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The Effect of Gambier Catechin Isolate on Cervical Cancer Cell Death (HeLa Cell Lines)

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Abstract

BACKGROUND: Cervical cancer is the second most common type of cancer in women worldwide. Human papillomavirus infection on the surface of the cervix is the most common causes which can cause abnormal growth of cervical cells.

AIM: This research was conducted *in vitro* which aims to determine whether catechin compounds can inhibit the growth and regulation of cervical cancer cells (HeLa cell line).

METHODS: This is an experimental research using the colorimetric assay method and qualitative observation of cervical cancer cell morphology (HeLa cell line) under a fluorescence microscope. The administration of catechin compounds was tested at different concentrations to HeLa cells, namely, 1000 g/ml, 500 g/ml, 250 g/ml, 125 g/ml, 62.5 g/ml, and 31.25 g/ml.

RESULTS: The smallest cell viability was obtained from a concentration of 1000 g/ml which was 5.98% while the largest cell viability was found at a concentration of 31.25 g/ml, which was 40.01%. The resulting IC_{50} value was 22.91 g/ml. Gambier catechin compounds have very high antioxidants because they contain an IC_{50} value < 50 g/ml. The effect of gambier catechin compounds on HeLa cell death can be found by increasing the percentage of dead cells. The difference in the fluorescence images of HeLa cells in the experimental group was assessed based on the percentage of the number of cells that died or underwent apoptosis, which was marked by a red or orange fluorescent image. At the concentration of IC_{25}, 31.87% of dead cells were found, the concentration of IC_{50} was 51.09% of dead cells, and the concentration of IC_{75} was 82.51% of dead cells. The test results showed that there was a significant difference in the average percentage of cells undergoing apoptosis in all study groups with $p < 0.05$.

CONCLUSION: Based on research, it can be concluded that catechin compounds could inhibit the growth and regulation of cervical cancer cells (HeLa cell line). Later, it has the potential to be developed as anticancer candidate for cervical cancer.

Introduction

Cervical cancer is the second most common type of cancer in women worldwide. The main caused of this disease is the infection of human papillomavirus (HPV) on the surface of the cervix which can cause abnormal cervical cell growth [1]. It is estimated that around 569,000 new cases and 311,000 deaths globally from cervical cancer were reported in 2018 [2]. Cervical cancer is the leading cause of cancer death among Indonesian women. According to Globocan data, there are at least 36,000 new cases and 21,000 deaths due to cervical cancer in Indonesia in 2020. This number continues to grow and makes cervical cancer the second largest cancer problem in Indonesia [3].

Various therapeutic strategies have been developed in the treatment of cervical cancer, including surgical therapy, radiotherapy, and chemotherapy. However, the results are not so optimal and tend to cause side effects that can harm. The failure of cancer treatment, especially chemotherapy, is caused by the low selectivity of anticancer drugs against normal cells and the resistance of cancer cells to chemotherapy agents who often cause disease recurrence. This agent has also been reported to be nephrotoxic and cardiotoxic for the body [4].

The therapeutic targets currently being developed are generally related to the proliferative activity of the HeLa cell line. HeLa cells are human epithelial cells originating from cervical cancer that has an active telomerase enzyme during cell division [5]. Therefore, further research is needed on candidate agents that are proven to be effective in inhibiting HeLa cell activity as a goal of developing therapy, especially in the exploration and utilization of natural ingredients which are considered to have minimal side effects [6].

The country of Indonesia has abundant biological wealth, one of which is the gambier plant which is generally found in the tropics, especially from

the West Sumatra area. Gambier is a specific superior commodity for this province with an export supply of around 95% per year and continues to increase every year [7]. The content of catechins in gambier is very high, around 7–33%, so it has great potential to be developed as medicinal raw materials. Catechins are active antioxidant compounds derived from flavonoids that have potential as anti-cancer [8]. Through the free radical scavenging mechanism, catechin compounds can induce apoptosis by increasing caspase activity and suppress cancer cell growth by changing the expression of cell cycle regulatory proteins [9].

In an *in vitro* study, it is known that catechin and eugenol isolates have antiproliferative activity against the growth of cervical cancer cells. Another study also stated that catechin along with curcumin compounds could induce apoptosis and inhibit proliferation in cervical cancer cells by regulating expression of VEGF [10]. Catechin compounds and their derivatives have been tested to inhibit the growth and regulation of cervical cancer cells using the MTT assay method. However, there is no literature that discusses the potential of catechin isolates from gambier to the pattern of cervical cancer cell death [11]. Therefore, it is hoped that further experimental research on the anticancer potential of gambier catechin isolates on the pattern of cervical cancer cell death (HeLa cell line) has the opportunity to become one of the candidates in the selection of cervical cancer treatment in the future.

Materials and Methods

Samples

The population used is cervical cancer cells (HeLa cell line) grown in the Biomedical Cell Culture Laboratory, Faculty of Medicine, Andalas University. The HeLa cell line used was 80% confluent, grew, and developed well, intact, and not contaminated. Samples were taken using the Federer formula: (t–1) (n–1) = 15. This study used four treatment groups so that the results obtained were n = 6 samples. To avoid the proportion of dropouts, the researcher used eight samples for each group.

Tools and materials

The tools used are 200 and 1000 mL micropipettes, small test tubes, small tube racks, lab tech chamber slide 4 wells, conical tubes, yellow tips and blue tips, pipette aids, 10 ml volumetric pipettes, incubators, autoclaves, and fluorescent microscopes. While the materials used in this study were gambier catechin isolate, phosphate-buffered saline (FBS), plate and culture media (RPMI), DMSO, consumables, distilled water, inverted, gambier catechin isolate, cervical cancer cells (HeLa cell line), trypsin 0,25%, fenstrep, streptomycin 10%, fungizone 1%, AO/PI kit, and health protocol requirements.

Production of catechin isolate

Pure gambier catechin isolate (99%) was obtained commercially produced by the Andalas Sitawa Fitolab, Padang, Indonesia. The calculation of the gambier catechin suspension to be used begins with suspending gambier powder into Aquadest until it has a liquid texture.

Observation of HeLa cell line fluorescence image

Qualitative observations were made by observing cell morphology under a fluorescent microscope after the administration of acridine orange and ethidium bromide. Live cells will fluoresce bright green, cells that undergo the early-stage apoptosis will experience chromatin condensation and are still green, and cells that undergo apoptosis in the late stages will be fragmented into smaller pieces and are orange in color while necrotic cells are normal in size and orange in color. Observation of the preparations was carried out using a fluorescent microscope which was then read using the ImageJ 2018 application version 1.52a.

Data analysis

Data collection techniques were carried out using experimental observation techniques. The data obtained are the percentage of HeLa cells undergoing apoptosis at the same place and time. The research data are compiled and tabulated according to the groups. The data are then presented in the form of tables and figures. Data analysis was performed with analysis of one-way ANOVA by variance with a 95% confidence level using SPSS software. After the data were analyzed, the analysis of the results of the data analysis was carried out on the pattern of HeLa cell death by looking at the fluorescence picture. The interpretation of the results of this analysis aims to draw conclusions from the research that has been carried out.

Results

Table 1 illustrates that the administration of different catechin isolates will produce different

Table 1: The results of the MTT assay of gambier catechin isolates on HeLa cells

Concentration (µg/ml)	Viability (%)	IC_{25} (µg/ml)	IC_{50} (µg/ml)	IC_{75} (µg/ml)
1000	5.98	128.825	22.91	3.89
500	7.34			
250	15.56			
125	25.36			
62.5	30.25			
31.25	40.01			

viability. The smallest cell viability was obtained from a concentration of 1000 g/ml which was 5.98% while the largest cell viability was found at a concentration of 31.25 g/ml, which was 40.01%. The relationship between the concentrations of catechin isolates on cell viability was presented using a graph of the concentration log with cell viability so as to produce the IC_{50} value from the regression equation.

Based on Figure 1, the resulting regression equation is y = –32,137x + 93,981. In this equation, the resulting IC_{50} value is 22.91 g/ml. The resulting IC_{75} value is 3.89 g/ml and the IC_{25} value is 128.825 g/ml. The lower the IC_{50} value of an active substance, the higher the antioxidant it has. Gambier catechin has very high antioxidant because it contains IC_{50} value <50 g/ml.

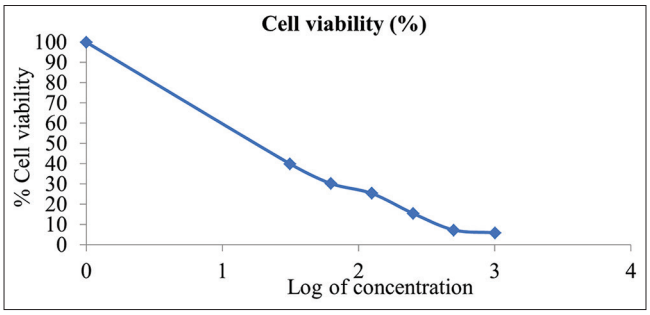

Figure 1: Graph of log concentration relationship with cell viability

Figure 2 shows the documentation of fluorescent micrographs of HeLa cell lines staining AOPI showing images of (a) control group, (b) Group P1, (c) Group P2, and (d) Group P3. Live cells appear green and dead or apoptotic cells appear red or orange.

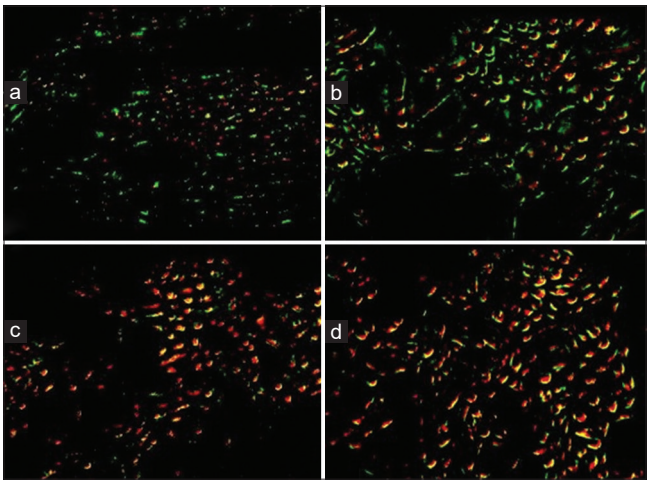

Figure 2: (a-d) Documentation of fluorescent micrographs of cervical cancer cell cultures

Table 2 shows the effect of gambier catechin isolates on cervical cancer cell death (HeLa cell lines) where an increase in the percentage of dead cells was found. The higher the concentration of catechins given, the greater the percentage of cell apoptosis.

Table 2: The results of the average calculation of HeLa cells undergoing apoptosis

Groups	Cervical cancer cell apoptosis percentage			
	K (n = 7)	P1 (n = 7)	P2 (n = 7)	P3 (n = 7)
Average	18.26	31.87	51.09	82.51

Discussion

Treatment of different concentrations of catechin isolates was tested against cervical cancer cells using the MTT assay. MTT assay was used to determine cell proliferation, viability, and activation. MTT can measure the ability of living cells to reduce the tetrazolium salt of MTT to formazan with NADH reductase and other enzymes resulting in a linear relationship between the number of living and dead cells. MTT enters the cell by endocytosis and is converted to formazan. The amount of formazan produced will describe the amount of cell viability. The number of active living cells and the intensity of the purple color formed have a linear relationship so that the MTT reagent will produce a low absorbance value in the absence of live cells [12].

The study used cervical cancer cell cultures (HeLa cell lines) cultured in 28-well plates consisting of control group, Group P1 with gambier catechin isolate concentration IC_{25}, Group P2 with catechin concentration IC_{50}, and Group P3 with catechin concentration IC_{75} where each totaling 7 wells. Groups P1, P2, and P3 were given catechin isolates on the 2nd day after the cells were incubated for 24 h, then incubated again for 72 h. Furthermore, incubation and staining were carried out and then observed using a fluorescent microscope. Observations were made on a representative section with a wavelength of 535 nm.

Differences in fluorescence images of cervical cancer cells (Figure 2) in the experimental group were assessed based on the percentage of the number of cells that died or underwent apoptosis, which were marked with red or orange fluorescent images. The color image is obtained from the test method with propidium iodide which is widely used as an apoptosis test in experimental models. This method is based on the apoptotic characteristics of cells, namely, DNA fragmentation and loss of DNA in the nucleus. This method uses a fluorescent dye propidium iodide reagent which can bind and label DNA. Propidium-iodide emits red or orange fluorescence when bound to nucleic acids, green fluorescence when bound to double-stranded DNA, and red fluorescence when bound to RNA or single-stranded DNA. Orange acridine can enter living or dead cells, while ethidium bromide and propidium iodide can only penetrate cell membranes that are destroyed [5].

Table 2 shows the effect of gambier catechin isolates on cervical cancer cell death (HeLa cell lines) where an increase in the percentage of dead cells was

found. At 72 h incubation period with IC_{25} concentration, 31.87% of dead cells were found, IC_{50} concentration was 51.09% of dead cells, and IC_{75} concentration was 82.51% of dead cells. This cell death caused by antioxidant properties of catechin which is also in accordance with the research of Anggraini *et al.* who found that diluted extract of gambier had strong antioxidant properties based on its IC_{50} value [13].

Based on the National Cancer Institute, a compound is considered to have an active cytotoxic effect if it has an IC_{50} value of <30 g/ml, moderately active if the IC_{50} value is 30 g/ml or IC_{50} <100 g/ml, and inactive if the IC_{50} value is >100 g/ml [14]. This result is in accordance with Menten theory which states that the effect of bioactive compounds arises due to their interaction with cell receptors, the intensity of the effect of the material is proportional to the fraction of the receptor that is bound, and the receptor fraction depends on the dose and duration of exposure [15]. The test results showed that there was a significant difference in the average percentage of cells undergoing apoptosis in all study groups with $p < 0.05$ after the administration of gambier catechin isolate.

The potential of catechins as an anti-cancer has been confirmed by several previous studies. Catechin is an active antioxidant compound derived from flavonoids and has anti-cancer potential [8]. The high content of catechin antioxidants is directly proportional to its anti-proliferative potential which is toxic to cancer cells. Catechin induces apoptosis by increasing caspase activity and inhibits cancer cell growth by changing the expression of cell cycle regulatory proteins in the free radical scavenging mechanism [9].

Recent studies have shown that catechin compounds can interfere with angiogenic cell signaling pathways in ovarian, lung, breast, and cervical cancer cells [16]. A recent study found that EGCG (catechin compounds) can inhibit HeLa cell proliferation through the depolymerization of cell microtubules and inhibit tubulin assembly in cells and cell-free systems (IC_{50} is 39.6 ± 0.63 M) [17]. EGCG can inhibit the migration and invasion of cervical cancer cells by regulating the activities of proteolytic enzymes, signal pathways, growth factors/receptors, and also the angiogenesis [16].

In addition, EGCG can inhibit the proliferation of HeLa cells so that their spherical shape will not become multiple filopodia and lamella peculiar to diffuse cells, thereby reducing the adhesion rate and migration ability of HeLa cells after 48 years, 40% and 68% h, respectively [18]. EGCG is not only a natural antioxidant in normal cells but also shows pro-oxidation and apoptosis-inducing properties in cancer cells [19]. High concentration of EGCG (50 μg–200 μg gallic acid equivalent GAE/mL) induces the formation of superoxide anion free radicals, H_2O_2, and hydroxyl free radicals in cells, which is the result of increased oxidative stress in HeLa cells [20].

Conclusion

The catechin compound has an active cytotoxic effect because it has an IC_{50} value of <50 g/ml, which is 22.91 g/ml. Based on the resulting viability value, treatment with a concentration of 1000 g/ml resulted in the lowest cell viability against cervical cancer, namely, 5.98%, while the concentration of 31.25 g/ml resulted in the highest viability, which was 40.01%. The average calculation of HeLa cells undergoing apoptosis at concentrations of IC_{25}, IC_{50}, and IC_{75} were 31.87%, 51.09%, and 82.51%. The test results showed that there was a statistically significant difference in the percentage of cells undergoing apoptosis in all study groups, which was statistically significant with $p < 0.05$.

Ethical Approval

This study was done *in vitro* and does not include human participants or animals.

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