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Research Report

A study of cytotoxicity and proliferation of *Cosmos caudatus* Kunth leaf extract in human gingival fibroblast culture

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ABSTRACT

Background: *Post-extraction dental sockets clinically resolve within a period of 3-4 weeks. However, complete healing and bundling of gingival fibers may require several months. Medication is therefore required to accelerate the healing process. Cosmos caudatus (C. caudatus), a local plant with antioxidant properties and high calcium content, has the potential to promote wound healing while also reportedly capable of strengthening bone. Previous studies have demonstrated the effectiveness of C. caudatus as an alternative treatment for post-menopausal osteoporosis by investigating the dynamic and cellular parameters of bone histomorphometry.* ***Purpose:*** *The study aimed to examine the citotoxicity and proliferation of human gingival fibroblast cells culture after the application of C. caudatus extract.* ***Methods:*** *Cultures of human gingival fibroblast cells with $5x10^4$ cell density were divided into two groups and placed in a 30-well culture dish. The control group contained human gingival fibroblast cell culture without extract, while the experimental group consisted of human gingival fibroblast cells culture with extract. The concentrations of extract were 1200 µg/ml, 600 µg/ml, 300 µg/ml, 150 µg/ml, and 75 µg/ml. A toxicity test was conducted and the optimum concentration evaluated using an MTT assay, while fibroblast numbers on were calculated days 1 and 2 by means of a hemocytometer. Research data was analyzed using a one-way ANOVA test.* ***Results:*** *No toxicity was found. The optimum concentration was 600 µg/ml and fibroblast proliferation was significantly higher in the experimental group compared to the control group, $p=0.002$ ($P<0.05$).* ***Conclusion:*** *C. caudatus leaf extract is non-toxic and increases the proliferation of human gingival fibroblast culture at an optimum concentration of 600 µg/ml.*

Keywords: *Cosmos caudatus leaf extract; human gingival fibroblasts culture; wound healing*

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INTRODUCTION

A wound is considered healed if tissue has regenerated to its original anatomic structure, function and within a reasonable period. Most wounds result from minor injuries, but some do not heal fully in a timely fashion. Several local and systemic factors can impede wound repair by disrupting the process of balance improvement, resulting in chronic wounds failing to healing.[1]

Fibroblasts, cells in the connective tissue that affect the wound healing process, enter the wound area three days after extraction and became dominant after 6-7 days.[2] Fibroblasts will experience certain changes to the phenotype and become myofibroblasts that serve to retract the wound. Fibroblasts produce extracellular matrix, collagen primer and fibronectin which promote cell migration and proliferation. Fibroblasts derived from the undifferentiated mesenchymal cells produce mucopolysaccharides, amino acid glycine and proline, a basic ingredient of collagen fibers, that binds wound edges. However, in cases of significant bone damage, the natural repair processes within the body cannot restore its function or promote clinical improvement.[3]

Cosmos caudatus (C. caudatus), known as kenikir in Indonesia, had been used in eastern regions of the country to reduce high blood pressure. As well as acting as an anti-

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hypertensive, it possesses anti-diabetic, anti-inflammatory, bone protective, anti-microbial and anti-fungal properties.[4] The polyphenol content of *C. caudatus* is high, as is the level of vitamin E, vitamin C, folic acid, β-carotene and polyphenols in the *C. caudatus* lycopene.[5] *C. caudatus* also promotes oral health, especially as an antibacterial, as it contains polyphenol derivates such as flavones, flavonols, flavonones, catechins and isoflavones. The catechin derivate has antioxidant and anti-inflammatory properties. Other substances contained in *C. caudatus* include phytochemical flavonoids. Previous studies have shown flavonoids to have anti-inflammatory and antioxidant properties, the capacity to accelerate tissue regeneration and to act as a natural antioxidant that enhances the wound healing process.[6] Another study stated that *C. caudatus* leaves contain terpenoids (essential oils), alkaloids and saponin.[7] *C. caudatus* was studied in this experiment because it is readily available, widely consumed and used in the manufacture of drugs to enhance the process of post-extraction healing.

Tooth extraction socket healing occurs through a secondary healing process that involves the formation of granulated tissue and subsequent covering by epithelium. *C. caudatus* leaf properties have proved capable of balancing the production of reactive oxygen species (ROS) and promoting cell growth, such as fibroblasts.[8]

This research was conducted as a preliminary study of the development of drugs applied to promote fibroblast proliferation. It followed the successive stages of drug development, namely: in vitro examination, experimentation on live subjects *(in vivo)*, clinical trials and a market launch.[9] The initial step of this study prior to analyzing the proliferation of human gingival fibroblast (HGF) culture was that of conducting a citotoxicity assay/ study.[10]

The study aimed to quantify the citotoxicity and proliferation of human gingival fibroblast cell culture after the application of *C. caudatus* extract. *C. caudatus* leaf properties were able to balance the production of ROS and promote cell growth such as fibroblast so that tooth extraction socket healing occurred through a secondary healing process that increased the proliferation of granulated tissue and the epithelium.

MATERIALS AND METHODS

This was an experimental laboratory study using a post-test control group design. The experiment involved *C. caudatus* leaf extract application to primary cell cultures of human gingival fibroblasts.

Preliminary cytotoxicity and optimum concentration tests were conducted using a colorimetric assay that measured the reduction of yellow 3-(4.5-dimethythiazol-2-yl) -2.5-diphenyl tetrazolium bromide (MTT) assay by mitochondrial succinate dehydrogenase. Five concentrations of extract, consisting of 1200 µg/ml, 600 µg/ml, 300 µg/ml, 150 µg/ml, and 75 µg/ml based on the $IC_{50,}$ were tested. The optimum concentration for cell growth was found to be approximately 504.840 µg/ml.[11]

The MTT results did not demonstrate toxicity after 24 hours with an ELISA reader used to calculate cells spectrophotometrically with a wavelength of 620-650 nm based on formazan crystal absorbance values.

Extract concentrations of 600 µg/ml were selected because, at this concentration, viable cell numbers of HGF were optimal with no bias in spectrophotometric absorbance. These concentrations correspond to the proliferation of fibroblasts compared to ones below 600 µg/ml because lower extract concentrations will not provide maximum results.

Two groups, a control group and an experiment group each containing five samples placed in 30-well culture disk, were used in this study, together with a monolayer HGF culture of $5x10^4$ cell density. A control group was HGF-cultured without extract application, while the experiment group was HGF-cultured with the application of 600 µg/ml extract. *C. caudatus* leaf extract was applied by means of a 1 ml pipette with the fibroblast numbers being counted after 24 hours and 48 hours.

Fibroblast cell counts were conducted using a hemocytometer after trypan blue staining. Cells were taken at the ratio 1:1 and resuspended with trypan blue stain and observed under a light microscope at 100x magnification. Cells were considered viable if they were single, clear and round. An overlap between two or more cells was counted as two cells. Non-viable cells were defined as those absorbing the color of trypan blue. The measurement results of the observations were added together and calculation values obtained for the subjects of research using the formula:[12]

$$Number\ of\ cells = \frac{(A+B+C+D)}{4} x10^4 x\ 2\ x\ sample\ dilution$$

Note: Count the number of cells – both viable (unstained) and non-viable (stained) in each of the four corner quadrants (A, B, C and D).

Data were then analised statistically using one-way ANOVA. A p-value less than 0.05 was considered statistically significant.

RESULTS

All concentrations increased the number of fibroblasts. There were differences in formazan crystal formation between each concentration. This was due to the difference in the number of living cells in each extract concentration as presented in Figure 1. The optimum concentration was 600 µg/ml which produced a high level of HGF proliferation as shown in Table 1. Extract concentrations of 1200 µg/ml produced a false positive result due to the dark color as shown in Figure 1A.

The proliferation of HGF culture in both the control group and experiment group at 600 µg/ml extract applications showed a higher density under light microscope

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examination in the experiment group on both the first and second days, as shown in Figures 2 and 3.

The number of fibroblast in experiment groups were higher compared to control groups. Anova statistical analysis showed that there were significant differences in average fibroblast numbers between groups, p=0.002 (p<0.05) as presented in Table 2.

Table 1. Toxicity test results of *C. caudatus* leaf extract

No.	Control well with medium	Control well with HGF	Well HGF + 1200 µg/ml extract	Well HGF + 600 µg/ml extract	Well HGF + 300 µg/ml extract	Well HGF + 150 µg/ml extract	Well HGF + 75 µg/ml extract
1	0.056	0.66	1.417	1.071	0.894	0.786	0.724
2	0.057	0.641	1.413	1.037	0.874	0.789	0.725
3	0.056	0.667	1.391	1.058	0.847	0.768	0.726
4	0.056	0.661	1.413	1.068	0.856	0.768	0.728
5	0.057	0.65	1.414	1.071	0.858	0.769	0.725
6	0.057	0.662	1.406	1.069	0.857	0.771	0.726
Total	0.339	3.941	8.454	6.374	5.186	4.641	4.354
Mean	0.057	0.657	1.409	1.062	0.864	0.774	0.726
Total living cell %			225.3	168	135	119	111

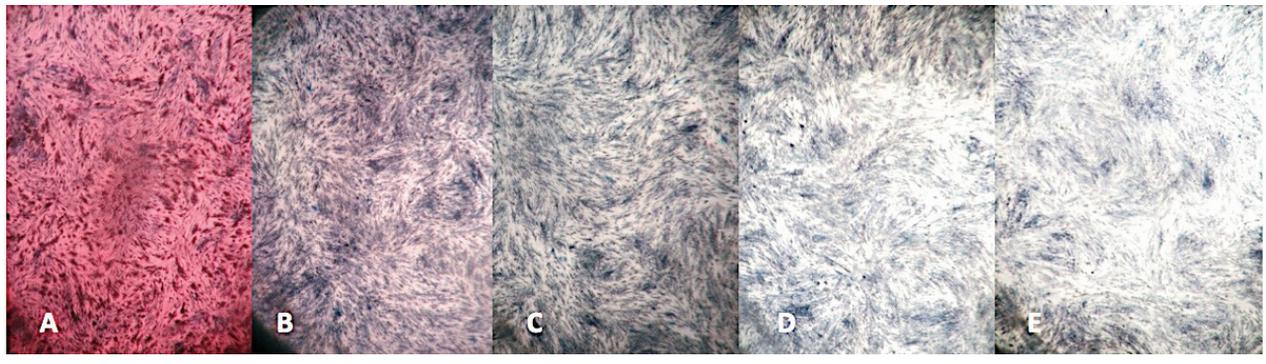

Figure 1. Figure fibroblasts after application of *C. caudatus* extracts for 24 hours under light microscope at 10x magnification. (A) Concentration of 1200 µg/ml. (B) Concentration of 600 µg/ml. (C) Concentration of 300 µg/ml. (D) Concentration of 150 µg/ml. (E) Concentration of 75µg/ml.

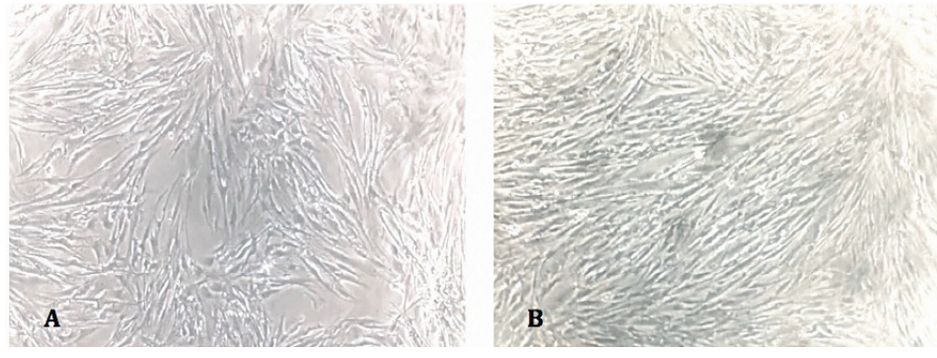

Figure 2. Microscopic picture of fibroblasts on the first day (40x magnification). (A) Control fibroblasts on the first day. (B) Treatment of fibroblast cells.

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DISCUSSION

This was an experimental *in vitro* study with an application of *C. caudatus* leaf extract on HGF cell culture. The study was carried out following a drug development sequence which comprised of *in vitro* examination, animal experiments *(in vivo),* clinical trials and preparation for marketing.[9] Cell culture was obtained from human gingival cells because these could be cultured into primary cell line culture demonstrating a similar pattern of behavior to in vivo reactions compared to cell lines.[13] Cell lines have been used for decades to produce culture, resulting in stronger cells often genetically and phenotypically different from their original networks which underwent morphological changes. Unlike cell lines, primary cells are isolated directly from the network, have a limited lifespan and expansion capacity. On the positive side, primary cells demonstrate normal cell morphology and retain many important observable markers and functions, and the data obtained from primary cells is more relevant for interpretation and can be generalized into an *in vivo* setting.[14]

This finding was in line with that produced by the research conducted by Wong et al[15] indicating that primary cells are more sensitive to drugs than cell lines and tend to survive at higher concentrations and proliferation. In contrast, the lifespan of primary cells was shorter at 16-24 hours after the administering of drugs at lower concentrations. The administering of 20 µM (6 µg/ml) cisplatin to primary cells resulted in a viability range of 64.0% cells, while the cell line with 33 µM (6.6 µg/ml) cisplatin concentration produced a viability level of 71.6% cells.[15]

Extract concentrations were set at 1200 µg/ml, 600 µg/ml, 300 µg/ml, 150 µg/ml and 75 µg/ml. Dose reduction was based on a geometric series of measurements to evaluate the dose-cell response relationship to the exposure of the extract.[11] All concentrations of *C. caudatus* extract were non-toxic as shown in Table 1. The concentration of 600 µg/ml was selected because it showed the highest number of proliferated living cells. Moreover, the absorbance value of formazan crystals at a concentration of 600 µg/ml could be read spectrophotometrically without bias caused by the extremely dark color. This concentration was suitable for fibroblast cell proliferation compared to those below 600 µg/ml because, according to the dose-response relationship, weaker extract concentrations will produce a lower number of living fibroblasts or will fail to produce optimum results. The concentration of 1200 µg/ml was rejected because it was found that significantly elevated proliferation rates would induce dense cell growth resulting in greater competition for nutrients in the media. As a result, the cells did not mature rapidly and experienced high mortality rates culminating in a false negative result. The possibility of such a result occurring also existed because the concentrated color of dense cells caused the spectrophotometric reading to indicate a higher level of absorbance.

Fibroblast proliferation was significantly higher in the experiment group compared to that in the control group, $p = 0.002$ ($p < 0.05$). This was due to the active ingredients in *C. caudatus* leaf extract containing catechins, polyphenols, flavonoids, vitamins C and E and saponins.[9] Schuck et al[16], citing Weisburg et al (2004), stated that catechins do not induce oxidative stress in normal human cells originating in the oral cavity because catechins in extracts can reduce ROS

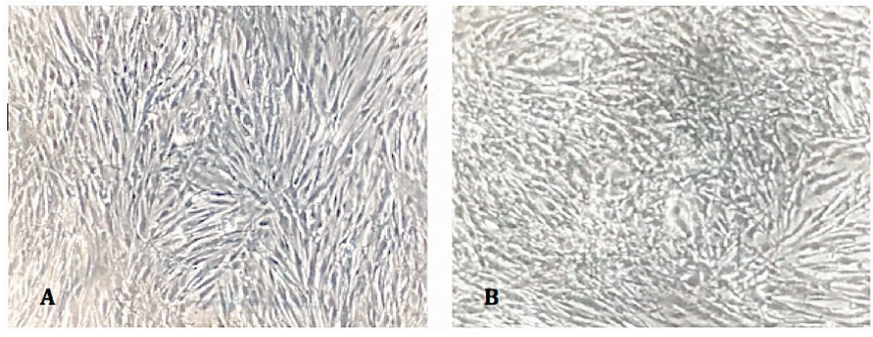

Figure 3. Microscopic picture of fibroblasts on the second day (40x magnification). (A) Control fibroblasts on the second day. (B) Treatment of fibroblast cells.

Table 2. Statistical analysis results of fibroblast numbers

Control and experiment group	Average	Standard deviation	Kolmogorov-Smirnov	Levenne	Anova
K1	82,000	9,082.95	0.2	0.782	0.002
P1	88,000	8,366.60	0.2		
K2	95,000	7,905.69	0.2		
P2	106,000	6,519.20	0.2		

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levels in normal cells, while causing intracellular oxidative stress in cancer cells. This indicates that the cathechin effect on normal and cancer cells worked in different biochemical pathways that induce normal cell proliferation. Polyphenol content promotes both anti-microbial and anti-fungal activities that play a role in maintaining the growth of cell cultures. The catechins present in polyphenols can reduce ROS levels in normal cells, while flavonoids stimulate fibroblast synthesis and are able to balance the production of ROS with antioxidant capacity that promotes fibroblast growth.[8]

Other ingredients found in *C. caudatus* include high levels of vitamin C and E. Flavonoids increase the effectiveness of vitamin C conducive to the formation of fibroblasts.[14] Ascorbic acid, also known as vitamin C, is necessary for normal responses to physiological stressors and its requirement increases during injury or stress. Studies have shown that physiological stress produces excessive ROS leading to vitamin C playing the role of activating intracellular signaling which regulates fibroblast proliferation.[17]

Terpenoids or essential oils, as well as polyphenol, promote antibacterial and antifungal activity. Fibroblasts are able to proliferate effectively because of the eugenol content of essential oils that damage the cell walls of bacteria causing damage to the cells themselves for example those of gram-positive bacteria, especially Streptococcus aureus. Phenolic compounds present in essential oils cause protein denaturation and bacterial cell death.[8]

Saponins, active substances that act as anti-microbes, increase cell membrane permeability and have antioxidant propoerties.[18] Saponins can stimulate the formation of a *cell-extracellular matrix* by stimulating fibronectin synthesis in fibroblasts capable of promoting the wound healing process.[19] The saponin content stimulating *transforming growth factor-beta 1* (TGF-β1) can affect *fibroblast growth factor* (FGF) in fibroblast cells thereby promoting the formation of collagen fibers. When FGF is stimulated by TGF-β1 fibroblast proliferation will be increased.[20]

Alkaloids represent one of the other active components in *C. caudatus* leaves with toxic properties that significantly affect physiological activity. Most alkaloids that have been isolated from plants are crystalline solids with a certain melting point.[21] The toxic content of alkaloids in *C. caudatus* leaf extract was at small levels, caused no effect on cell culture and does not increase ROS but induced oxidative stress in normal cells. In this study, cell death occurred but there were also fibroblast proliferation that did not effect the results.[22]

The wound healing process is evident from several indicators, including an increase in the number of fibroblasts that will stimulate the collagen forming extra-cellular matrices. The proliferation of fibroblasts promoted by *transforming growth factor-β* (TGF-*β*) will increase collagen and fibronectin synthesis and promote extracellular matrix deposition.[20]

In the next stage, there is a decrease in endothelial cell proliferation and the number of fibroblast cells, but fibroblasts become more progressive in synthesizing collagen and fibronectin involving changes in the composition of *extra-cellular matrix* (ECM).[19] The ECM provides a sub-layer for cell attachment and effectively regulates cell growth, movement and differentiation propagated by growth factors and cytokines namely *platelet-derived growth factor*, FGF, TGF-β and *interleukin-1*, *interleukin-4*, *immunoglobulin-g* produced by leukocytes and lymphocytes during collagen synthesis. Remodeling will commence after an extracellular matrix is formed.[20]

An inherent weakness of this study was the fact that dense fibroblasts in the culture plate can perish because of an over-proliferation of their cells. Further research needs to be undertaken by reducing the number of cells to less than $5x10^4$. Further study will be conducted to develop a herbal drug which promotes post-extraction tooth socket healing. It can be concluded that *C. caudatus* leaf extract is non-toxic to human gingival fibroblast culture resulting in a significant increase in fibroblast numbers. The optimum concentration of *C. caudatus* leaf extract to increase fibroblast proliferation is 600 µg/ml.

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