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Original Article

Viability Test of Ethanol Extract of *Beluntas* (*Pluchea indica*) Leaves on *In vitro* Fibroblast Cells

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Abstract

Background: Tooth extraction is the most frequently conducted dental care procedure. In Indonesia, there is an extremely high utilization of dental and oral health services for tooth extraction, reaching 79.6%. Pain is a side effect of extraction. Pain due to extraction wounds can be treated with analgesic drugs, but alternative drugs with minimal or no side effects are still being researched. An herbal active *beluntas* leaf substance can reduce pain from extraction wounds. The *beluntas* plant not only aids in healing wounds but also exhibits anti-inflammatory and antipyretic effects. **Objectives:** In this study, the aims were to determine the 50% inhibitory concentration (IC_{50} value) and examine the viability effect of an ethanol extract of *beluntas* leaves on fibroblast cell cultures *in vitro*. **Methods:** Laboratory experiments were carried out. *Beluntas* leaves were obtained; their leaf extracts were prepared using ethanol as the solvent; phytochemical tests were performed. Triplicate measurements for the viability of 3T3 BALB/c fibroblast cells were carried out using the Methylthiazol sulfophenyl (MTS Assay) method. The extract concentrations were 500, 250, 125, 62.50, 31.25, 15.63, and 7.81 µg/mL. **Results:** Data analysis was carried out by one-way analysis of variance statistical test. Analysis results revealed that extract concentrations of 500, 31.25, 15.63, and 7.81 µg/mL exhibit a significant difference in the effect of cytotoxicity ($P < 0.05$) on fibroblast cells, and the IC_{50} value is 265.388 µg/mL. **Conclusion:** A significant difference in the cytotoxicity effect between the concentrations of the ethanol extract of *beluntas* (*P. indica*) leaves on the fibroblast cell cultures *in vitro* was observed. The *beluntas* leaf extract at an IC_{50} value of 7.81 µg/mL did not affect cell viability; hence, it is considered safe.

Keywords: *Beluntas leaves, cytotoxicity, ethanol extract, fibroblast cells, MTS assay, viability*

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Background

Tooth extraction is common in dental practice. In Indonesia, there is extremely high utilization of dental and oral health services for tooth extraction, reaching 79.6%.[1] Dental extraction is a surgical procedure that deals with soft and hard tissues in the oral cavity.[2] Pain is a side effect of extraction. Pain starts to disappear during the proliferation phase in the proliferation process of fibroblast cells. The healing process of extraction occurs in several phases, viz., inflammatory phase, proliferation phase (complete wound closure and epithelial formation occur in this phase), and maturation. The wound-healing process is strongly affected by the migration and proliferation of fibroblasts in the wound area, and fibroblasts play a key role in the repair process.[3]

Pain due to extraction wounds can be treated using analgesic drugs, but alternative drugs with minimal or no side effects are remained unclear. Flavonoids are herbal

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active substances that can reduce pain caused by extraction wounds. Besides flavonoids, *beluntas* leaves also contain other active compounds that can accelerate wound healing, including alkaloids, saponins, polyphenols, tannins, sterols, sodium, citrus oils, amino acids, fats, calcium, magnesium, phosphorus, Vitamin A, and Vitamin C. The *beluntas* plant can not only aid in wound healing but also exhibit anti-inflammatory and antipyretic effects.[4,5]

Flavonoids can stop the production of prostaglandin; therefore, it reduces pain, renders antibacterial effects, functions as an anti-inflammatory agent, and exhibits a working mechanism for the inhibition of lipid peroxidation, which serves to reduce reactive oxygen species; hence, it can slow tissue death, increase vascularity and collagen, prevent cell damage, and increase DNA synthesis.[6]

Tannins also exhibit an effect that can stimulate not only the production of fibroblast cells but also the formation of collagen tissues in wound healing. Tannins exhibit antimicrobial and antioxidant effects that can help prevent infections and fight free radicals; therefore, tannins accelerate the wound-healing process.[7]

Beluntas leaves can be used as therapeutic agents for oral mucous tissue damage. To serve as therapeutic agents, *beluntas* leaves must satisfy the requirements for biocompatibility, i.e., must not cause irritation and toxicity to the body. Therefore, it is imperative to conduct a standardized study that can determine the effect of the *beluntas* leaves extract on cell viability involved in tissue healing, including fibroblast cells.

In this study, the 50% inhibitory concentration (IC_{50}) value was determined, as well as the cytotoxicity effect of the ethanol extract of *beluntas* (*P. indica*) leaves on fibroblast cell cultures *in* was determined.

Materials and Methods

Ethanol extract of *beluntas*

A ± 10-year-old *beluntas* leaf sample was obtained from the experimental garden in Manoko Spice and Medicinal Plant Research Institute (Balittro), which was determined in the laboratory of identification and determination of the Faculty of Life Sciences, Institut Teknologi Bandung (ITB).

A crude *beluntas* leaf extract was prepared by the maceration method using 70% ethanol, which was carried out at the Aretha Medika Utama, Biomolecular and Biomedical Research Center.

The dilution of the ethanol extract of the *beluntas* leaf started with the preparation of a stock solution, which had a concentration of 500 µg/mL, followed by the preparation of a series of working solutions with concentrations of 250, 125, 62.50, 31.25, 15.63, and 7.81 µg/mL.

Phytochemical test

Phytochemical analysis by the Farnsworth method was carried out to identify the chemical groups of alkaloids, saponins, tannins, flavonoids, terpenoids, phenols, and steroids/triterpenoids.[8]

MTS assay

The MTS assay was performed to measure the viability of fibroblast cells, which was based on the conversion of tetrazolium salts to colored formazan by the mitochondrial activity of living cells. The amount of the produced formazan depends on the number of cells that are feasible in the culture, which is measured in triplicate by a spectrophotometer at 490 nm, with 24 h of incubation.[9]

The viability test of the *beluntas* leaf extract was carried out using confluent 70%–80% 3T3 BALB/c fibroblast cells and planted with a density of 5000 cells/well in 96 wells/plate using the cell culture medium of the Dulbecco's modified Eagle medium, 10% fetal bovine serum, and 1% antibiotic-antimycotic solution, followed by incubation for 24 h in an incubator at a temperature of 37°C and a CO_2 level of 5%.

The old medium was removed and replaced with 200 µL of a new medium, and 2 µL of the extract with various series of concentrations was added to each well, followed by incubation for 24 h with a temperature of 37°C and a CO_2 content of 5%. After 24 h, 20 µL of the MTS reagent was added to each well and incubated for 3 h at the same temperature and CO_2 levels, and the cells were calculated on the basis of their absorbance and curve integration. Based on the number of viable cells, the cytotoxicity of a material can be classified [Table 1].

IC_{50} was calculated from the plot of concentration as a function of the percentage of viability.[10]

$$\% \text{ Viability} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of control}} \times 100\%$$

$$IC_{50} = \frac{\text{Absorbance of control} - \text{absorbance of the sample}}{\text{Absorbance of control}} \times 100\%$$

The obtained data were first processed by a normality test, i.e., the Kolmogorov–Smirnov test. Second, one-way analysis of variance with $\alpha = 0.05$ and an advanced test, i.e., the *post hoc* test (Tukey), were carried out. Finally, probit analysis was performed to process the IC_{50} data.

Results

Phytochemical test results of the *beluntas* leaf extract revealed the presence of alkaloids, saponins, flavonoids, phenols, terpenoids, steroids, and tannins [Table 2]. These results are in agreement with those reported previously.[11]

Table 2 and Figure 1 summarize the results obtained from the viability test. Based on the classification of cell viability, the table data revealed that concentrations of 500, 250, and 125 µg/mL are classified as extremely toxic. By contrast, lower concentrations of 62.5, 31.25, and 15.63 µg/mL are classified as slightly toxic. A concentration of 7.81 µg/mL is classified as nontoxic.

Then, the probit IC_{50} test was performed, and the IC_{50} value (safe concentration) of the ethanol extract of the *beluntas* was 265.388 µg/mL [Table 3 and Figure 1].[10]

Table 1: Classification of material cytotoxicity

Percentage of viability cells	Classification
>90% of the cells are viable	Nontoxic
60%-90% of the cells are viable	Slightly toxic
30%-59% of the cells are viable	Quite toxic
<30% of cells are viable	Extremely toxic

Table 2: Phytochemical examination results of *beluntas* leaves

Phytochemical compounds	Results
Flavonoids	+
Saponins	+
Phenols	+
Tannins	+
Steroids/triterpenoids	+
Terpenoids	+
Alkaloids	+

+: Present active compound

Table 3: Viability tests for the 3T3 BALB/c fibroblast cells

Concentration (µg/mL)	Absorbance	Average		Number of viable cells
		Viability (%)	Inhibition (%)	
Cell control	2.185	100	0.00	5000
DMSO control	1.939	97.23	2.77	4.861
7.81	1.934	91.16±0.95	8.84	4.558
15.63	1.843	79.01±1.72	20.99	3.950
31.25	1.945	65.37±2.55	34.63	3.268
62.50	2.025	59.18±1.13	40.82	2.959
125	2.145	57.18±2.38	42.82	2.859
250	2.234	55.68±1.84	44.32	2.784
500	2.348	45.53±2.04	54.47	2.276

DMSO: Dimethyl sulfoxide

Discussion

Healing occurs in several phases, viz., homeostasis, inflammation, proliferation, and maturation. The wound-healing process is strongly affected by the migration and proliferation of the fibroblasts in the wound area, and fibroblasts play a key role in the repair process.[12,13]

Flavonoids in the *beluntas* leaves play a role in the anti-inflammatory process because it can shorten the inflammation time; therefore, proliferation can occur immediately and inhibit bleeding. The flavonoid activity can accelerate the wound-healing process, and it is supported by antioxidant mechanisms for the inhibition of the free radical activity.

Antioxidants can block the initiation of free radical and trigger the proliferation of fibroblast cells. Besides flavonoids, *beluntas* leaves also contain saponins, which can not only stimulate collagen formation but also increase the fibroblast density via the activation of TGF-β.[4,14,15]

Phenols, alkaloids, and tannins can serve as antibacterial agents, and terpenoids are active ingredients that help to accelerate the formation of collagen fiber produced by fibroblast cells.[14]

Hence, to develop materials for the natural treatment of postextraction wounds, materials must be subjected first to biocompatibility tests in accordance with the material requirements in dentistry, especially those used in the mouth. One test to determine the various properties of a dental material is a cytotoxicity test on tissues. To determine the cytotoxicity of the ethanol extract of the *beluntas* leaves (*P. indica*), a fibroblast cell was tested using the MTS assay.

Parameters for the cytotoxicity test are based on the absorbance value, i.e., if the cell color becomes thicker (purple), the absorbance value is higher, implying that more cells are alive; however, if the cell

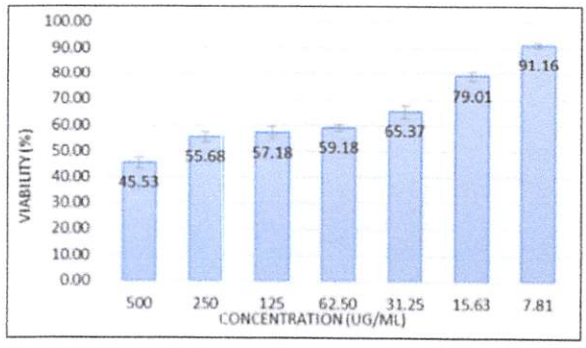

Figure 1: Relationship between the concentrations of the *beluntas* leaf extract on the viability of 3T3 BALB/c fibroblast cell

color fades, the absorbance value is lower, implying that several cells die.[16]

The cell viability decreased after the administration of the extracts to cells, and significant differences between concentrations were related to the differences in the cell response to the concentration, including the number of active ingredients at different concentrations, indicating that the extract exhibits cytotoxic properties on fibroblast cells. The mechanism and intensity of cell death depend on material content or cell contact. *Beluntas* leaves contain eugenol, which is a phenol derivative.[17] Eugenol can cause cell cytotoxicity, which can damage the protein structure by a number of physical and chemical elements.[18]

Cells exposed to materials or extracts exceeding the peak of exposure cause cell death. It can cause toxicity to cells via different mechanisms, such as the destruction of cell membranes; prevention of protein synthesis; irreversible binding to receptors; inhibition of the polydeoxynucleotide elongation; and other enzymatic reactions.[9,18,19]

In a particular cell, cytotoxic agents can also be metabolized without any observable effect although most cells experience necrosis when confronted with toxic compounds. Necrosis can occur when cells are exposed to conditions that are extremely different from their physiological conditions or when the compound content of the extracts can damage cell membranes. Necrosis starts from the disruption of the cell's ability to maintain homeostasis because it can cause the entry of water and extracellular ions. Intracellular organelles, especially mitochondria and all other cells, can swell and rupture (cell lysis).[20]

As a result of the disruption of the plasma membrane, the cytoplasmic contents, including the lysosome enzyme, are released into the extracellular fluid. The activity of these enzymes in extracellular media can be used to determine the level of necrosis. Besides these factors, the dose, exposure duration, and mechanism of cytotoxic agents are other factors that can cause cell death.[9,21,22]

The IC_{50} value for the ethanol extract of *beluntas* leaves on fibroblast cells is 265.388 μg/mL, which is categorized as safe because it does not interfere with the viability of fibroblast cells; however, further research is required to investigate the effect of the *beluntas* leaves extract on the proliferation of fibroblast cells.

Conclusion

A significant difference in the cytotoxicity effect between the concentrations of the ethanol extract of *beluntas* (*P. indica*) leaves on fibroblast cell cultures *in vitro* was observed. The *beluntas* leaf extract at an IC_{50} concentration of 7.81 μg/mL did not affect cell viability; hence, it is considered safe.

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Conflict of interest

There are no conflicts of interest.

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