*) in inducing apoptosis of HeLa cells in cervical cancer.
Methodology: The method used includes ethanol extraction from maja fruit flesh and treatment of HeLa cells with various concentrations of the extract. Identification of compound content in *Aegle marmelos* was carried out by phytochemical tests. Apoptosis measurements were carried out using the MTT Assay method and flow cytometry analysis.
Results: The results showed that 96% ethanol extract of *Aegle marmelos* contained secondary metabolites in the form of alkaloids, saponins, tannins, and flavonoids. The IC_{50} value of the MTT Assay results of maja fruit flesh extract was 7,573 µg/mL, with an apoptosis percentage of 17.6% and had an effect on inhibiting the growth of HeLa cells in the S phase as measured using the flow cytometry method.
Value: The flavonoid content in 96% ethanol extract of maja fruit flesh (*Aegle marmelos*) has an apoptotic effect on HeLa cells. This is evidenced by the IC_{50} value index of 7,573 µg/mL, an apoptosis percentage of 17.6%, and the presence of inhibition in the S phase in the cell cycle.

Keywords: *Aegle marmelos; apoptosis; HeLa cells; cervical cancer; ethanol extract*

Introduction

Cancer is one of the words that describes a pathological condition in which the cells of the body in the tissues undergo excessive or uncontrolled proliferation, and undergo deformation until it is not recognized by the local immune system and can metastasize to distant places. According to data from the International Agency for Research on Cancer (IARC) in 2020, there were 19.3 million new cases of cancer worldwide and 10 million deaths from cancer. Where cancer is one of the world's second-largest causes of death after heart disease, this number will continue to increase over time (American Cancer Society, 2023).

One of the many types of cancer that exists is cervical cancer. Cervical cancer (cervical cancer) is a condition in which abnormal cell growth occurs in the cervix (the lowest part of the uterus attached to the top of the vagina). According to the Indonesian Ministry of Health in 2019, the incidence of cervical cancer was 23.4 per 100,000 population with an average mortality of 13.9 per 100,000 population, meaning that almost 50% of cervical cancer sufferers ended in death. While in 2018 it was estimated that 311,000 women died from cervical cancer, more than 85% of deaths occurred in low- and middle-income countries (WHO, 2019).

So far, cervical cancer therapy has been obtained in the form of surgical therapy, chemotherapy, radiotherapy and palliative therapy. The purpose of the therapy is to kill and inhibit the growth of cervical cancer cells, namely HeLa cells. HeLa cells or also known as *HeLa cell lines* are continuous *cell lines*, which are immortal and non-dead cells found in cervical cancer.

The use of anticancer compounds can be used to induce apoptosis mechanisms in cancer cells, so that specific apoptosis mechanisms in cancer cells become more effective and toxicity in normal cells is minimal (Fatmawati et al., 2018). Assessment of cell proliferation and apoptosis can be used to evaluate the growth or reduction of tumor mass in response to chemotherapy and radiotherapy given. However, chemotherapy and radiotherapy have often been unaffordable to the public. Apart from the limited health facilities in Indonesia and the price of the therapy itself which is still relatively expensive, sometimes people turn to traditional medicine for treatment. One of the plants that is known to be used for anticancer therapy is the maja plant (*Aegle marmelos*).

Aegle marmelos or maja plant or plant commonly known as mojo is one of the plants that contains a lot of secondary metabolites. This plant belongs to the *Rutaceae* family which has been widely used in the traditional medicine system in India (Seemaisamy et al., 2019). This plant is a type of subtropical plant that is easy to grow and develop in almost all regions of Indonesia. Phytochemical compounds contained in this plant include alkaloids, terpenoids, polyphenols, saponins, tannins, flavonoids, citronelals, auraptene, eugenol, fagarine, D-limonene, plobatanin, etc. Flavonoid compounds, auraptene, and alkaloids are antioxidants that have cytotoxic effects on cancer cells (Monika et al., 2023).

The cytotoxic, antitumor and anticancer activities of *Aegle marmelos* auraptene have been discussed in many in vivo and in vitro studies (Tayarani et al., 2021). *Aegle marmelos* leaves have a high antioxidant component. Essential oil extracted from *Aegle marmelos (L.) Corr leaves.* showed significant anticancer, antioxidant, and anticariogenic effects. In particular, volatile metabolites in the oil inhibited DPPH and ABTS-induced free radicals as well as the growth of Streptococcus mutans bacteria in in vitro studies (Aodah et al., 2023). *Aegle marmelos* hydroethanol leaf extract is also known to have chemotherapy potential by suppressing the growth rate of lung tumors. It also has a hepato-kidney protective effect, so it can be targeted as a new anticancer drug and safe against lung cancer A549 (Kumar et al., 2021). Aegle *marmelos* leaf hydroethanol extract has great anticancer activity against lung cancer cells treated with mRNA expression caspase 3 and caspase 9 (Sukanth et al., 2021).

According to research conducted by Seemaisamy et al., (2019) *Aegle marmelos* showed significant anticancer potential from *Aegle marmelos* extract (methanol and acetone) in tumor cells such as (MDA-MB-231 and HEp-2) by leaving normal vero cells. *Aegle marmelos* can be used as a new innovative anticancer drug by Karetana, able to inhibit the proliferation of liver cancer cells by reducing the expression of MMP 2 and MMP 9 and having anti-cancer activity (Susmitha et al., 2021).

Aegle marmelos meat ethanol extract has antiproliferating activity by suppressing the growth rate of breast tumors in a mouse model (Akhouri et al., 2020). *Aegle marmelos* has an effect on the MCF-7 breast cancer cell line which shows anti-inflammatory and anti-proliferation activity so that it can be used as a drug to treat breast cancer (Dhivya et al., 2021).

Based on the above background, the researchers analyzed the potential of ethanol extract from the pulp of maja fruit (*Aegle marmelos*) as an inhibitory agent for cancer cell growth. The study will focus on increasing the percentage of apoptosis in HeLa cells, which are cervical cancer cells, as a key indicator of the variable. Thus, it is hoped that researchers can reveal the effectiveness of ethanol extract from the pulp of maja fruit (*Aegle marmelos*) as an anticancer therapy that has the potential to be an alternative to traditional medicine.

Materials and Methods

1. Tools and materials

The test material for ethanol extract of 96% maja pulp with raw materials for maja pulp powder was obtained from UPT Materia Medika Batu Malang. The chemicals used are 96% ethanol solvents (PT. Cahya Intan Medika), doxorubicin, concentrated H2SO4, ethanol pro analysis, *aqua pro injection,* DMSO (SIGMA), silica gel, MTT in PBS (*phosphate –Buffer Saline)* 20% RPMI 1640 culture media, FBS (*Fetal Bovine Serum)* 10% v/v, Trypsin EDTA 0.25% and SDS (*Sodium Dodecyl Sulfate*) 10% in HCL 0.01N, yellow tips, blue tips, tissue culture flask (Nulcon), and *membrane filters*, PE Annexin V Apoptosis detection kit (BD Biosciences). The test cells used in this study were HeLa cells obtained from the Biomedical Laboratory, Faculty of Medicine, Sebelas Maret University.

The tools needed in this study are glassware, test tubes, *rotary* evaporators, centrifuges (Sorvall), incubators, *inverted microscop* (Tropmed), weighing instruments (Sratorius), maceration vessels, micropipettes, 5% CO2 incubators, watch glasses, paraffin blocks, glass objects, *Laminar Air Flow* (German Science), ultraviolet lamps, *96-well plates*, transparent glass jars, sterile scapels.

2. Methods

2.1. Extract creation

One kg of maja fruit powder is weighed, then macerated with 96% ethanol solvent in a ratio of 1:5 for 14 days with occasional stirring. The maceration results are then evaporated with a rotary evaporator at a temperature of 50°C. If the extract begins to thicken, transfer the extract to a *water bath* to further thicken the extract. After the consistency of the extract is weighed, the percentage of rendem is calculated.

2.2. Phytochemical screening

2.2.1. Identification of alkaloids

Weigh 0.5 g of the extract then put it in a test tube then add 96% ethanol then shake. Add 5mL of HCl 2N and then heat it using *a water bath.* Once cool, strain the filtrate then add 5 drops of meyer reagent. The formation of red deposits indicates the presence of alkaloid content in the sample.

2.2.2. Saponin identification

Weigh 0.5 g of the extract then put it in a test tube then add 2 mL of 96% ethanol then shake. After that, add 20 mL of aquades, then shake then let it sit for 15-20 minutes. After that add 0.5 g of magnesium powder and 3 drops of concentrated HCl. The appearance of a steady foam as high as 1cm for 10 minutes indicates the presence of saponin content.

2.2.3. Tannin identification

Weigh 0.5 g of the extract then put it in a test tube then add 2 mL of 96% ethanol then shake. Add 3 drops of FeCl3. The formation of blue, black-blue, or bluish-green colors and sediments indicate the presence of tannins.

2.2.4. Identification of flavonoids

Weigh 0.5 g of extract then put it in a test tube then add 2 mL of 96% ethanol then shake. After that add 0.5 g of magnesium powder and 3 drops of concentrated HCl. The formation of orange to red color indicates the presence of flavonoid content.

2.3. Preparation of test solution

Ethanol extract of maja pulp is weighed 10 mg then dissolved in 100 mL of DMSO solvent, then sonicated for 15 minutes until homogeneous then add media up to 1 mL. Then it vortex for 10 minutes. Then make a concentration series of 32.25; 61,5; 125; 250; and 500 ug/mL with culture media.

2.4. MTT Test

Determination of cytotoxic activity of maja fruit extract (*Aegle marmelos*) started by planting cells that had been planted in a 96-well plate with a density of 17,000 cells/well incubated and observed using an inverted microscope until the cells reached 80% confluence. If a flurent has been obtained, discard the culture media and replace it with 50 µL of new culture media. Extract *Aegle marmelos* (5 mg) dissolved in 1 mL of 2.5% DMSO then stratified dilution. A total of 50 µL of diluted samples, doxorubicin positive control with concentrations (31, 25; 62.5; 125; 250; 500 µg/mL), and solvent negative control were placed in 96-well plates and then incubated for 24 hours at 37°C and 5% CO2 gas (Purwanto *et al.*, 2015).

After incubation, remove the supernatant with a sterile micropipette, then add 20 µL of MTS reagent, then incubate in a dark room for 4 hours at 37°C and 5% CO2 gas. After incubation, add 25 µL of 10% SDS in each well to dissolve the formed formazan crystals, then measure the absorbance using *microplate reader* at a wavelength of 550 nm. The corrected absorbance that has been obtained from the result of the reduction of the absorbance of the sample to the absorbance *background*, then the value is converted in percentage of living cells (Purwanto *et al.*, 2015).

2.5. Flow cytometry Test

Flow cytometry is a laboratory instrument used to analyze the characteristics of cells or particles (Mulki, 2020). This instrument allows analysis techniques to be carried out quickly in a short time. The basic principle of *flow cytometry* is that light scattering and fluorescence emission occur due to the presence of an excitation source that hits moving particles (Ibrahim, 2019).

Flow cytometry begins with the selection process of fluorescent-labeled antibodies that are specific to cell surface markers used to characterize the desired cell population. Samples can be processed by enzymatic degradation, centrifugation, and/or filtration to isolate the desired cells, and the resulting cellular suspension is stained with fluorescent antibodies. The single-cell suspension is then fed into a *flow cytometer* into a cell-free buffer solution called enveloped fluid, which flows towards a laser aimed at the solution path. Because the flow of liquid through the pipe is laminar, or sheet-like, and the diameter of the pipe narrows along the path, the cells are made to line up in a single beam as they approach the laser. If the cell has a selected marker on its surface, the bound fluorophore antibody will absorb the laser energy and further release it in the form of a specific wavelength of light as the cell passes through the laser. The emitted light is detected by an optical system that is sensitive to various wavelengths, allowing information about multiple surface markers to be read simultaneously and collected by a connected computer (Mulki, 2020).

The result of this instrument is specific multiparameter data of cells and particles with diameters between 0.5-40 µm.

2.6. Data Analysis

The cytotoxic test data obtained in the form of absorbance data to obtain the calculation of the percentage of living cells, calculated using the cell viability percentage formula as follows:

$$Viability\ (\%) = \frac{Treatment\ OD\ -\ media\ control\ OD}{Cell\ control\ OD\ -\ media\ control\ OD}\ x\ 100\%$$

The results of the percentage of living cells were then analyzed using probit analysis to determine the relationship between the percentage of living cells and the log concentration so that the regression equation Y = BX + A was obtained, the calculation of the IC_{50} value was calculated by substituting the value of 50 in Y to obtain the value of X and the IC_{50} value is anti log x (Nurfiana et al., 2021).

Results and Discussion

1. Extraction of Maja Pulp Powder (*Aegle marmelos)*

Extraction was carried out using the maceration method. Maceration is an extraction technique by soaking and stirring plant material in a solvent at room temperature. Extraction is one of the steps to obtain compounds from natural sources, such as alkali from banana stems. In addition, maceration is used in the initial filtration of secondary metabolites in plant extracts (Dwi, 2022).

The solvent used in the extraction is 96% ethanol, 96% ethanol is a type of ethanol solution with a high concentration of 96%, commonly used in various extraction processes to obtain bioactive compounds from plants (Yuri *et al.,* 2023). Therefore, the use of ethanol (specifically 96%) for extraction purposes may provide advantages in obtaining bioactive compounds such as catechins, alkaloids, and flavonoids from *Aegle marmelos*, demonstrating its significance in pharmaceutical and medicinal applications (Ines *et al.,* 2017).

After maceration of 1000 grams of *Aegle marmelos* meat powder with 10 liters of 96% ethanol, a yield of 488.91 grams was obtained with a yield percentage of 48.891%.

2. Phytochemical Tests

Maja fruit (*Aegle marmelos*) is rich in various phytochemical compounds with significant medicinal properties. Studies have identified that *Aegle marmelos* contains phytochemical compounds such as carotenoids, phenolic compounds, alkaloids, pectins, tannins, coumarins, flavonoids, and terpenoids, which indicate a diverse chemical composition and potential health benefits. In addition, (Dhamne & Agarwal, 2024)*Aegle marmelos* fruit extract has been found to contain abundant amounts of flavonoids and phenolics, which contribute to its antioxidant activity. (Thaware et al. , 2020)

Table 1. Phytochyma test results

Metabolit	Result	Sign
Alkaloid	(+)	Red
Saponins	(+)	Foam 1 cm for 10 minutes
Tannins	(+)	Bluish green
Flavonoid	(+)	Reddish orange

Phytochemical screening was carried out by the tube method on ethanol extracts of maja fruit pulp including alkaloids, saponins, tannins, and flavonoids. Flavonoids in *Aegle marmelos* have shown significant anticancer properties. The flavonoids in *Aegle marmelos* contribute to anticancer potential by inducing apoptosis, inhibiting cell cycle development, and reducing cell proliferation in various cancer cell lines such as lung, breast, colon, and leukemia (Sagarika, 2023). Flavonoids along with other bioactive compounds such as alkaloids and terpenoids, play an important role in the therapeutic applications of plants, including anti-inflammatory, antioxidant, and anticancer activities (Ena, 2019).

3. Cytotoxic Tests

The cytotoxic test was carried out to determine the potential cytotoxic activity contained in 96% ethanol extract from the flesh of maja fruit (*Aegle marmelos*) against HeLa cells. This study uses the MTT *assay* method, in the form of a colorimetric test of redox reactions in the mitochondria of living cells. The cell will reduce the yellow tetrazolium salt with the help of the enzyme succinate dehydrogenase so that it produces purple formazan crystals. The absorbance of formazan crystals was read using an ELISA reader with a wavelength of 550 nm.

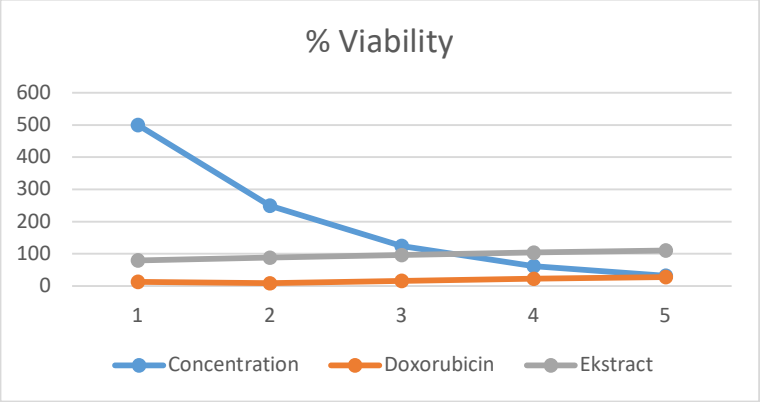
Then the IC_{50} value is calculated to measure the concentration of a drug or compound needed to inhibit certain biological or biochemical processes by 50% (Ugwu & Conradie, 2023). The level of the cytotoxic criteria of a compound is, high activity: $IC_{50} < 5$ µg/mL, active: 5 µg/mL ≤ $IC_{50} < 50$ µg/mL, medium activity: 50 µg/mL ≤ $IC_{50} < 100$ µg/mL, and inactive: $IC_{50} > 100$ µg/mL (Yamen et al., 2023).

The results of data processing from the IC_{50} value of 96% ethanol extract from the pulp of maja (*Aegle marmelos*), can be seen in the following table.

Table 2. Cytotoxic test results

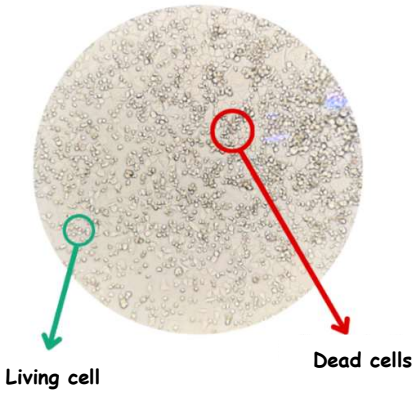
Treatment	Concentration	%Viability	IC_{50} (µg/mL)
Doxorubicin	500	13.86	3.08
	250	9.36	
	125	16.25	
	62.5	23.19	
	31.25	28.41	
Esktrak	500	79.6	7.573
	250	88.026	
	125	96.341	
	62.5	104.135	
	31.25	110.42	

Chart 1. %Viability



The above data shows that 96% ethanol extract of *Aegle marmelos* meat has cytotoxic activity, seen from the same decreasing trend as doxorubicin (graph 1). Along with the increasing dose of *Aegle marmelos* meat extract given, there was a decrease in the percentage value of cell viability at each dose tested. The calculation results obtained the IC_{50} value of *Aegle marmelos* meat extract of 7.573 µg/mL. However, because the IC_{50} value produced <100 µg/mL, the extract has anticancer activity.

This can be proven from the presence of cells that undergo apoptosis when viewed using a microscope.



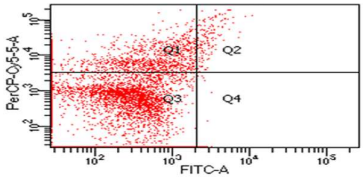
Picture 1. HeLa cells live and die

4. F*low Cytometry Tests*

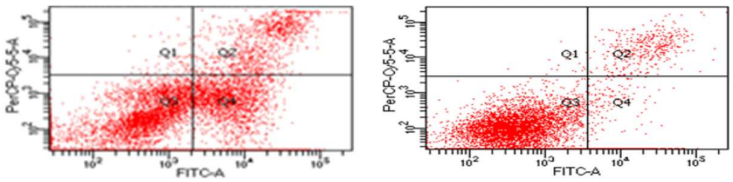
Maja pulp extract (*Aegle marmelos*) that has been tested by MTT and is known to have cytotoxic activity against HeLa cells in cervical cancer was then analyzed using *the flow cytometry* method to see the cell cycle and apoptosis induction that occurred. Annexin V is used in detecting cells that are undergoing apoptosis. Propidium iodide is used as a marker to distinguish *viable*, apoptosis, and necrosis.

4.1 Percentage of apoptosis

The results of the apoptosis percentage test using *the flow cytometry* method are shown as follows.



(a)



(b) (c)

Picture 2. Apoptosis test results (a) Control Cells (b) HeLa Cells + Doxorubicin, (c) HeLa Cells + Maja Pulp Extract

Table 3. Test results *flow cytometry* apoptosis sel

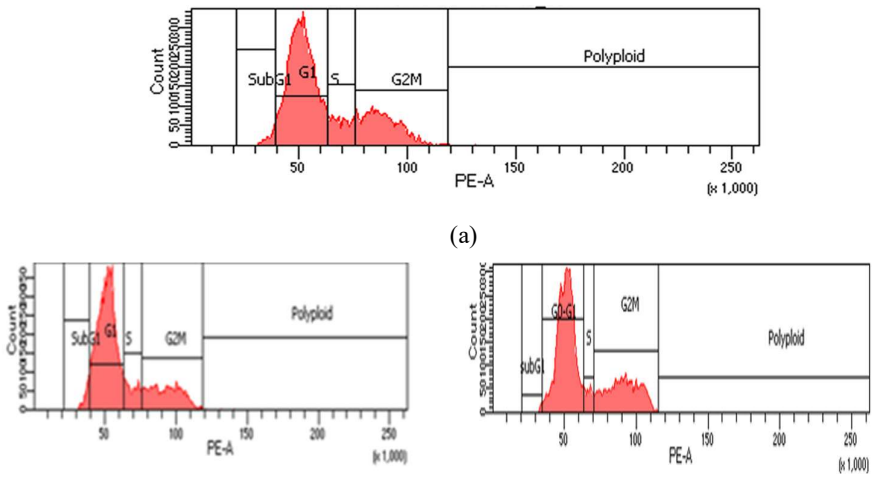
Treatment	Q2 (%)	Q4 (%)	Total percentage of apoptosis (%)
Kontrol Sel	1.5	0.2	1.7
Doxorubicin	11.6	32.9	44.5
Esktrak	4	13.6	17.6

Apoptosis is a programmed cell death that is very well regulated and characterized by morphological and biochemical changes. The apoptosis process allows cells to destroy themselves. Apoptosis test data using the flow cytometry method showed that the extract of maja fruit flesh (*Aegle marmelos*) was able to kill HeLa cells by 17.6%. This can be seen from the sum of Q2 which is late apoptosis and Q4 which is early apoptosis.

4.2 Cell cycle

Flow cytometry *data* was analyzed with *the cell quest program* to see the distribution of cells in the cell cycle phases of sub-G1 *(growth),* S (synthesis), G2/M (preparation and mitosis), and cells undergoing polyploidy. The inhibition of cell cycle that occurs can be determined by comparing the effect of the test solution treatment with the control.

The results of the cell cycle test using *the flow cytometry* method are shown as follows.



Picture 3. Test results *cycle cell* (a) Control Cells (b) HeLa Cells + Doxorubicin, (c) HeLa Cells + Maja Pulp Extract

Table 4. Cell cycle test results

Treatment	G1 (%)	S (%)	G2/M (%)
Kontrol Sel	60.3	10.9	24.7
Esktrak	61.5	4.9	32.4
Doxorubicin	62.9	8.9	22.5

Based on these data, there was an increase in the number of cells in the S phase in each treatment. This indicates that abnormal cells are inhibited in the S phase. These cells are inhibited to be examined

before entering the G2/M phase, resulting in an increase in the percentage of cells in the S phase. This was proven when only maja pulp extract was given without a combination, a percentage of 4.9% was obtained. While the administration of doxorubicin obtained a percentage in the S phase, which was 8.9%.

Conclusion

Ethanol extract of 96% *Aegle marmelos* contains secondary metabolites in the form of saponins, tannins, alkaloids, and flavonoids. Based on the results of the cytotoxic test with MTT assay, the IC_{50} value of the ethanol extract of maja pulp was 7.573 µg/mL, which means it shows cytotoxic activity, namely actively inhibiting hela cells. This is further strengthened by the flow cytometry test to see the percentage of cells undergoing apoptosis. The results of the flow cytometry test of the extract showed a percentage of cell apoptosis of 17.6%. Programmed cell death occurs in the S phase as seen from the percentage ratio of each phase in each treatment.

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Declaration of Conflict of Interest

All researchers stated that there was no conflict of interest with the research, authorship, and publication of this article.

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