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The Effect of Alpha Mangostin on The Expression of TGF-β1, SMAD3, Type I Collagen, Proliferation and Migration of Keloid Fibroblasts

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ABSTRACT

Background: Keloid is a process of abnormal collagen thickening during wound healing in skin tissue accompanied by the formation of new blood vessels. Many keloid therapy modalities have been developed but the recurrence rate of those treatment still ranged 1-70%. Herbal plants have been developed for various types of treatment, one of which is for treating keloids. **Purpose:** The alpha mangostin content in mangosteen peel is known to have antifibrotic properties, further research is needed regarding the administration of alpha mangostin on the process of keloid occurrence. **Methods:** The investigation was conducted in vitro on phase III keloid fibroblast cells. There were two groups, which divided into the control groups and the treatment groups. The control groups and treatment groups were given alpha mangostin extract in concentrations of 20 μM; the sample of this study was 16. For each group after 24h of the incubation, fibroblast cell proliferation was measured by Microtetrazolium (MTT) assay, fibroblast cell migration was measured by scratch assay, SMAD3 expression was measured after immunocytochemical staining, and type 1 collagen was measured by Enzyme-Linked Immunosorbent Assay (ELISA). The Ethics Committee at the Research Ethics Commission of Faculty of Medicine Andalas University has reviewed this research. **Result:** Alpha mangostin can reduce the average expression of TGF-β1, SMAD3 expression, type 1 collagen, proliferation, and migration. However, only keloid fibroblast cell proliferation showed significant results ($p<0.05$). **Conclusion:** At concentration of 20 μM, alpha mangostin suppressed TGF-β1 expression, SMAD 3, collagen type 1, proliferation, and migration in keloid fibroblast cell.

Keywords: alpha mangostin, ELISA, keloid, keloid fibroblast cell, PCR.

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BACKGROUND

Keloid is a process of abnormal collagen thickening during wound healing in skin tissue accompanied by the formation of new blood vessels. The main symptom in keloid patients is cosmetic disturbance at the location where the keloid is present, followed by complaints of itching, pain, and psychological barriers. In general, keloid disease can cause a decrease in quality of life both physically and mentally.[1,2]

Several keloid therapy modalities that are being developed can provide therapeutic response of up to 90%. Pressure dressings, silicone gel, corticosteroids, verapamil, bleomycin, 5-fluorouracil, topical mitomycin C irradiation, cryotherapy, and excision are some of the methods that can be used. This therapy can be a single or multimodal one. However, current keloid therapy also has recurrence rate, ranging from 1-70%. Therefore, it is necessary to develop safer and more effective treatments for keloids.[3,4]

Recently, in addition to chemical therapy, treatment using plant extracts has received more attention, Asian countries have many types of fruits and their own natural ingredients. Mangosteen is one of the tropical fruits that are widely found in Southeast Asia countries including Indonesia. This plant is usually found in lowlands with an altitude of 500-600 meters. There are several centers spread throughout the region including West Sumatra, West Java, and Bali.[5,6]

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Mangosteen peel contains various polysaccharides, xanthrones, procyanidins, benzophenones, bioflavonoids, and triterpenoids. These chemicals act as antioxidants, anti-inflammatories anti-tumors, anti-bacterials, and many other effects. Xanthon is a flavonoid compound which is the main content of mangosteen peel. The alpha mangostin type of xanthone which found most often in mangosteen skin has many health benefits, such as anti-inflammatory, anti-oxidant, anti-cancer, anti-bacterial and others.[7,8] This is what underlies this research to explore the effects of alpha mangostin in preventing keloids.

METHODS

This is an experimental in vitro study with a post-test control group design. This study was conducted in the Biomedical Laboratory Faculty of Medicine, Andalas University, after receiving ethical approval from the Faculty of Medicine's ethics committee. The sample consists of keloid fibroblast cells that meet the inclusion and exclusion criteria and are representative of the population. The inclusion criteria were keloid fibroblast cells with a cell count of $1x10^5$ cells/ml that could grow in culture. The exclusion criteria were keloid fibroblast cells that were unable to proliferate or were contaminated throughout the investigation's duration (turbid media, turbid cell cytoplasm/containing bacteria or fungal spots). The sample size in the study follows the calculation by Federer, namely (t-1) (n-1) ≥15, where t is the number of treatment groups and n is the number of samples. This study devided into two groups, which added up to 16 samples: 8 group were used as controls and 8 group were treated with 20 µM of alpha mangostin. The research ethics committee of the Faculty of Medicine at Andalas University approved the study, which ran from January to December 2023. The statement number for this study was 544/UN.16.2/KEP-FK/2023. The study took place in the Biomedical Laboratory of the Faculty of Medicine of Andalas University.

The high-performance liquid chromatography (HPLC) method was used by PT. Andalas Sitawa Fitolab to standardize and making alpha mangostin, which was 98% pure. These chemicals were used: phosphate buffered saline (PBS), trypsin EDTA 0.25%, povidone iodine 10%, hematoxylin-eosin dye, methanol, aquades (H2O), DMSO, hematoxylin–eosin dye, penicillin–streptomycin, trypsin EDTA, hematoxylin–eosin dye, and MTT [3–4-dimethylthiazol–2–yl)–2, 5-diphenyltetrazolium bromide]. This study also used RNAse-free water, a cDNA kit, primers for the TGF-1 gene, SMAD3 gene, and type I collagen, as well as an ELISA kit for TGF-1 and type I collagen.

The Shapiro-Wilk test was used to establish whether the data were normally distributed or not at the beginning of this research. If the data are not normally distributed, a log10 transformation is applied, then followed by other normality test. The parametric test (ANOVA) is used if the data exhibit regular distribution, and a non-parametric test if they do not. If $p<0.05$, statistical results are considered significant. The Ethics Committee at the Research Ethics Commission of the Faculty of Medicine Andalas University has reviewed this research.

RESULT

The PCR test results for TGF-β1 expression in keloid fibroblast cell cultures were normally distributed in both the treatment and control groups, meeting the requirements for a one-way ANOVA comparison test; the results were significant (p=0.023). Table 1 and Figure 1 illustrate a comparison of the mean TGF-β1 expression of keloid fibroblast cells in the control group and the treatment group.

Table 1. Mean expression of TGF-β1 in keloid fibroblast cells in the control and treatment groups by PCR examination

Group	TGF-β1 expression	p-value
Control	1.946±1.332	0.087
Alpha Mangostin 20 µM	0.700±0.010	

PCR: Polymerase Chain Reaction

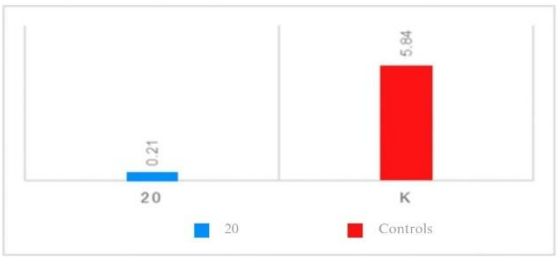

Figure 1. Mean expression of TGF-β1 in keloid fibroblast cells in the control and treatment groups by Polymerase Chain Reaction (PCR) examination.

The results of the Bonferroni post hoc test with RT-PCR showed that the average expression of TGF-β1 in keloid fibroblast cells in the 20 μM concentration group was lower than the control group, but there was no statistically significant difference (p>0.05).

Results on TGF-β1 expression in keloid fibroblast cell cultures by ELISA showed normal data distribution in all treatment and control groups, therefore meeting the conditions for the one-way ANOVA comparison test; the results obtained were not significant (p=0.385). Table 2 and Figure 2 illustrate a comparison of the mean TGF-β1 expression of keloid fibroblast cells in the control group and the treatment group. It was found that keloid fibroblast cells in the 20 μM concentration group had less TGF-β1 than cells in the control group, but there was no statistically significant difference.

Table 2. Mean expression of TGF-β1 in keloid fibroblast cells in the control and treatment groups by ELISA* examination

Group	TGF-β1 expression	p-value
Control	1114.006±122.889	1.000
Alpha Mangostin 20 μM	1025.658±17.526	

*ELISA: Enzyme-Linked Immunosorbent Assay

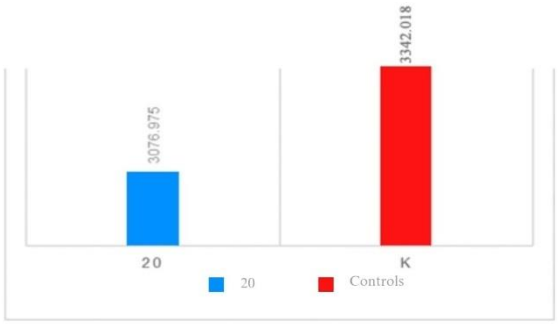

Figure 2. Mean expression of TGF-β1 in keloid fibroblast cells in the control and treatment groups by ELISA examination.

The data of SMAD3 expression in keloid fibroblast cell cultures was normally distributed in all treatment and control groups according to the Shapiro-Wilk normality test. This meant that the conditions were met for a one-way ANOVA comparison test, but the results were not significant (p=0.056). Table 3 and Figure 3 illustrate a comparison of the mean SMAD3 expression of keloid fibroblast cells in the control group and the treatment group. The mean expression of SMAD3 in keloid fibroblast cells in the 20μM concentration group was lower than the control group, but there was no statistically significant difference.

Table 3. Mean expression of SMAD3 in keloid fibroblast cells in the control and treatment groups

Group	SMAD3 expression	p-value
Control	1.376±0.471	0.460
Alpha Mangostin 20 μM	0.910±0.180	

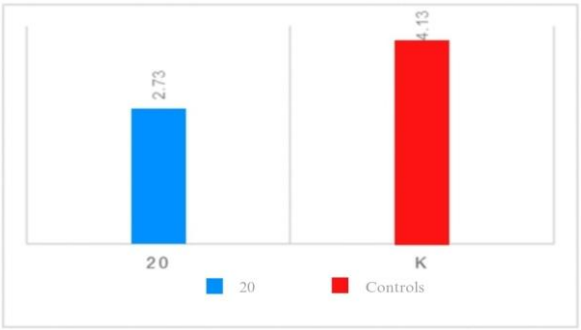

Figure 3. Mean expression of SMAD3 in keloid fibroblast cells in the control and treatment groups.

The PCR test results for type 1 collagen expression in keloid fibroblast cell cultures were normally distributed in both the treatment and control groups, meeting the requirements for a one-way ANOVA comparison test and giving results that were not statistically significant (p=0.202). Table 4 and Figure 4 illustrate a comparison of the mean type 1 collagen expression of keloid. The mean expression of type 1 collagen in keloid fibroblast cells in the 20μM concentration group was lower than the control group.

Table 4. Mean expression of type 1 collagen in keloid fibroblast cells in the control and treatment groups by PCR examination

Group	Type 1 collagen expression	p-value
Control	1.375±0.579	0.581
Alpha Mangostin 20 µM	0.869±0.032	

PCR: Polymerase Chain Reaction

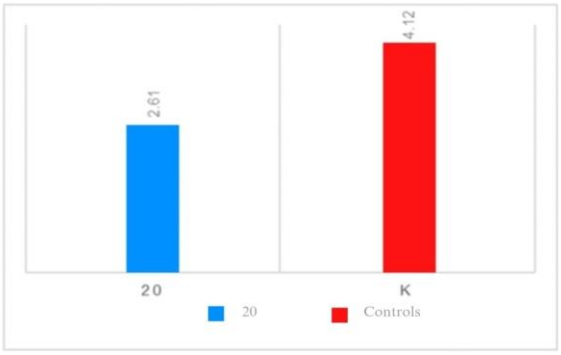

Figure 4. Mean expression of type 1 collagen in keloid fibroblast cells in the control and treatment groups by Polymerase Chain Reaction (PCR) examination.

Results on type 1 collagen expression in keloid fibroblast cell cultures by ELISA showed normally distributed data in all treatment and control groups in the Shapiro-Wilk normality test, therefore meeting the conditions for a one-way ANOVA comparison test; significant results were obtained (p=0.026). Table 5 and Figure 5 illustrate a comparison of the mean type 1 collagen expression of keloid fibroblast cells in the control group and the treatment group.

Table 5. Mean expression of type 1 collagen in keloid fibroblast cells in the control and treatment groups by ELISA examination

Group	Type 1 collagen expression	p-value
Control	17.542±0.168	0.268
Alpha Mangostin 20 µM	14.160±2.144	

ELISA: Enzyme-Linked Immunosorbent Assay

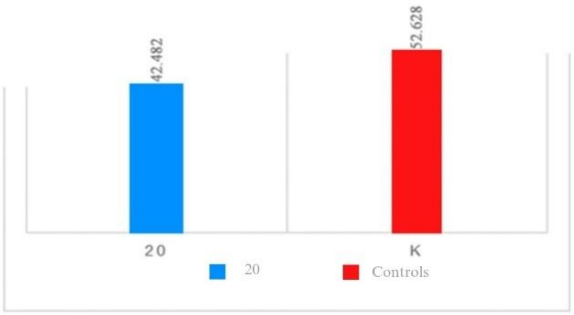

Figure 5. Mean expression of type 1 collagen in keloid fibroblast cells in the control and treatment groups by Enzyme-Linked Immunosorbent Assay (ELISA) examination.

The results of the Bonferroni post hoc test showed that the average expression of type 1 collagen in keloid fibroblast cells using ELISA in the 20 µM concentration treatment group was lower than the control group, but statistically there was no significant difference (p>0.05).

The Shapiro-Wilk normality test showed that the proliferation expression in keloid fibroblast cell cultures was normally distributed in both the treatment and control groups. This meant that the conditions were met for a one-way ANOVA comparison test, which gave results that were significantly different (p<0.001). Table 6 and Figure 6 illustrate a comparison of the mean proliferation expression of keloid fibroblast cells in the control and the treatment groups.

Table 6. Mean expression of proliferation in keloid fibroblast cells in the control and treatment groups

Group	Proliferation expression	p-value
Control	0.683±0.008	<0.001
Alpha Mangostin 20 µM	0.415±0.004	

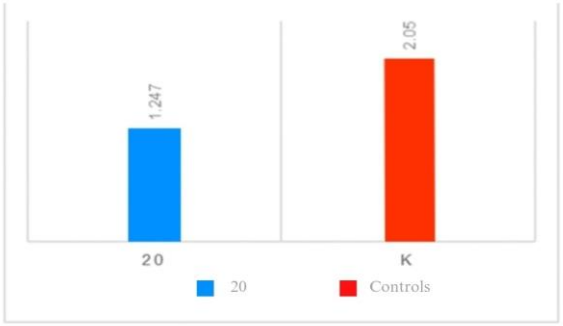

Figure 6. Mean expression of proliferation in keloid fibroblast cells in the control and treatment groups.

The Bonferroni post hoc test with the MTT assay revealed that keloid fibroblast cells in the 20 µM concentration group proliferated less on average than those in the control group. This result was a significant difference ($p<0.05$).

The migration expression data in keloid fibroblast cell cultures were normally distributed in all treatment and control groups according to the Shapiro-Wilk normality test. This meant that the conditions were met for a one-way ANOVA comparison test, and the results were significant ($p=0.002$). The mean migration expression of keloid fibroblast cells in the control group and the treatment group is shown in Table 7 and Figure 7.

Table 7. Mean expression of migration in keloid fibroblast cells in the control and treatment groups

Group	Migration expression	p-value
Control	399.700±41.181	0.103
Alpha Mangostin 20 µM	315.393±38.840	

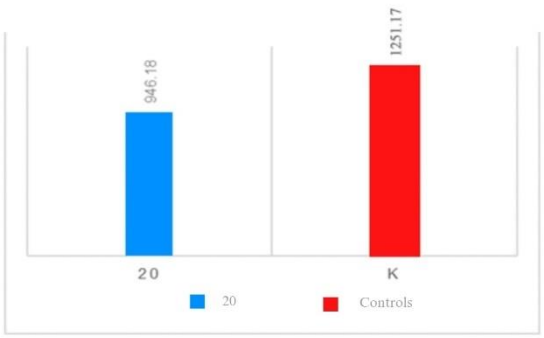

Figure 7. Mean expression of migration in keloid fibroblast cells in the control and treatment groups

The results of the Bonferroni post hoc test with the scratch test showed that the average migration of keloid fibroblast cells in the 20 µM concentration treatment group was lower than the control group, but statistically there was no significant difference ($P>0.05$).

DISCUSSION

Mangosteen skin has various effects, such as anti-inflammatory, anti-oxidant, anti-cancer, anti-bacterial, and others.[7,8] This 20 µM concentration of alpha mangostin expected to have better effect than other studies that used lower concentrations in stopping cell growth.[11]

Based on the research results, it was found that alpha mangostin at concentration 20µM could reduce the expression of TGF-β1 in keloid fibroblast cells compared to the control group, although it was not statistically significant either by PCR or ELISA examination. The pathogenesis of keloid formation cannot be explained with certainty, but TGF-β1 is one of the main cytokines that contributes to the wound healing process; specifically TGF-β1 plays a role in fibroblast cell proliferation and collagen secretion.[9,10] This is supported by other research, which states that alpha mangostin has been shown to influence TGF-β1, showing the same results that alpha mangostin with concentrations of 1, 2, and 4 µM in oral submucous fibrosis can reduce the expression of TGF-β1 and alpha-smooth muscle actin (α-SMA), which are myofibroblast markers compared to the control group. These results make it clear that alpha mangostin has anti-fibrotic effects and inhibits TGF-β1-induced signaling pathways [11]

SMAD3 expression in keloid fibroblast cells was not significantly different between the 20 µM group and controls, but there was a decrease in the mean expression of SMAD3 as seen in Figure 3. The elevated expression of phosphorylated SMAD2/3 resulting from the increased TGF-β1 expression would lead to a rise in keloid fibroblast cells compared to normal fibroblasts.[11] This is in line with similar research regarding alpha mangostin doses of 0.5 and 1 mg/kg, which can inhibit the phosphorylation of TGF- β1-SMAD3 expression for cardiac and liver fibrosis.[12]

In this study, alpha mangostin was given to type 1 collagen of keloid fibroblasts. PCR and ELISA tests showed that the mean went down in the 20 µM group compared to the control group, as shown in Figure 4. Other related research reported a significant formation of fibrotic tissue between the normal and keloid

groups. Administration of 5 μM alpha mangostin suppressed the expression of COL1A1 and HAS2; COL1A1 and HAS2 was decreased in keloid fibroblasts compared to normal fibroblasts. Collagen is the main group of extracellular matrix in keloids, especially collagen types 1 and 3, where increasing TGF-β1 will trigger the proliferation and migration of keloid fibroblast cells, which can increase the synthesis of collagen types 1 and 3 in keloid formation.

Another study indicates that a 10 μM dose of alpha mangostin can inhibit the formation of hydroxyproline (HYP), which is crucial for amino acid synthesis in collagen. Additionally, it significantly reduces the levels of type 1 collagen, TGF-β1, α-SMA, and SMAD2/3 in lung proliferation.[13,14]

This study showed that 20 μM alpha mangostin can slow the growth of keloid fibroblast cells compared to the control group. Data from this study are similar with research by McFarland and colleagues, who assesing the impact of administering alpha mangostin on keloid fibroblast cells in an anti-proliferation test. Cells were treated with doses ranging from 1 μM to 10 μM. The results revealed that administration of alpha mangostin caused a decrease in keloid fibroblasts and normal skin cells proliferation, especially at concentrations of 5, 7.5, and 10 μM. In particular, keloid fibroblast cells showed a much greater reduction in proliferation compared to normal fibroblast cells when treated with alpha mangostin at a concentration of 10 μM. Interestingly, when alpha mangostin was administered to keloid fibroblast cells at a concentration of 10 μM with an incubation period of 24 hours, there was a 99.39% reduction in proliferation.[13]

TGF-β1 has an important role in differentiation, proliferation, apoptosis, and cell migration. This is because TGF-β1 is widely related to cellular processes as a fibrosis factor in cells. Previous studies have shown that increasing TGF-β1/SMAD3 independent signaling can increase cell proliferation. Administration of alpha mangostin at a dose of 10 μM can suppress the occurrence of fibrosis cell proliferation.[15]

Decreased migration of keloid fibrosis cells happened with the administration of 20 μM alpha mangostin compared to the control group. In another study, it was found that administration of alpha mangostin at concentrations of 5, 10, and 20 μM can inhibit the PI3K/Akt pathway and inactivate the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2), p38, and jun N-terminal kinase (JNK) pathways. In other studies, treatment with 15 μM of alpha mangostin can reduce ERK5 expression. Activation of MAPK and ERK1/2 pathways can worsen the wound healing process. Suppression of these pathways and decreased TGF-β1, PDGF, bFGF, and VEGF expression can increase migration, proliferation, differentiation, and protein synthesis in wounds.[16,17]

In this study, alpha mangostin with concentration of 20 μM can lower the number of TGF-β1, SMAD3, and type 1 collagen cells by blocking the pathways including the PI3K/Akt, MAPK, ERK1/2/5, p38, and JNK pathways. It can also lower the number of proliferations and migrations, which is reduce the formation of keloid fibroblasts. The statistically insignificant results in this study may have occurred due to the small number of samples, the selection of alpha mangostin concentration, or factors during the preparation of alpha mangostin. It is necessary to carry out more in vitro research and further examination regarding the effectiveness of other alpha mangostin in order to define the lowest dose that can have the maximum results in formation of fibroblast cells in keloids.

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