

Original Article

Green tea leaf extract reduces viability and migration of cholesteatoma fibroblast of chronic suppurative otitis media cultured in vitro

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ABSTRACT

Background: Chronic Suppurative Otitis Media (CSOM) is still a health problem, especially in developing countries. CSOM with cholesteatoma is a dangerous type. Cholesteatoma in CSOM sufferers can cause various complications. Therefore, alternative therapies are needed, such as green tea leaf extract. Green tea leaf extract can be an antioxidant and anti-inflammatory, but its effectiveness in treating CSOM has not been studied before, so research is needed.

Purpose: This research aimed to determine the effect of green tea leaf extract that could reduce viability and migration in cholesteatoma fibroblast of CSOM.

Methods: This research was an in vitro experiment with a post-test-only control group design. The sample for this research was cholesteatoma fibroblast cells obtained from the isolation of patients with CSOM. The method used is Hoechst staining for viability and scratch techniques for cell migration with eight groups of cholesteatoma fibroblasts consisting of a negative control group (DMEM+FBS), DMEM group, two positive control groups dexamethasone (10 μ M and 100 μ M), and four green tea leaf extract group (10 μ g/ml, 40 μ g/ml, 80 μ g/ml, and 160 μ g/ml). Data analysis used One-Way ANOVA and Kruskal Wallis tests.

Results: The results showed that the highest average cell viability was in the negative control group (165.33), and the lowest was in green tea leaf extract at a dose of 160 μ g/ml (70.88). Cell migration at 72 hours showed that in the negative control group, there was faster closure of the scratch area (97,78%) and the slowest closure on green tea leaf extract at a dose of 160 μ g/ml (13,81%).

Conclusion: Green tea leaf extract can reduce the viability and migration in cholesteatoma fibroblast of CSOM. It shows the potential of green tea extract as an alternative to prevent cholesteatoma.

INTRODUCTION

The World Health Organization (WHO) estimates that 65-330 million people are sufferers of CSOM worldwide, 94% are in developing countries, and 60% (39-200 million people) die from CSOM.¹ The prevalence of CSOM in Indonesia is 2.6% of the total population of Indonesia.² The incidence of CSOM varies in each developing country and is generally influenced by factors such as socioeconomic. Low socioeconomic life, such as poor nutritional health status and a slum environment, are risk factors that underlie the increased prevalence of CSOM in developing countries.³ CSOM is divided into tubotympanic CSOM (without https://doi.org/10.30595/medisains.v21i2.17049 cholesteatoma) and attico-antral CSOM (with cholesteatoma).⁴ CSOM with cholesteatoma is a dangerous type that compares the incidence of CSOM, which reaches 9 out of 100,000 people or 95% of the incidence of CSOM cholesteatoma.⁵

Cholesteatoma, characterized by hyperproliferation, is associated with chronic inflammation and bone destruction.⁶ Many Factors that can increase the severity of CSOM include late therapy, inadequate therapy, high virulence of germs, weak immune response, and bad hygiene.⁷ Pharmacological therapy carried out so far still has several drawbacks, including a long treatment time, patients who

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do not routinely control, repeated symptoms, and high recurrences, so surgery is needed many times. Therefore, alternative therapies are needed, such as giving extracts derived from herbal ingredients. Several studies report that the content of polyphenols (catechins) in green tea extract has many benefits for the body, such as anti-carcinogenic, anti-inflammatory, antimicrobial, and antioxidant. In addition, the polyphenols in green tea leaf extract also have growth-inhibitory effects on cancer cells.⁸

Viability is the ability of cells to grow normally under optimal conditions. Cell viability is the ratio between living cells and the total number of cells. Cell viability was analyzed in cell culture to evaluate the effect of in vitro drugs on cellmediated cytotoxicity tests to monitor cell proliferation.9 Cell migration is a process involved in various biological mechanisms, such as cancer progression. Migration ability in cancer cells plays a vital role in cell invasion and metastasis.¹⁰ Research on green tea leaf extract's effect on cholesteatoma fibroblast has never been done. However, previous research mentioned that epigallocatechin-3-gallate (EGCG) content in green tea is known to reduce the viability of peripheral blood mononuclear cells in people with psoriasis.¹¹ In another study, EGCG in green tea leaves reduced the proliferation and migration of bladder cancer.12 EGCG in green tea leaves is also known to reduce viability and inhibit breast cancer cell migration.¹³ From various previous studies, no one has examined the effect of green tea leaf extract on the viability and migration of cholesteatoma fibroblast of CSOM. So, this research aimed to determine the effect of green tea leaf extract can reduce the viability and migration of cholesteatoma fibroblast of CSOM.

METHOD

Study Design

This research was an in vitro experiment with a post-testonly control group design.¹⁴

Study Site

This research was conducted from November 2022 to January 2023. Green tea leaf obtained from plantation tea Pangelangan Bandung. Cholesteatoma fibroblast cells isolated from chronic suppurative otitis media patients stored in Biorepository YARSI University. Viability and migration tests were conducted at the Stem Cell Laboratory, Integrated Research Laboratory of YARSI University in Jakarta.

Materials

The materials used were dry green tea leaves, aquadest as a solvent in the manufacture of extracts, Dulbeco's Modified Essential Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS), Antibiotics-Antimycotics (AA), Dexamethasone, Dimethyl sulfoxide (DMSO), Trypsin 0,25%, Trypan Blue, and Hoechst Staining.

Green Tea Leaf Extraction

Green tea leaf extract used the maceration technique. First, green tea leaf is washed and aerated in a temperature room until dry. After drying, green tea leaves are made Simplicia with a blender to form powder rude. Green tea leaf was weighed and done immersion using aquadest for 72 hours. Then filter, it with a paper filter, so substance liquid (filtrate) and dregs Simplicia (debris) were obtained. Then it evaporated with a rotary evaporator at 40°C for 1×24 hours.¹⁵

In Vitro Procedure

Cell Preparation

Cryotubes containing cholesteatoma fibroblast cells from nitrogen cylinders were taken it out. Then, thaw the cryotube in a water bath until melted (1-2 minutes), and move the whole cell suspension in cryotubes to a 15 ml tube containing 9 ml of complete DMEM + FBS + AA. After that, they were centrifuged at 1500 RPM for 7 minutes. Discarded supernatant and pellet, resuspension with 1 ml of complete medium, and subsequent cell enumeration. The expansion was carried out on the flash T75. After cell confluent > 80%, then harvest. Then cells were seeded on 96 and 24 healthy plates for given treatment t.¹⁶

Experimental Procedure

The treatment in this research involving eight groups of cholesteatoma fibroblasts consisting of a negative control group (DMEM+FBS), DMEM group, two positive control groups dexamethasone (10 µM and 100 µM), and four green tea leaf extract groups (10 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml). Cholesteatoma fibroblast cells are cultured in 96 healthy plates for the cell viability test and 24 well plates for the cell migration test. After the cells attach and grow stable for the next 1x24 hours, cells can be given treatment. We weighed as much as 0.03925 grams of powder dexamethasone (Sigma-Aldrich) and dissolved it into 10 ml of complete DMEM to obtain a dose stock of 10.000 µM. Then dilute as much as 25x so the dose stock to 400 µM and can dilute return to get the dose dexamethasone under treatment. Green tea leaf Extract weighed as much as 10 mg and dissolved in DMEM as much as 10 ml. The dose solution is 1.000 µg/ml. Then, adjusted return the dilution in accordance need dose treatment. After the dexamethasone and green tea leaf extract dose was ready, then added 100 µl group treatment dexamethasone and green tea leaf extract on each well that contains 100 µl of cells.

Cell Viability Test

It prepared culture cells that have been given treatment. Prepared solution 35 μI of Hoechst staining with 3.465 μI

of DMEM (1:99 dilution) so that the total mixture obtained is 3.500 μ l. Then, the medium contained in the 96 healthy plates was thrown away, and a solution mixture of 100 μ l was added to each healthy plate. Incubated for 5-10 minutes and protected from the light. Observed with microscope fluorescence and counted the amount. The viable cells are blue. Cell viability is the number of living cells, usually expressed as a percentage of the number in the control group.¹⁷

Cell Migration Test

Cells were grown to a density of 80% in 24 healthy plates and then scratched the cell colonies using a sterile yellow tip to break the connection between the cell colonies. They were rinsed using PBS. After that, they are given treatment and evaluated cell migration ability by measuring streaks and taking micrographs using a digital microscope. Migration micrograph proportion measurements were at 0 hours, 8 hours, 24 hours, 48 hours, and 72 hours. Images are always taken in the same area on all test samples. The area of the scratches was measured using the Image J Application.¹⁸

Statistical Analysis

The data obtained were analyzed with One-Way ANOVA and Kruskal Wallis.

Ethical Considerations

This research has been approved by the research ethics committee of YARSI University, number 328/KEP-UY/BIA/XI/2022.

RESULTS

Figure 1 shows that cell viability in green tea leaf extract at doses of 10 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml was less than the negative control group (DMEM+FBS). The higher the green tea leaf extract dose, the lower the cell viability. Table 1 shows the highest average cholesteatoma cell viability in the negative control group (DMEM+FBS) with an average of 165.33 and the lowest average in the green tea leaf extract group at a dose of 160 µg/ml with an average of 70.88. Figure 2 shows that the green tea leaf extract groups at doses of 10 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml were significantly different from the negative control group. It can be seen that green tea leaf extract at doses of 10 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml can reduce the viability of cholesteatoma fibroblast cells. The ability to reduce the viability of cholesteatoma fibroblast cells was statistically significant (p<0.05).

Figure 3 shows that in the green tea leaf extract group at doses of 10 μ g/ml, 40 μ g/ml, 80 μ g/ml, and 160 μ g/ml was slower closing of the scratch area than the negative control group (DMEM+FBS). Table 2 shows that at 8, 24, 48, and 72 hours in the negative control group, there was faster closure of the scratch area and the slowest on green tea leaf extract at a dose of 160 μ g/ml. This shows that there was no cell migration inhibitory activity against cholesteatoma fibroblast cells in the negative control group, there was cell migration inhibiton activity. Figure 4 shows the best-



Figure 1. Comparison of Cholesteatoma Fibroblast Viability in the Treatment Group Using Fluorescence Microscopy. A (DMEM+FBS); B (DMEM); C (Dexamethasone 10 µM); D (Dexamethasone 100 µM); E (Green Tea Leaf Extract 10 µg/ml); F (Green Tea Leaf Extract 40 µg/ml); G (Green Tea Leaf Extract 80 µg/ml); H (Green Tea Leaf Extract 160 µg/ml).

cell migration inhibition activity with the green tea leaf extract group at a 160 μ g/ml dose. In contrast, the negative control group had no cell migration inhibitory activity against cholesteatoma fibroblast cells.



Figure 2. Graph of Comparison of Cholesteatoma Fibroblast Viability in the Treatment Group. (#) The negative control group; (*) The green tea leaf extract group, which reduced cell viability and was significantly different from the negative control group (p<0.05).



Figure 3. Treatment of Dexamethasone 100 μ M (Dex-100) and Green Tea Extract 160 μ g/ml (GTE-160) Inhibit Migration Cell



Figure 4. Graph Comparison of Cell Migration in the Treatment Group

DISCUSSION

Cell viability is the ratio between living cells and the total number of cells. Cell viability is the number of living cells, usually expressed as a percentage of the number in the control group.¹⁷ Cholesteatoma fibroblast cell viability showed the highest average in the negative control group (DMEM+FBS) with an average of 165.33 and the lowest

average in the green tea leaf extract group at a dose of 160 µg/ml with an average of 70.88. The value of the negative control group was much more extensive and significantly different compared to the positive control group (dexamethasone at doses of 10 µM and 100 µM) and the green tea leaf extract group at doses of 10 µg/ml, 40 µg/ml, 80 µg/ml, and 160 µg/ml. Cell migration at 72 hours showed that in the negative control group, there was faster closure of the scratch area and the slowest on green tea leaf extract at a dose of 160 µg/ml. Green tea contains >200 bioactive compounds, including polyphenols, caffeine, theanine, vitamins, and minerals. The most abundant bioactive compounds in green tea are catechins.¹⁹

The most significant bioactive components contained in tea are polyphenols such as epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG).20 The content of EGCG in green tea is known to reduce the viability of peripheral blood mononuclear cells in people with psoriasis.11 Cell migration is a process involved in various biological mechanisms, such as cancer progression. Migration ability in cancer cells plays an essential role in cell invasion and metastasis.¹⁰ EGCG in green tea extract has been shown to inhibit the inflammatory response (in vitro) in different cell types in animal model studies for arthritis therapy development. EGCG, which is the most polyphenol in green tea, can inhibit the activity of enzymes and signal transduction pathways that play an essential role in the inflammatory process.²¹ Dexamethasone is a drug that belongs to the corticosteroid class which has anti-inflammatory activity.22

Based on the results of the research in Table 1, when compared between the dexamethasone group at doses of 10 μ M and 100 μ M with the green tea leaf extract group at doses of 80 μ g/ml and 160 μ g/ml, the viability test results showed that green tea leaf extract was better at reducing the viability of cholesteatoma fibroblast cells. If seen from the statistical test of green tea leaf extract at the dose of 160 μ g/ml, it was significantly different compared to the negative control group and dexamethasone at doses of 10 μ M and 100 μ M. This shows that green tea leaf extract at the dose of 160 μ g/ml has a better ability to reduce cell viability and is thought to have lower side effects than dexamethasone.

Cell migration assay shows that the dexamethasone group did not differ significantly in reducing the migration of cholesteatoma fibroblast cells when compared to the green tea leaf extract group. This shows that green tea leaf extract has the same effect as dexamethasone in inhibiting the migration of cholesteatoma fibroblast cells. Besides being easy to obtain, herbal plants also have relatively low side effects and are safe for disease prevention and treatment.²³

Treatment Group	Average	Percentage	Standard	
	Viability	Viability	Deviation	
DMEM+FBS	165.33	100%	15.50	
DMEM	141.66	85.69%	4.90	
Dex-10 (Dexamethasone 10 µM)	123.32	74.59%	8.64	
Dex-100 (Dexamethasone 100 µM)	106.77	64.58%	2.83	
GTE-10 (Green Tea Leaf Extract 10 µg/ml)	120.44	72.85%	1.52	
GTE-40 (Green Tea Leaf Extract 40 µg/ml)	117.88	71.30%	0.42	
GTE-80 (Green Tea Leaf Extract 80 µg/ml)	84.22	50.94%	1.38	
GTE-160 (Green Tea Leaf Extract 160 µg/ml)	70.88	42.88%	1.88	

Table 1. Cholesteatoma Fibroblast Viability (n=3)

Table 2. Percentage of Closure Area of Cell Migration 8 Hours, 24 Hours, 48 Hours, and 72 Hours

Treatment Group	% Closure of Cell Migration			
	8 Hours	24 Hours	48 Hours	72 Hours
DMEM+FBS	11.54	23.18	61.22	97.78
DMEM	7.22	17.45	37.44	53.94
Dex-10 (Dexamethasone 10 µM)	3.40	7.26	16.41	22.49
Dex-100 (Dexamethasone 100 µM)	1.32	3.50	9.75	16.18
GTE-10 (Green Tea Leaf Extract 10 µg/ml)	3.23	7.13	16.20	22.09
GTE-40 (Green Tea Leaf Extract 40 µg/ml)	2.92	7.08	14.49	19.98
GTE-80 (Green Tea Leaf Extract 80 µg/ml)	1.06	3.18	9.43	14.55
GTE-160 (Green Tea Leaf Extract 160 µg/ml)	0.99	2.34	9.11	13.81

The negative control group did not significantly compare to the group given DMEM alone. This can happen because DMEM is a suitable medium for tumor cells, so the cholesteatoma fibroblast cells can proliferate without requiring serum. DMEM is a suitable medium for tumor cells with fast growth rates.²⁴ EGCG is reported to function as an antibacterial, antitumor, antioxidant, anti-inflammatory, and antiviral.²⁵ Aside from being an antioxidant, the active substances contained in green tea also stop the pathogenesis of cancer cells before they cancer cells undergo the process of metastasis. EGCG is the most considerable polyphenol in green tea, which can cause apoptosis (programmed cell death) and stop the cell cycle in cells that have experienced DNA damage (cancer cells).²⁶ In line with previous research, EGCG is known to reduce cell viability and increase apoptosis which triggers mitochondrial damage in HCT116 and HT-29 cancer cells.²⁷

Based on the results of this research, there was a significant decrease in cell viability (living cells) in the group given green tea leaf extract. This is probably due to apoptosis in cholesteatoma fibroblast cells caused by the EGCG content in the green tea leaf extract. Green tea plays a role in cancer chemoprevention by triggering cell apoptosis, preventing cell hyperproliferation, detoxifying carcinogens, preventing signaling for cell hyperproliferation, and preventing oxidation-reduction (redox) reactions. EGCG plays a role in causing apoptosis by activating the caspase-3 enzyme, a protease that can break down proteins, causing DNA fragmentation and depolarization of the mitochondrial membrane so that it can release cytochrome c into the cytosol. Cytochrome c will then form bonds with cytosolic proteins that will cause caspase activation, which causes apoptosis.²⁸

EGCG has the potential to an anti-carcinogenic and can reduce the migration of breast cancer cells. The role of green tea on p53 is mediated by cytotoxicity and inhibition of tumor cell migration.²⁶ In line with previous research, EGCG can modulate p53 levels and reduce the viability and migration of breast cancer cells.¹³ Cell viability and migration relate to the inflammatory response. When cell viability and migration decrease, it is hoped that the inflammation in cholesteatoma fibroblast cells can be suppressed. EGCG belongs to the nutraceutical group suggested as a safe alternative for osteoarthritis (OA) therapy against inflammation, aging, and cancer.²⁹ EGCG in green tea can inhibit the activity of enzymes and signal transduction pathways that play an essential role in the inflammatory process.²¹ Flavonoids can suppress macrophage activity by inhibiting inflammatory enzymes, namely cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The inhibition of the COX-2 inflammatory enzyme causes inhibition of inflammation.30

CONCLUSIONS AND RECOMMENDATION

Green tea leaf extract has been shown to reduce the viability and migration in cholesteatoma fibroblast of CSOM. Green tea leaf extract can be used as an alternative for cholesteatoma prevention. In future studies, it is necessary to test for apoptosis and pro-inflammatory cytokines to assess the ability of green tea leaf extract to cause programmed cell death and reduce inflammation.

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