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Effects of Green Tea Leaf Extract on Viability, Apoptosis, and Expression of Interleukin-6 in Keloid Fibroblasts

Mariah Shofwah Fasihah[1], Restu Syamsul Hadi[2]*, Samsul Mustofa[2]
[1]Faculty of Medicine, YARSI University, Jakarta, Indonesia
[2]Department of Biology-Anatomy, Faculty of Medicine, YARSI University, Jakarta, Indonesia

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***CORRESPONDENCE:**
restuhadi@gmail.com

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Abstract: Green tea leaf (*Camellia sinensis*) extract contains polyphenols, anti-inflammatory and anti-carcinogenic agents that may effectively to as keloid therapy. This study aims to examine the effect of green tea leaf extract on viability, apoptosis, and *interleukin*-6 expression in keloid fibroblasts. Keloid fibroblast cultures were divided into groups that were cultivated with green tea extract at dosages of 100, 200, 400, and 800 µg/mL, negative control (10% Fetal Bovine Serum/FBS), and positive control (100 µM dexamethasone). This study used post-test-only control group design. The highest mean IL-6 expression was in the GTE 200 (275,942.7 pg/mL), while the lowest was in the GTE 800 (-2,890.6 pg/mL). The viability and apoptosis was analized by using one-way ANOVA, continued with *Least Significant Difference* (LSD) with a significance of p <0.05 in the GTE 800 treatment group compared to the entire intervention group. Kruskal-Wallis followed by a Mann-Whitney was carried out for *interleukin*-6 (IL-6) with a significance of p <0.05 in the extract therapy group at dosages of 200, 400, 800 µg/ml and 100 µM dexamethasone. In conclusion, green tea leaf extract at 800 µg/ml could reduce viability *interleukin*-6 expression and increase apoptotic *caspase*-3 expression in keloid fibroblast cells.

Keywords: keloid fibroblast; green tea; anti-inflammatory; *caspase*-3; apoptotic cell

INTRODUCTION

Keloid is a dermal fibroproliferative disorder caused by abnormal wound healing and excessive collagen deposition.[1] Keloid cases can be found worldwide, with an incidence of 0.09-16%.[2] Keloids are more common in people aged 20-30 and those with high hormone levels, such as during puberty and pregnancy.[3] Besides causing appearance disturbances, keloids are accompanied by complaints of itching and pain.[4]

Existing keloid treatments have painful procedures, are expensive, are only used on keloids of small size, and require repeated procedures. Thus, new therapies, especially with natural ingredients, are needed to overcome keloids. Research by Park *et al*. stated that *Epigallocatechin-3-Gallate* (EGCG) in green tea could suppress collagen production and proliferation in keloid fibroblasts through Phosphoinositide 3-kinases (PI-3Ks), *Mitogen-activated protein kinase* (MEK)/ *extracellular signal-regulated kinase* (ERK), and *signal transducer and activator of transcription* 3 (STAT-3) signaling pathways.[5] Other studies also revealed that EGCG can inhibit cell viability and induce apoptosis in several cancer cell lines, such as human hepatocellular carcinoma (HCC) HepG2 cells.[6] Several previous studies have not examined the effect of green tea leaf extract on viability, apoptosis, and interleukin-6 expression in keloid fibroblasts.

Keloids develop during the proliferative phase of the wound-healing process. Fibroblast and myofibroblast cells lead to overproduction of extracellular matrix proteins.[7] During the normal maturation phase of wound healing, the connective tissue segments shrink after three weeks. In keloids, collagen synthesis is about 20 times higher than in normal skin and about three times higher than in hypertrophic scars. Not only that, the ratio of collagen types I and III is also increased.[8]

Some studies report that green tea (*Camellia sinensis*) contains high catechin-containing polyphenols, known as anti-oxidant, anti-carcinogenic, anti-microbial, and anti-inflammatory agents.[9] Green

tea is known to contain relatively high EGCG. It can reduce proinflammatory cytokines such as Tumor Necrosis factor alpha (TNF-α), Interleukin 1beta (IL-1β), IL-8, and IL-6, which are crucial in the inflammatory process. Based on previous research, there was an increase in the expression of IL-6 and its receptors in keloid fibroblasts, with a concomitant increase in collagen biosynthesis[10]. Aberrant regulation of apoptosis is also researchers' hypothesis on how keloids appear. Researchers showed a decrease in gene-related apoptosis in human keloid tissue and a reduction in apoptotic activity in keloid-derived fibroblasts compared to normal scars.[11,12]

In the inflammatory phase of wound healing, IL-6 increases and initiates excessive signaling of Janus Kinase (JAK)/ Signal Transducer and Activator of Transcription (STAT) and Mitogen Activated Protein Kinase (MAPK) pathways in fibroblast migration to the wound area[13], leading to fibroblast hyperproliferation and collagen accumulation.[14] Fibroblasts that differentiate into myofibroblasts can survive in the wound area, cause contracture, and resist apoptosis through anti-apoptotic Bcl-2 signaling.[15,16] As a result, fibroblasts in keloids experience decreased apoptosis and increased viability. Thus, this study aims to examine the effect of green tea leaf extract on viability, apoptosis, and *interleukin*-6 expression in keloid fibroblasts *in vitro*.

MATERIAL AND METHOD

Cell Preparation

This study was conducted using experimental methods in vitro in the cell culture lab of the Integrated Research Institute of YARSI University (LPTUY). The sample was fibroblast cells from human keloid tissue isolation. Keloid fibroblast (KF) cell cultures were grown in 96 and 24 well culture dishes that were given growth media, namely Gibco *Dulbecco's Modified Eagle Medium* (DMEM), which has been mixed with *Fetal Bovine Serum* (FBS) 10% and *Antibiotic-Antimicotic* (AA) 1% and then incubated in a 5% Co_2 incubator, 37°C with a cell count of 10,000 cells/cm^2. [17] Each was divided into six treatment groups with four replicates, namely: DMEM-serum control (KS) as negative control, dexamethasone 100 µM (Dexa 100) as positive control, and four groups of green tea leaf extract 100 µg/mL (GTE 100), 200 µg/mL (GTE 200), 400 µg/mL (GTE 400) and 800 µg/mL (GTE 800).

Extract Preparation

Weighed thick green tea leaf extract of 0.02 g was dissolved with 10 mL of complete DMEM.[18] A stock solution of 2000 ppm of green tea leaf extract was obtained, then continued to make working solutions for treatment at concentrations of 100 µg/mL; 200 µg/mL; 400 µg/mL; 800 µg/mL.[19]

Cell Viability Test

A prepared solution 300 µL of Hoechst was made in 2700 µL DMEM (1:9 dilution) for a 3000 µL solution. The DMEM was discharded in the wells, and cells were washed with Gibco *Phosphate Buffer Saline* (PBS). Cells were added with 100 µL of Hoechst dye to each well and then covered with aluminum foil. Cells were incubated at room temperature for 5-10 minutes then cells were washed with PBS (optional). Live cells will look blue and be counted using an EVOS fluorescence microscope. [20]

Caspase-3 Apoptosis Test

This test using the colorimetric assay kit (Caspase 3 Activity Assay Kit, by Colorimetric, Elabscience). One million KF cells per treatment group should be detached with trypsin and then collected sedimentary cells. Collect the cells, centrifuge at 2000 rpm for 5 minute and discard the supernatant. Cells were resuspended with PBS for 1 mL, then cells were counted. The cells were centrifuged after counting at 2000 rpm for 5 minute and the supernatant was discarded. 50 µL cold Lysis Buffer working solution was added to resuspend the cells. Incubate in ice bath for 30 minute and oscillate 3~4 times during incubation.

The sample was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed and stored in a new tube. In the new tube, 50 µL 2x Reaction working solution, 45 µL sample, 5 µL Ac-DEVD-pNA for a total of 100 µL solution was added, and one tube was provided for a blank solution containing 50 µL 2x Reaction working solution, 45 µL lysis working solution, and 5 µL Ac-DEVD-pNA (100 µL total). At 37°C for 2~4 hours. The samples were incubated and the OD value (A405) was measured at 405 nm using a 100 µL cuvette spectrophotometer.[21]

Interleukin-6 Test

Reagents and samples were prepared at room temperature (18 - 25°C) before use. It was recommended that all standards and samples run in duplo. 100 µL of each standard and sample was added

into the appropriate well. Wells were capped and incubated for 2.5 hours at room temperature or overnight at 4°C with gentle shaking. The solution was discarded and washed with 1x Wash Solution four times. We washed each well with Wash Buffer (300 µL) using a multi-channel Pipette. After the last wash, the remaining Wash Buffer was removed by aspirating or pouring.

The plate was tipped over and cleaned with a clean paper towel. 1x detection antibody of 100 µL was added into each prepared well and incubated for 1 hour at room temperature, and the well was shaken gently and covered with aluminum foil. The solution was discarded and washed with 1x Wash Buffer (300 µL). 100 µL of prepared Streptavidin solution was added to each well. Incubation was carried out for 45 minutes at room temperature, shaken gently, and the well was closed. The solution was discarded and washed with 1x Wash Buffer (300 µL). The dose 100 µL of TMB One-Step Substrate Reagent was added. The well was capped and incubated for 30 minutes at room temperature in the dark, shaking gently. 50 µL Stop Solution was added to each well. Absorbance was immediately read at 450 nm.[22]

Data Analysis

This research is a quantitative study, with an experimental method of post-test-only control group design used as a data collection method.[23] All data obtained were then described with mean ± SD. In the viability and apoptosis testing, one-way ANOVA statistical analysis was carried out using SPSS because the data obtained was normally distributed and continued with post-hoc *Least Significant Difference* (LSD) analysis. The data obtained in the *interleukin*-6 (IL-6) test was not normally distributed. Therefore, Kruskal-Wallis statistical analysis was used, followed by the Mann-Whitney test.

RESULT

The following are the results of studies conducted on viability, apoptosis, and IL-6 expression in keloid fibroblasts. Based on the Figure 1 presented, the study's results showed the GTE 100, GTE 200, GTE 400, and Dexa 100 treatment groups did not significantly decrease viability against negative control (KS). It can be seen from the histogram that those who experienced a significant decrease in viability after treatment were the GTE 800 group, but the Dexa 100 treatment group, which was expected to decrease against KS, showed no decrease in viability.

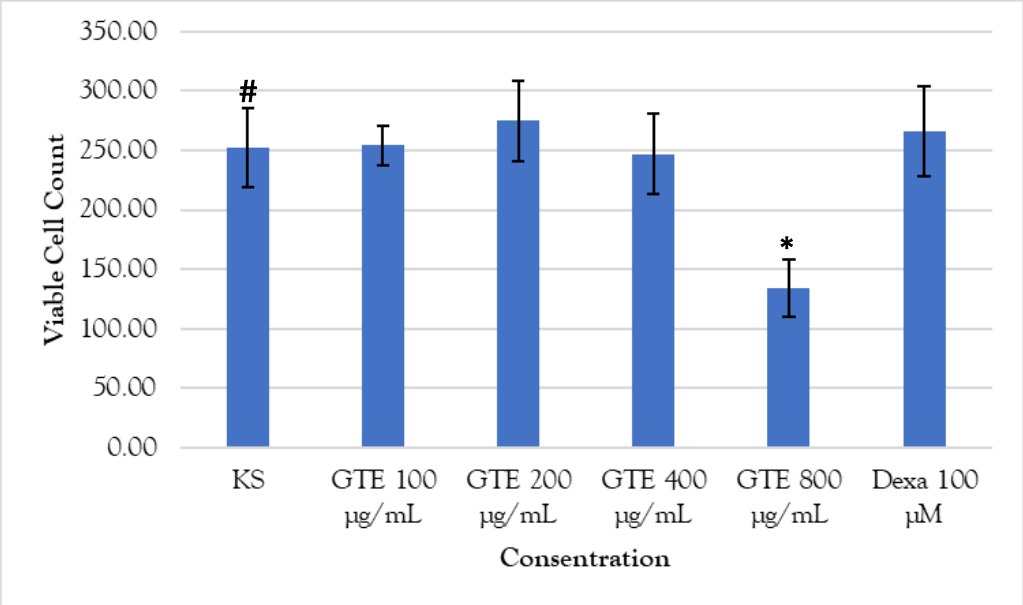

Figure 1. Graph of Green Tea Leaf Extract (*Camellia sinensis*) on Viability of Keloid Fibroblasts

Figure 2 shows viability using fluorescence microscopy when compared between treatment groups. KS, GTE 100, GTE 200, GTE 400, and Dexa 100 showed no significant difference in cell viability. Conversely, in the GTE 800 treatment group, the number of living cells looks less when compared to negative control (KS) and all existing treatments.

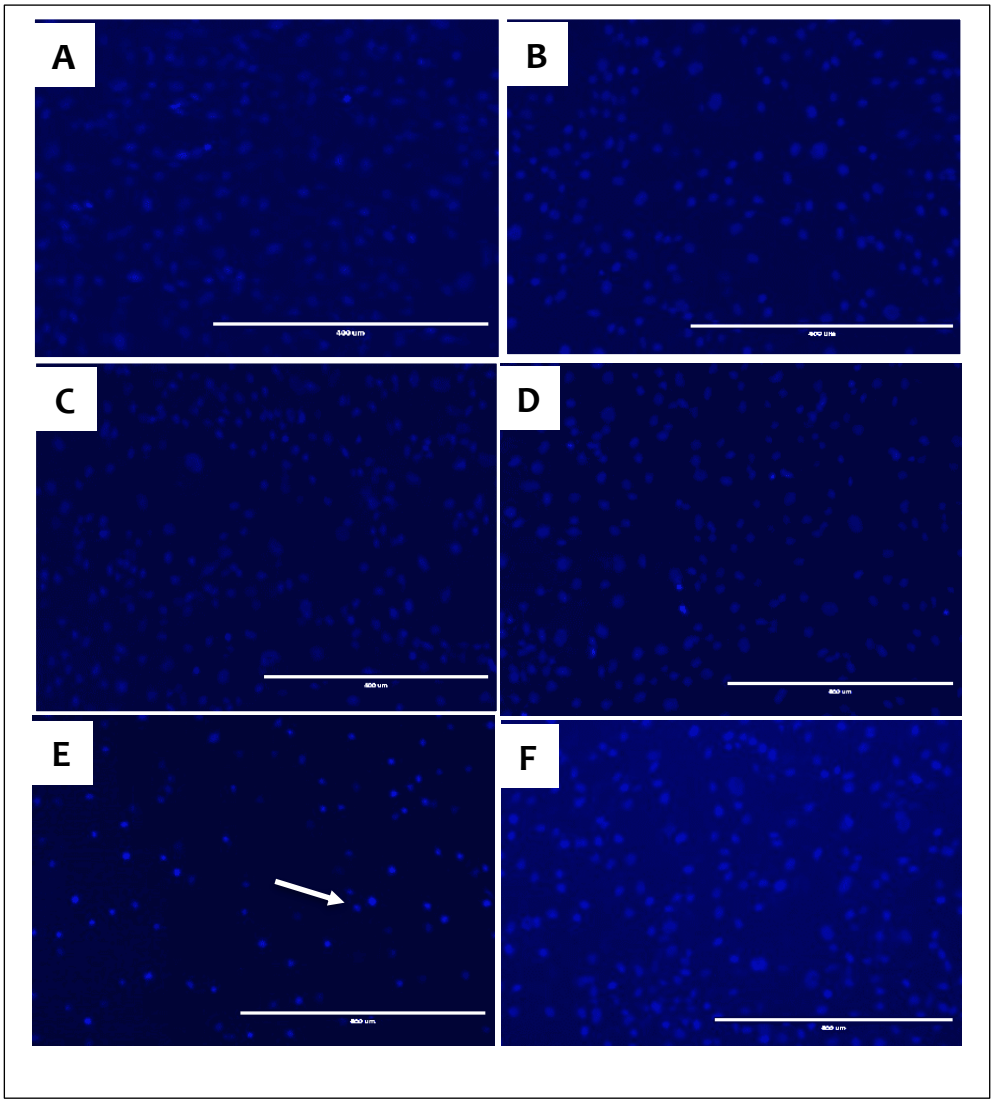

Figure 2. Comparison of Keloid Fibroblast Viability in Each Treatment Group Using Fluorescence Microscopy (400 µm). Live cells appear as blue-coloured spheres, indicated by white arrows. A (Negative Control / KS); B (Green Tea Leaf Extract 100µg/mL); C (Green Tea Leaf Extract 200 µg/mL); D (Green Tea Leaf Extract 400 µg/mL); E (Green Tea Leaf Extract 800 µg/mL); F (Dexamethasone 100 µM).

The apoptosis test result can be noted in the histogram presented that the expression of *caspase*-3 produced for 100.000 cells is highest to lowest in the GTE 800 treatment group, followed by the GTE 200 treatment group, then GTE 400, then GTE 100, and Dexa 100. Finally, the least expressing *caspase*-3 is the KS (negative control) group. Hence, we can conclude that the GTE 800 treatment group significantly increased *caspase*-3 expression compared to the KS group (negative control). Based on the data obtained, there is a significant difference in caspase-3 expression produced by 100.000 cells to the GTE 800 treatment group, with all other treatment groups, indicating that the 800 µg/mL dose green tea extract group can increase *caspase*-3 expression in 100.000 keloid fibroblast cells.

The results of viability testing were carried out using statistical tests using the One-Way ANOVA test and continued with the Post Hoc LSD test. The following are the results of statistical tests on all treatment groups.

Table 1. Statistical Results of Green Tea Leaf Extract on The Viability of Keloid Fibroblasts

Treatment Groups	n	Average	SD	*One-Way* Anova (*p-value*)	*Post-Hoc* LSD (*p-value*)					
					KS	GTE 100	GTE 200	GTE 400	GTE 800	Dexa 100
KS	4	251.83	33.34	0.000		0.923	0.327	0.832	0.000*	0.550
GTE 100	4	254.08	16.55				0.376	0.757	0.000*	0.616
GTE 200	4	274.83	33.63					0.238	0.000*	0.696
GTE 400	4	246.92	33.86						0.000*	0.421
GTE 800	4	134.17	24.04							0.000*
Dexa100	4	256.75	37.70							

Note:
* = There is a significant difference with a p-value <0.05

The statistical results in Table 1 show a p-value <0.05, which means Ho is rejected or there is a significant difference in viability between the GTE 800 treatment group and all existing treatment groups (KS, GTE 100, GTE 200, GTE 400, and Dexa100) with the average viability amounting to 134.17. The GTE 100 treatment group, compared to other treatment groups, had a p-value> 0.05, which indicates that there was no significant difference in viability between the GTE 100 treatment group and the KS (negative control), GTE 200, GTE 400, and Dexa100 treatment groups. The GTE 200, GTE 400, and Dexa100 treatment groups, compared to other treatments, also had a p-value> 0.05, indicating that there was no significant difference in viability.

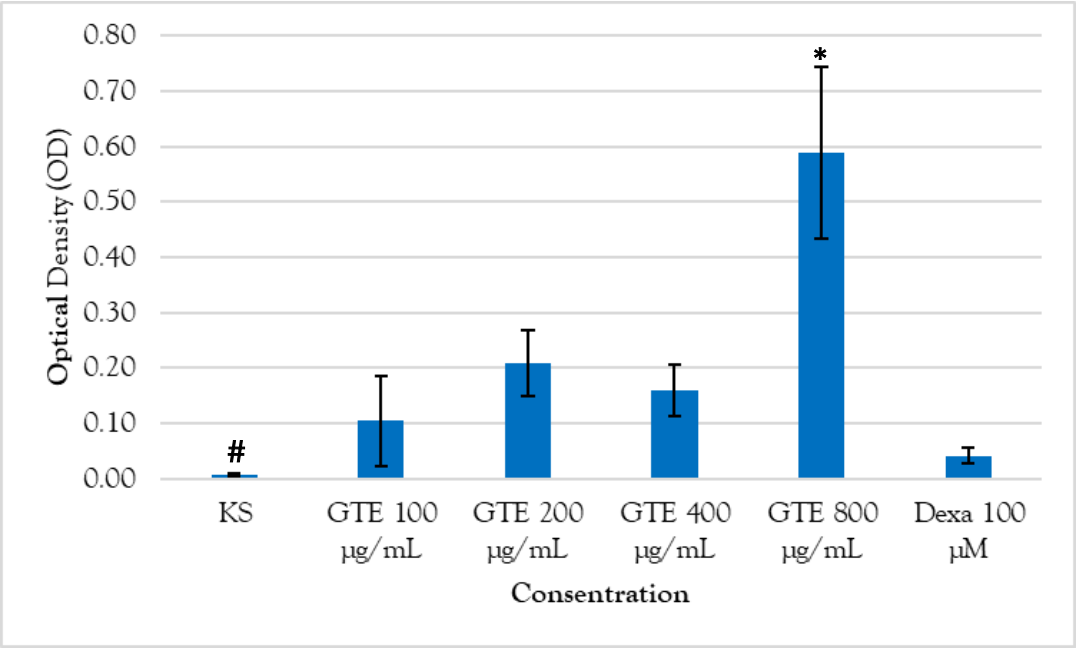

Figure 3. Graph of Green Tea Leaf Extract (*Camellia sinensis*) on *Caspase*-3 Expression of Keloid Fibroblasts

The results from the graph presented demonstrated that testing of IL-6 expression revealed that the GTE 800 and Dexa 100 treatment groups significantly decreased against KS. In the GTE 100, GTE 200, and GTE 400 treatment groups, IL-6 expression increased compared to the KS group.

Table 2. Statistical Results of Green Tea Leaf Extract on *Caspase*-3 Apoptosis of Keloid Fibroblasts

Treatment Group	n	Average	SD	*One-Way* ANOVA (*p-value*)	*Post-Hoc* LSD (*p-value*)					
					KS	GTE 100	GTE 200	GTE 400	GTE 800	Dexa 100
KS	2	0.006	0.003	0.003		0.256	0.041*	0.097	0.000*	0.670
GTE 100	2	0.104	0.082				0.227	0.504	0.001*	0.450
GTE 200	2	0.209	0.059					0.549	0.003*	0.075
GTE 400	2	0.160	0.046						0.002*	0.180
GTE 800	2	0.589	0.155							0.000*
Dexa 100	2	0.041	0.014							

Note:
* = There is a significant difference with a p-value <0.05

Based on the data presented in Table 2, the results of statistical tests, it can be seen that the highest mean of caspase-3 expressed per 100,000 cells from the GTE 800 treatment group was 0.589, with a significance value of $p < 0.05$, which means that the GTE 800 treatment group had a significant difference. Statistically for all treatment groups (KS, TH100, TH200, TH400, Dexa100). When compared with other treatment groups, GTE 100, GTE 400, and Dexa100 show a significance value of $p > 0.05$, which means there is no significant difference in the KS (negative control) group.

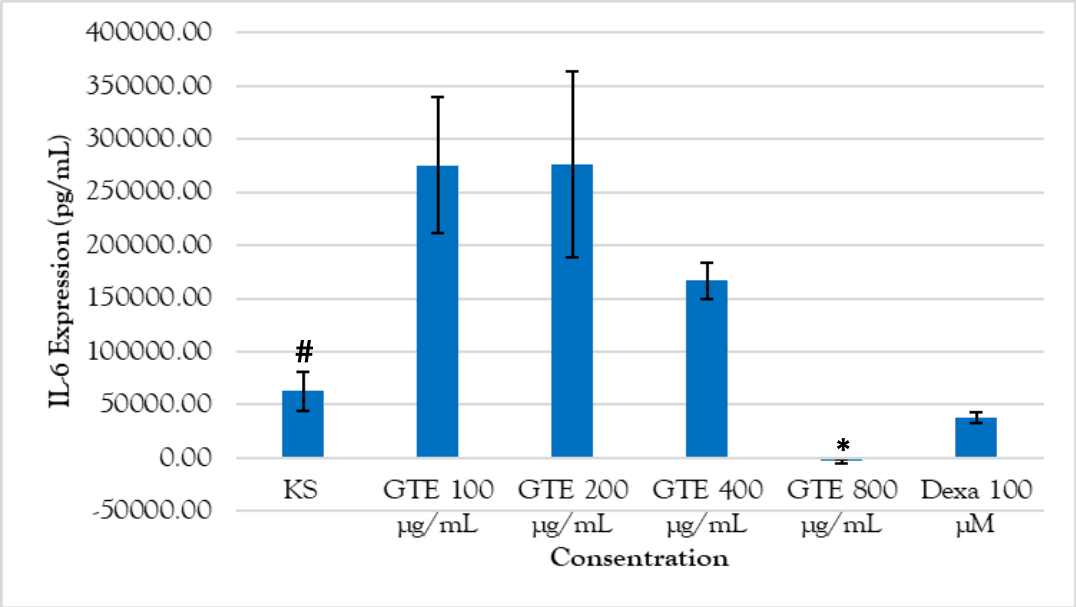

Figure 4. Graph of Green Tea Leaf Extract (*Camellia sinensis*) on *Interleukin*-6 Expression of Keloid Fibroblasts

Table 3. Statistical Results of Green Tea Leaf Extract on *Interleukin*-6 (IL-6) Expression of Keloid Fibroblasts

Treatment Groups	n	Average (pg/mL)	SD	Kruskal Wallis (*p-value*)	Mann-Whitney (*p-value*)					
					KS	GTE 100	GTE 200	GTE 400	GTE 800	Dexa 100
KS	4	62,682.3	18796,38	0.000		0.021*	0.021*	0.021*	0.021*	0.021*
GTE 100	4	275,078.1	63780,57				0.773	0.021*	0.021*	0.021*
GTE 200	4	275,942.7	87274,80					0.021*	0.021*	0.021*
GTE 400	4	166,588.5	17112,93						0.021*	0.021*
GTE 800	4	-2,890.6	1982,01							0.021*
Dexa 100	4	37,791.7	4883,274							

Note:
* = There is a significant difference with a p-value <0.05

Based on the statistical tests that have been carried out, the GTE 200, GTE 400, GTE 800, and Dexa100 treatment groups have a significance value of $p < 0.05$, which means there is a significant difference to the KS (negative control) group. From the data above, it can be seen that the highest mean IL-6 expression was in the GTE 200 treatment group at 275,942.7 pg/mL, while the lowest IL-6 mean was in the GTE 800 treatment group with a mean value of -2,890.6 pg/mL. Based on the statistical tests that have been carried out, the GTE 100 and GTE 200 treatment groups have a significance value of $p > 0.05$, which means there is no significant difference between the two groups compared to the other treatment groups.

From the data presented, the GTE 100, GTE 200, and GTE 400 treatment groups showed increased IL-6 expression and had a higher mean compared to the KS (negative control) group. The 100 µM dexamethasone treatment group experienced a decrease in IL-6 expression when compared with the KS group, with a statistical test result of $p < 0.05$, which means there was a significant difference between the Dexa100 group and all treatment groups (KS, GTE 100, GTE 200, GTE 400, GTE 800).

DISCUSSION

In several laboratory studies, polyphenols in green tea have been shown to have anti-cancer activity, which could be mediated through antioxidant or pro-oxidant mechanisms. Supported by previous research, green tea leaf extract used in this study can inhibit growth, proliferation, and differentiation in HepG2 cells and induce cell apoptosis.[6,24] A significant decrease in viability after treatment was the GTE 800. This study aligns with research conducted by El Naga *et al.* [6] and Lambert *et al.* [25], that EGCG induces apoptosis in cell culture by binding to anti-apoptotic proteins Bcl-2 and Bcl-xL. Increased apoptotic activity resulted in decreased viability in keloid fibroblasts.

In viability test result, it was seen that the Dexa 100 treatment group, which acted as a positive control group, showed a higher mean compared to the GTE 100 and GTE 400 treatment groups. In line with previous research by Syed *et al*., it showed that dexamethasone induces CTGF overexpression. Therefore, dexamethasone also exerts undesirable effects by stimulating the expression of keloid genes and proteins that promote CTGF. Connective Tissue Growth Factor (CTGF) is a signaling molecule. It plays a central role in various biological processes, such as cell proliferation, angiogenesis, and wound healing, as well as in multiple pathologies, such as tumor development and tissue fibrosis. [10,26]

In wound healing, there should be a balance between cell proliferation and apoptosis. However, apoptosis and proliferation were altered and unbalanced in keloid fibroblasts.[27] The caspase family has been the subject of intensive research in drug design for many years. Currently, 14 mammalian caspases have been identified, including caspases -2, -3, -6, -7, -8, -9, and -10, called apoptotic caspases.[28] One of the executor caspase proteins involved in the apoptotic mechanism is caspase-3, which is crucial in processing the cell apoptotic cascade through intrinsic and extrinsic pathways.[29]

The increased expression of caspase-3 in this study is thought to be due to polyphenols in green tea extract, especially EGCG, which is induced into keloid fibroblast culture and initiates apoptosis, which is detected by death receptors, one of which is CD 95 (Fas) on the cell surface which will activate caspase-8 as an initiator. This caspase works by blocking the work of Bcl-2 and inducing Bax into the mitochondrial membrane, then releasing cytochrome c, which acts as a pro-apoptotic into the cytoplasm.[30]

Based on the data obtained, the highest IL-6 levels were shown in the GTE 200 treatment group. The lowest IL-6 levels were found in the GTE 800 treatment group; there were significant differences in the results of IL-6 testing in keloid fibroblasts between the KS group and the GTE 800 treatment group. Thus, in this study, the GTE 800 treatment group is thought to reduce IL-6 expression in keloid fibroblasts.

Epigallocatechin-3-gallate (EGCG) in its natural form or wound dressings inhibits the production of specific pro-inflammatory cytokines released into cell supernatants such as TNF-α, IL-1β, and IL-8. In the inflammatory phase (first 48 hours), the wound involves many hemostasis responses, including recruiting neutrophils, monocytes, and macrophages and activating inflammatory factors such as cytokines and chemokines. Neutrophils recruited during wounding regulate the expression of chemokine genes such as TNF-α, IL-1β, IL-6, and IL-8, recruiting more macrophages, T cells, and neutrophils, which in turn increase angiogenesis and proliferation of fibroblasts and keratinocytes.[31] Therefore, EGCG plays a role in inhibiting neutrophil infiltration, migration, and adhesion of monocytes. The anti-inflammatory effects of EGCG were also confirmed in animal studies when levels of IL-1β, TNFα, and IL-6 in wound tissue decreased.[32,33]

In this study, the GTE 100, GTE 200, and GTE 400 treatment groups had higher IL-6 expression than the negative control group (KS). Green tea leaf extract at 100, 200, 400 µg/mL doses may increase interleukin-6 expression. This study is in line with the research of Haghighatdoost & Hariri [34] revealing that green tea can increase IL-6 expression at specific doses. The research conducted by Luo *et al* unexpectedly found a hormetic effect on CES1 given EGC and EGCG on S9 rat liver in vitro. [35] Hormetic effects are stimulatory effects at low concentrations and inhibitory effects at high concentrations. Hormetic responses are reported in more than 100 highly diverse chemical and physical agents, with some common dietary supplements, such as resveratrol and EGCG, showing a biphasic dose response in various stem cell types. [36]

CONCLUSION

It can be concluded that the dose of green tea leaf extract at 800 µg/mL could reduce viability, increase caspase-3, and reduce the expression of interleukin-6 in keloid fibroblast cells.

CONFLICT OF INTEREST

We stated that there is no conflict of interest between the authors.

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