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Original Article

Viability and Tyrosinase Inhibition of Catechins Isolated from *Uncaria gambir* on B16F0 Cells

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Abstract *- Catechins are found in various plants such as tea, gambier, apples, persimmons, chocolate, grapes and so on. Catechins are compounds that determine the quality of gambier. The content of catechins in gambier can reach more than 90%. Catechins isolated from gambier have not been tested for their activity. Research on the activity of catechins derived from Uncaria gambir, which has activity as an inhibitor tyrosinase, has not been widely carried out. This study aimed to test the catechins isolated from the Uncaria gambir plant against cytotoxic effects on B16F0 cells using an MTT assay with gambir catechin concentrations of 25-500 µg/ml. Furthermore, its activity against the tyrosinase enzyme was determined using mushroom tyrosinase. From the results obtained with the purity of catechins (C90 and C98), increasing the concentration and the length of the test time gave a significant difference in reducing the viability of B16F0 cells ($p<0.05$) with IC_{50} values of C90 and C98 after 72 hours of 171.995 and 204.486 µg/mL. The IC_{50} values for tyrosinase enzyme inhibition from C90 and C98 were 19.047 and 11.772 µg/mL, respectively. Gambier catechins have a depigmenting agent effect without being toxic to cells in vitro.*

Keywords *- Melanogenesis, Flavonoids, Gambier, Cytotoxic B16F0 cell, Inhibit tyrosinase.*

1. Introduction

Catechins derived from the term 'catechu', which is an extract from the plant *Acacia catechu* L., are 3,3',4'5,7-pentahydroxyflavans with two steric and enantiomeric forms. Catechins represent the term from a class of chemical compounds with a catechin group. Catechins are found in various plants, such as tea, apples, persimmons, chocolate, grapes and so on [1]. Catechins in plants can be in the form of isomers and have different pharmacological activities. Research on the pharmacological activity of catechins has mostly been carried out on catechins derived from tea plants, which contain many isomeric compounds of catechins in the form of EGCG [2]. EGC and EGCG, which are isolated compounds from tea, affect the viability of MCF-7 cells by decreasing the activity of the tyrosine phosphatase enzyme that regulates oxidative stress and is involved in prooncogenic pathways that lead to the formation of breast cancer cells [3]. While EGCG (Sigma) could kill cancer cells at a concentration of 10 µM, catechins isolated from gambier with a purity of 99% did not kill cancer cells in SiHa-F3 cells up to a concentration of 100 µM. This suggests that EGCG is more toxic to SiHa-F3 cells than catechins [4]. Catechins (Sigma) at 50 µM significantly decreased the cell viability of nearly 68% of human pancreatic cancer cells after 48 hours [5]. EGCG isolated from green tea has an IC_{50} value of 36.0 µM in cell A549 using the MTT assay [6]. When EGCG was added to MCF-7 cells, its cytotoxicity was notably greater than that of HT-29 cells. The IC_{50} values of EGCG were 11.2 ± 1.4 µM in MCF-7 cells and 136.3 ± 2.1 µM in HT-29 cells [7].

Catechins are the main bioactive compounds in gambier and are used to determine the quality of gambier. The catechins contained in gambier are in the form of (+)-catechins and (+)-epicatechins and have the potential as antioxidants, anthelmintics, antibacterials, antidiabetics, and to relieve osteoarthritis [8]. Catechins as purified extracts from gambier have levels of 92.69% antioxidant activity with an IC_{50} value of 11.76 µg/mL using the DPPH assay [9]. Catechins exhibit antioxidant and proantioxidant effects in microsomal CYP2EI-dependent oxidative stress [10]. Catechins have antioxidant activity with an IC_{50} value of 2.0 ± 0.2 µM [11]. Catechins isolated from tea at 0–100 µM concentrations can protect against oxidative stress-induced cell death in fibroblasts, thus showing potential as therapeutic agents to prevent premature skin aging [12]. EGCG in murine melanoma B16 cells exposed to ultraviolet A (UVA) showed that at a concentration of 100 µg/mL had a cell viability of 67.09 ± 3.27% [13]. Catechins protect keratinocytes against ultraviolet B (UVB) radiation and Reactive Oxygen Species (ROS) [14].

UV radiation causes the start of the process of melanogenesis to produce melanin. Melanin is a natural photoprotector in the skin, but as a result, it produces a brownish tint to the skin. If the melanin formation is uneven, it will cause spots on the skin [15]. Depigmenting agents generally have a mechanism of action to inhibit the formation of melanin by several mechanisms, one of which is by inhibiting the work of the tyrosinase enzyme [16]. Depigmenting agents used, such as hydroquinone in some countries, are used with certain concentration limits and under the supervision of a dermatologist. Benzoquinone, which can be hazardous to melanocyte cells and result in contact leukoderma, is produced when the tyrosinase enzyme oxidizes hydroquinone, which is how it works as a depigmenting agent [17].

Measurement of cell viability in vitro to determine whether a compound or extract has biologically toxic properties to cells is often referred to as a cytotoxicity test in cell culture. One method commonly used to measure cell viability is the MTT assay [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] [18]·[19]. Cell viability is directly proportional to the formazan formed. The concentration of the test substance with the percentage of viability was made a linear regression equation to determine the IC_{50} value of the test substance against cell culture [20]. The cell line melanoma mouse model is used to perform basic testing to see the effect of a compound or material as a depigmenting agent [21]. A number of cell line mouse melanoma features are derived from its mortality and capacity for metastasis. B16F0 cells are often used to test the effects of depigmenting agents on the skin because they perform better with in vitro experiments than B16 cells do. [22].

Extraction methods and isolation results from various plants that produce the same phytochemical compound can affect the pharmacological activity of these compounds [23]·[24]. The purity of the isolation of phytochemical compounds also has significant differences in antioxidants and antibacterials [25]. The use of catechins as depigmenting agents will later be made in the form of topical preparations used on the skin, so that it does not affect the bioavailability of catechins systemically. This study aimed to examine the effect of the purity of the catechin compound isolated from gambier on the cytotoxic effect on B16F0 cells using kojic acid as a positive control using the MTT assay and its activity as a depigmenting agent by inhibiting the tyrosinase enzyme.

2. Materials and Methods

2.1. Reagents

Catechins 90% (C90) (PE-001) and catechin 98% (C98) (PE-003) isolated from gambier in Andalas Sitawa Fitolab, West Sumatra, Indonesia. Kojic acid (KA) (K3125) was obtained from Sigma Aldrich. B16F0 cells from the European Collection of Cell Cultures (ECACC). Cell media, supplements and other reagents were obtained from Sigma Aldrich.

2.2. Cell Culture

In DMEM media supplemented with 10% fetal bovine serum (FBS), 2% penicillin-streptomycin, and 0.5% amphotericin B, mouse melanoma B16F0 cells were grown. The culture was kept in an environment with 5% CO_2 and 37ºC. The density of the cell culture was 5 x 10^4 cells/mL. To achieve the appropriate density, fresh media is added every two days, and cells are counted and replanted.

2.3. Viability Test

B16F0 cells were added to the 96-well plate at a density of 5 x 10^3 cells/well. Incubate in an environment with 5% CO_2 for 24 hours at 37ºC. Change the medium and allow it to incubate for another 24 hours. Distilled water was used to dissolve C90, C98, and KA, resulting in a range of values from 25 to 500 µg/mL. The test solution was added to the well, and the cells were then incubated for 24, 48, and 72 hours at 37ºC in an environment of 5% CO_2. Throw away the cell medium, give each well a PBS wash, and then add the MTT reagen, along with the media control (cell-free). If formazan has clearly formed after 2 to 4 hours of incubation at 37ºC in an environment with 5% CO_2, then add a stopper. After an overnight incubation period, use a microplate reader to detect the absorbance at 595 nm. Equation can be used to compute the percentage of cell viability [18]: % viability = [Mean OD_{sample}] / [Mean OD_{blank}] x 100% Compute the IC_{50} value, find the linear regression equation, and create a concentration graph with cell viability.

2.4. Determination of Tyrosinase Activity

Distilled water dissolves C90, C98, and KA, resulting in a range of values from 5 to 80 µg/mL. After adding 200 U/mL mushroom tyrosinase to the test solution and letting it sit in a dark area, the well was filled with 10 mM L-DOPA solution and again placed in a dark area. The wavelength at which absorption was measured was 475 nm. Using blank, the percentage of inhibition was computed. Determine the linear regression equation, plot the concentration against tyosinase inhibition, and compute the IC_{50} value.

2.5. Statistical Analysis

Each measurement was reported as the group mean ± SD, with four replications. One way ANOVA was used to process the data statistically. To elucidate the shift group mean, Tukey's post hoc test was utilized; statistical significance was established at $p<0.05$.

3. Results and Discussion

3.1. Viability Test

Research conducted on the effect of gambir catechins on the viability of B16F0 cells showed that increasing concentrations and the length of time for determining C90,

C98, and KA significantly affected the decrease in viability of B16F0 cells, $p<0.05$. The data obtained from the test were then graphed with a straight-line equation of the concentrations of C90, C98, and KA with the percentage of cell viability. The result is a straight-line equation. Based on the straight-line equation, the IC_{50} value is calculated, which is shown in Table 1.

Table 1. Linear regression equations of C90, C98, and KA, and IC_{50} value

t (h)	test	Equation	R^2	IC_{50} (µg/mL)
24	C90	y = -0.049x + 86.831	0.9112	748.598
	C98	y = -0.055x + 82.201	0.9522	583.351
	KA	y = -0.096x + 82.599	0.9834	338.515
48	C90	y = -0.065x + 73.444	0.9393	358.471
	C98	y = -0.081x + 74.187	0.9255	298.237
	KA	y = -0.095x + 72.736	0.9792	240.338
72	C90	y = -0.078x + 63.450	0.9563	171.995
	C98	y = -0.105x + 71.471	0.9842	204.486
	KA	y = -0.100x + 50.585	0.9591	5.844

Test methods regarding the toxicity of a test substance to cells carried out on cell cultures have been developed and broadly classified as dye exclusion assays, colorimetric assays, fluorometric assays, luminometric assays and flow cytometric assays. MTT assay is part of colorimetric assays and is a simple test with significant results on cell viability as an illustration of the cytotoxicity of the test substance on cells. This test is based on the conversion of MTT reagent to formazan which indicates a live cell, which indicates the function of the cell's mitochondria[18].

Tests using the MTT method are also used to test the potential of a substance or compound as an anti-cancer material at the initial screening of in vitro research using cell culture before the next research stage in vivo[26]. Research involving melanoma cell culture to see the effect of depigmenting agents uses KA as a comparison. KA inhibits the tyrosinase enzyme competitively, which is the same mechanism of action as hydroquinone. KA can be used to treat patients who do not respond to hydroquinone. KA can take the place of hydroquinone's carcinogenic effect [27]. The concentration of KA that is often used to see the cytotoxic effect ranges from 5 to 500 µg/mL[28].

The IC_{50} value shows that the longer the test duration, the smaller the IC_{50} value. After 72 hours, KA showed a more toxic effect than C90 and C98, which had a much smaller IC_{50} value of 5.8442 g/mL, while C90 and C98 were 171.9949 and 204.4857 µg/mL, respectively. Based on this, C90 and C98 were not toxic to B16F0 cells up to 200 µg/mL. Based on this, it can be seen that the viability of B16F0 cells at 200 µg/mL decreased significantly with the length of time of the test compared to control cells (Figure 1). Control cells without treatment were only given 3-isobutyl-1-methylxanthine (IBMX) to stimulate melanogenesis. Catechins derived from green tea at a concentration of 20 M (~ 5.8 g/mL) had cell viability ranging from 60 to 80% against B16 cells for 5 days [29]. Extracts of black, green, and white tea were tested for cell viability at 50 µg/mL on Melan-A cells for 48 h, and cell viability was 62 to 76% [30]. Catechins derived from gambier with a purity of 99% did not kill cancer cells up to 100 µM (~27 µg/mL) in SiHa-F3 cells [4]. (+)-catechin (Sigma) at 400 µM (~ 116 µg/mL) had a cell viability value of 70 to 80% in PC12 cells [31]. Catechins (Sigma) at 50 µM (~14.5 µg/mL) resulted in a significant reduction of nearly 68% of human pancreatic cancer cells after 48 h[5]. The determined anti-melanogenesis effect of green tea compound EGCG in murine melanoma B16 cells irradiated with ultraviolet A (UVA) showed at 100 µg/mL showed cell viability of 67.09 ± 3.27%[13]. This shows that gambier catechins are lower in toxicity than catechin compounds derived from other plants.

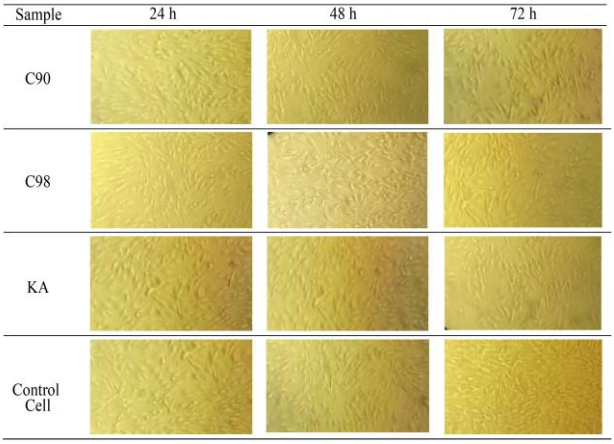

Fig. 1 Cell viability of C90, C98, and KA at concentrations of 200 µg/mL, duration 24, 48, and 72 h

Uncaria gambir Roxb leaf extract was tested for cell viability using T47D cells; the IC_{50} value was 1.086 mg/mL [32]. This indicates that the isolation of catechins from gambier has a better anti-cancer effect than in the form of extracts. EGCG derived from green tea had an IC_{50} value of 36.0 µM (~ 16.5 µg/mL) in A549 cells using the MTT assay[6]. EGCG caused significantly higher cytotoxicity in MCF-7 cells compared to HT-29 cells, which had an IC_{50} value of 11.2 ± 1.4 µM (~ 5.13 µg/mL) for MCF-7 cells and 136.3 ± 2.1 µM (~62.48 µg/mL) for HT-29 cells[7]. Catechins derived from Annona reticulata Linn, after 72 h, had an IC_{50} value of 584.00 µg/mL against MCF-7 breast cancer cells using the MTT assay[33]. The catechins found in green tea have IC_{50} values of epicatechin, EGC, ECG and EGCG, which are 113.2 ± 22.6 µM, 35.9 ± 10.6 µM, > 125 µM and 13.9 ± 3.1 µM[3]. Research conducted on KA at 25 µg/mL for 72 h had an inhibitory effect of 66.305%, and this is almost the same where KA at 200 µM (~ 28.42 µg/mL) had an inhibitory effect of 70%[34]. Research shows that cancer cell types, catechin isolates from different plants,

concentration and exposure time of the test substance have important effects on cell viability. Cell density also affects the results of the study. The IC_{50} value is used to determine the potency of a drug in certain cell cultures. Based on tests on the same cells with the MTT assay obtained at 24 h, a hydroalcoholic extract of *Rhodiola rosea* L. with 80 µg/mL showed the viability of B16F0 cells was around 65%[35]. The fermented extract of *Cordyceps militaris* with 1 mg/mL showed B16F0 cell viability of 83.7% after 72 h[34]. 1-O-methyl-fructofuranose isolate from *Schisandra chinensis* fruit at 40 µg/mL for 24 h showed B16F0 cell viability was around 25%[36]. Daidzein derived from fermented soymilk at 200 µM for 72 h showed the viability of B16F0 cells was around 62%[37]. Based on this, the effect of C98 on the toxicity of B16F0 cells was stronger than in the form of extracts from other plants and weaker than those isolated from other plants.

3.2. Determination of Tyrosinase Activity

Determination of C90, C98 and KA on the tyrosinase enzyme by looking at the dopachrome content formed compared to the blank, which is an initial test to see the activity of a test substance as a depigmenting agent. From graph concentration versus percentage of inhibition, a line equation was made, and the IC_{50} values of C90, C98 and KA were determined. The complete results are in Table 2.

Table 2. Concentrations of C90, C98 and KA on tyrosinase inhibition and IC_{50} values

Conc. (µg/mL)	Enzyme Inhibition (%)		
	C90	**C98**	**KA**
5	40.268 ± 0.225	43.903 ± 0.225	43.124 ± 0.225
10	44.942 ± 0.390	49.487 ± 0.225	49.357 ± 0.225
20	53.902 ± 0.225	54.681 ± 0.225	55.590 ± 0.225
40	61.823 ± 0.225	69.095 ± 0.225	70.264 ± 0.225
80	77.535 ± 0.225	86.365 ± 0.225	88.833 ± 0.225
equation	y = 0.476x + 40.928	y = 0.557x + 43.444	y = 0.596x + 42.967
R^2	0.969	0.983	0.982
IC_{50}	19.047 µg/mL	11.772 µg/mL	11.806 µg/mL

Tyrosinase, an enzyme involved in melanogenesis, catalyzes the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and subsequently to dopaquinone in melanocytes. Tyrosinase activity was directly measured to determine the depigmenting agent's activity because these reactions are the rate-limiting steps in the melanogenesis process. Tyrosinase activity measurement can also be used to gauge the melanogenesis of pigment cells grown in culture. Due to its capacity to catalyze the first rate-limiting synthesis reactions, hydroxylation of tyrosinase to DOPA and its subsequent oxidation to dopaquinone-tyrosinase plays a pivotal role in the biosynthesis of melanin. These reactions essentially result in the spontaneous formation of melanin. Numerous additional factors, including the activity of additional enzymes (dopachrome tautomerase, peroxidase, and so forth) and specific metal ions, particularly copper and iron, have been found to regulate melanogenesis. Even before tyrosinase is involved, melanin synthesis is subject to an additional degree of genetic regulation. Tyrosinase activity is, therefore, a significant, but not the only, factor in determining the amount of melanin produced [38],[39].

Research conducted using the tyrosinase enzyme obtained IC_{50} values of C90, C98 and KA, respectively, 19.047 µg/mL, 11.772 µg/mL and 11.806 µg/mL. The IC_{50} C98 value with KA is almost the same. The obtained results are within the range of IC_{50} values for catechin, which are approximately from 20 to 150 µM (~ 5.8 to 43.5 µg/mL) [24]. In this study, C98 derived from gambier outperformed catechins derived from tea, with an IC_{50} value of 57.12 µM (~16.565 µg/mL), but not C90 [40]. Tyrosinase inhibition was observed at KA concentrations ranging from 3.91 to 250 µg/mL. The inhibitory effect on the enzyme was not dose-dependent [28].

4. Conclusion

Catechins are the main bioactive compounds in gambier (*Uncaria gambir* [Roxb]) and are used to determine the quality of gambier. Most of the catechins contained in gambier are (+)-catechin compounds. In this study, the increase in concentration and duration of C90 and C98 gave a significant difference in decreasing the viability of B16F0 cells ($p<0.05$). C98 is better at reducing cell viability than C90. C90 and C98 were not toxic to B16F0 cells up to a concentration of 200 µg/mL during the 72 h test time. The IC_{50} value of C98 and KA is almost the same in terms of inhibiting the action of the tyrosinase enzyme, so it can be concluded that catechins derived from gambier have potential as depigmenting agents without causing toxicity to B16F0 cells in vitro. The work's major conclusions and implications should be succinctly explained in the conclusions section, emphasizing the significance and applicability of the research.

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