# Effect of Sisal Nanofiber (Agave Sisalana) as a Filling Material for Root Canal Sealer on Confluensity and Viability of Fibroblast Cells Nih-3t3

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**Abstract**

The dental caries index in Indonesia is still at 4.5. Dental caries infection can extend to the pulp and periapical tissue, so tooth requires Root Canal Treatment. Root Canal Treatment need gutta percha and sealer as obturation material. Epoxy resin sealers are known to be widely used today and the addition of a filler in the sealer will improve the quality of the sealer. The addition of sisal nanofiber (Agave Sisalana) as an organic material is being developed as a filler in root canal sealers. The purpose of this study was to determine the effect of sisal nanofiber as a filling material for root canal sealers on the confluency and viability of fibroblast cells NIH-3T3.

The experimental laboratory was starting to extract sisal nanofibers through alkylation, neutralization, bleaching, ultrasonication and freeze dried. Preparation of 1% sisal nanofiber sealer in epoxy resin root canal sealers with a diameter of 5 mm and a thickness of 3 mm as a specimen. Incubation of fibroblast cells NIH-3T3 with specimen was done for 24 hours. The specimen immersion medium (supernatant) was used as a treatment. Supernatant was made serial concentrations of 12.5%, 25%, 50% and 100% as well as control cells. Incubation was continued for 24 hours. Viability cell was measured using the MTT assay and absorbance was read at 570 nm. The results obtained from observations of cell confluency and cell viability calculations.

Statistical analysis: Data were analyzed using ANOVA and Tukey's Post Hoc Test. The highest cell viability was obtained at a concentration of 12.5% (89.46%). The ANOVA result exhibited that there was a significant difference in the effect of adding sisal nanofiber as a filling material for root canal sealers on the confluency and viability of fibroblast cells NIH-3T3 ($p<0.01$). Tukey's Post Hoc Test showed a significant difference in the comparison cell viability of all concentrations to the control ($p<0.05$).

The addition of sisal nanofiber material (Agave Sisalana) as a filling material for root canal sealer significantly affected the confluency and viability of fibroblast cells NIH-3T3, which was marked by a decrease in cell cytotoxicity.

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## Introduction

The results of Riskesdas in 2018 exhibit the average DMFT (Decay Missing Filling Teeth) value of the Indonesian population is 7.1 with a DT (Decay Teeth) number of 4.5.[1] This means that the number of permanent teeth or primary teeth that have caries and have not been treated or filled is 4.5 per person. These results indicated that the numbers are still high. In the event of untreated dental caries, there is a risk of expanding the infection to the root canal of the tooth and can continue to other body tissues. Root Canal Treatment is needed to sterilize root canals from microorganisms that cause infection. Filling material (obturation) after root canal treatment should be carried out hermetically using durable materials to prevent the spread of microorganisms to the periapical area and prevent recurrent infection.[2,3] The dental root canal obturation materials that have become the

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standard for clinicians are gutta percha and sealer. Sealer is needed as a binding material between gutta percha and the root canal wall and serves to seal the root canal from periapical and lateral so that it is expected to prevent apical leakage. [2,3,4]

The sealer material that is often used by clinicians is an epoxy resin-based sealer have the advantages of good resorption resistance and dimensional stability and providing a good seal. However, this material still has disadvantages, including high toxicity, can shrinkage that cause microleakage in the apex area.[2,4] Many studies continue to be developed to improve of sealer material by adding fillers.

Sisal (Agave Sisalana) is a natural fiber plant that grows a lot on the island of Madura and the southern part of Java. Sisal plants are also known to be biodegradable and environmentally friendly. Sisal fiber have potential as a filler to strengthen composite filling materials. Nano-size sisal fiber is known to be a filler in composite filling materials and shows good mechanical properties such as increasing tensile strength and thus increasing polymer strength.[5,6,7]

The development of sisal as a filling material for root canal sealers has been carried out in a preliminary test. Some results indicate that the addition of sisal as a filler for epoxy resin sealers produces good physical properties, including apical density,[8] microhardness,[9] push-out adhesion strength[10] and *wettability*.[11] Antibacterial ability against S. mutans has also been tested in vitro.[11] The study showed sisal nanofibers have the potential to improve the physical properties of root canal sealers. The criteria for root canal sealer materials require non-toxic materials as indicated by the large number of viable cells and the achievement of cell confluency.

The sealer application to the root canal allows the material to contact the periapical tissue which is always moist.[12] The contact of sealer material to the periapical tissue will cause a response from the tissue. This study is important to evaluate the sisal nanofiber as a filling material for root canal sealers on the confluency and viability of fibroblast cells NIH-3T3. The results of this study will be used for the development of sisal nanofibers as the first organic material that can be used as a sealer material based on epoxy resin.

## Materials and methods

The research was conducted after obtaining ethical clearance from the Ethics Commission of the Faculty of Dentistry, Universitas Gadjah Mada No. 0049/KKEP/FKG-UGM/EC/2022 dated April 5, 2022. The identification of sisal plants was carried out by the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada in accordance with Letter No. 065/S.Tb/IV/2022 dated April 11, 2022.

### 1. Manufacture of sisal nanofiber

The process of extraction of sisal nanofibers through alkylation, neutralization, bleaching, ultrasonication and freeze dried followed the procedure of Sosiati et al.[13] which has been modified according to study by Amanda et al.[10] and Arini et al.[8] The manufacturing phase of sisal nanofiber was carried out at the Integrated Research Laboratory of the Faculty of Dentistry and the Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Gadjah Mada.

Alkalization process was done by soaking dry sisal fiber in a beaker containing 6% NaOH solution and repeated 3 times. Neutralization by means of the alkalized fiber was washed by soaking the fiber in acetic acid solution. The bleaching process used 3% $H_2O_2$ and 1% NaOH, the process was repeated 3 times. Neutralization using distilled water was carried out after bleaching. The process continued by ultrasonication at 10.000 rpm for 2 hours at the Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, Universitas Gadjah Mada, then freeze dried using a freeze dryer.

### 2. Specimen study

The specimens were made at the Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada. The specimen study was a mixture of epoxy resin sealer (AH Plus, Dentsply) with the addition of 1% sisal nanofiber. Specimens were prepared with a diameter of 5 mm and a thickness of 3 mm. The specimens were immersed in the media for 24 hours as supernatant. The supernatant was used as a treatment with serial concentrations of 100%, 50%, 25% and 12.5%. As control used media control and cell control.

### 3. Fibroblast Cell NIH-3T3 Culture

Procedure of fibroblast cell NIH-3T3 culture and MTT assay were carried out at the Integrated Research and Testing Laboratory

(LPPT) Universitas Gadjah Mada. Fibroblast cells NIH-3T3 were grown in DMEM High Glucose medium (Gibco, CA, USA), supplemented with 10% Bovine Calf Serum (Sigma, MA, USA), Pen Strep 2% (Gibco, CA, USA) and Fungizone 0.5 % (Gibco, CA, USA).

**a. Cell Harvest**

The cells were observed using an inverted microscope. Cells were ready to be harvested if they were 80% confluent. The media was poured into the flask and then 3-4 ml of PBS 1x was added to the flask and closed. The process was carried out by washing the cells from the remnants of the media then 1x PBS was removed. Trypsin EDTA (Gibco, CA, USA) 0.25%, 0.5-1 ml was added, and incubation was done for 4 minutes in a $CO_2$ incubator. The flask was shaken to remove the cells from the artificial matrix of the flask. Then 10 ml of complete media was added to inactivate trypsin EDTA 0.25% and rinsed to remove the cells that were still attached. Flushing of the flask walls to remove any adhering cells was followed by resuspension and transfer to a 15 ml sterile conical tube. Centrifugation was carried out at a speed of 2500 rpm for 5 minutes then the supernatant was removed, and 1 ml of complete media was added and resuspended until homogeneous. Suspension cell as much as 10 µl were taken for observation using a microscope and hemocytometer.

b. **Cultivation of cells in 96-well plate (Nunc, MA, USA)**

In each well on a 96-well plate, 100 µl of cell suspension was added with a cell number of $10^4$ cells and then incubated for 24 hours. The treatment was given nanofiber supernatant (from the result of immersion of sisal nanofiber for 24 hours) according to the serial concentration. Incubation was carried out according to the treatment time (24 hours).

**4. MTT Assay**

The media in each 96-well plate was aspirated slowly using a pipette. MTT reagent (Biobasic, NY, USA) was prepared with a concentration of 0.5 mg/ml. In each well, 100 µl MTT 0.5 mg/ml was added then incubated for 4 hours and stopped using DMSO (Merck KGaA, Darmstadt, Germany) 100 µl/well. The absorbance was read using Tecan Spark® (Tecan Trading AG, Switzerland) at a wavelength of 570 nm. The calculation of cell viability is carried out by the formula:

$$\% \text{ Viability} = \frac{\text{OD Treatment} - \text{OD Media Control}}{\text{OD Cell Control} - \text{OD Media Control}}$$

**5. Data Analysis**

The mean value of cell viability with nanofiber supernatant as a treatment was calculated by Microsoft Excel and One Way ANOVA using GraphPad Prism 7 software (GraphPad Software, CA, USA).

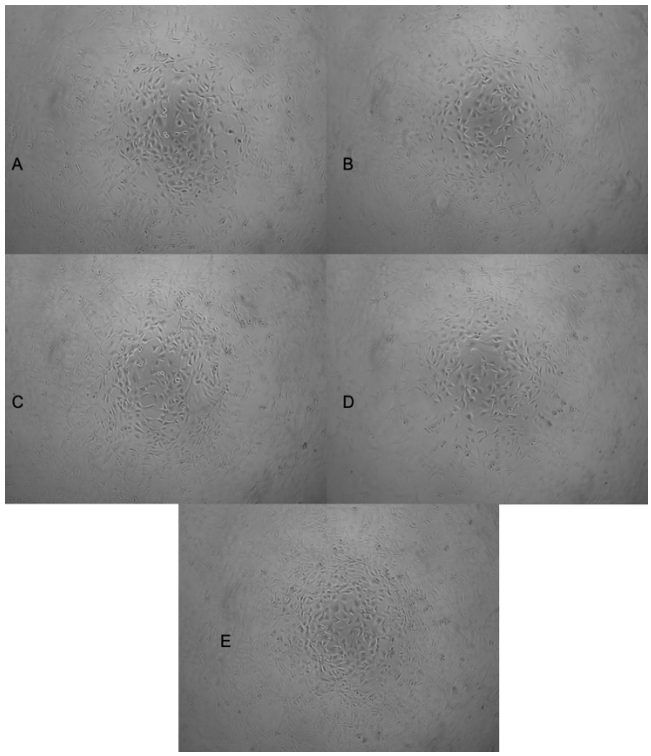


**Figure 1.** Observation confluency of fibroblast cells NIH-3T3 each concentration of sisal nanofibers in the root canal sealer of 12.5% (A); 25% (B); 50% (C); 100% (D) and Control Cells (E). It appeared that the confluency of cells is denser at a concentration of 12.5% compared to the concentration above and it was seen that the cell density decreases as the concentration increases.

In Figure 1 of the concentration used 12.5%; 25%; 50% and 100% showed that the cell confluency was denser at a concentration of 12.5% than the other concentration above. Cell density was decreases with increasing concentration. The results of the effect of sisal nanofibers in root canal sealers at various concentrations on the viability of fibroblast cells NIH-3T3 were shown in Figure 2.

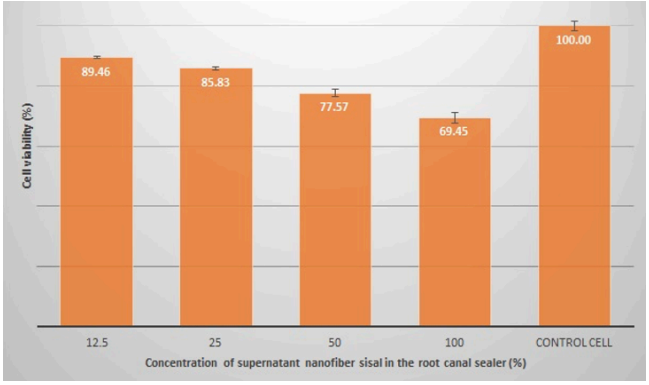


**Figure 2.** Effect of sisal nanofiber in root canal sealer on fibroblast cells NIH3T3 showed the highest viability at 12.5% concentration and the lowest at 100% concentration.

Statistical calculations of normality and homogeneity of cell viability data on the effect of sisal nanofiber in root canal sealer against fibroblast cells NIH-3T3 were shown in Table 1.

| Concentration of supernatant nanofiber sisal in the root canal sealer | Shapiro-Wilk | | | Levene |
|---|---|---|---|---|
| | Statistik | df | Sig. | Sig. |
| 12,5% | 0.750 | 3 | 0.050 | 0.123 |
| 25% | 1.000 | 3 | 0.975 | |
| 50% | 0.767 | 3 | 0.058 | |
| 100% | 0.877 | 3 | 0.315 | |
| Control cell | 0.908 | 3 | 0.413 | |

**Table 1.** Shapiro-Wilk and Levene test results to determine the normality and homogeneity of data on the effect of sisal nanofibers in root canal sealers on the viability of fibroblast cells NIH-3T3.

| Concentration of supernatant nanofiber sisal in the root canal sealer | 12,5% | 25% | 50% | 100% |
|---|---|---|---|---|
| 12,5% | | | | |
| 25% | 0,026 | | | |
| 50% | 0,000 | 0,000 | | |
| 100% | 0,000 | 0,000 | 0,000 | |
| Control cell | 0,000 | 0,000 | 0,000 | 0,000 |

**Table 2.** Post Hoc Tukey test results the effect of sisal in root canal sealer on fibroblast cell viability NIH-3T3 each concentration.

The results of Table 1 indicated that the data viability was normal ($p>0.05$) and homogeneous ($p>0.05$). The next test was performed with parametric statistical tests ANOVA and Post Hoc Tukey to determine the effect of sisal in the root canal sealer each concentration on fibroblast cells NIH-3T3. ANOVA test results showed $p = 0.000$ or it assumed that the effect of sisal in the root canal sealer showed a significant difference ($p<0.05$) on the viability of fibroblast cells NIH-3T3. The test was continued to determine the significance of each concentration shown in Table 2.

Tukey's Post Hoc test results showed that there was a significant difference in the comparison of all concentrations and controls ($p<0.05$). These results indicated that the addition of sisal material to the root canal sealer significantly affected the viability of fibroblast cells NIH-3T3.

## Discussion

The results showed that the confluency of cells was denser at a concentration of 12.5% compared to the concentration above (25%, 50%, and 100%) and the cell density was seen to be less frequent as the concentration increased. This is in line with the results of cell viability (Figure 2) which showed the highest fibroblast cell NIH-3T3 viability was obtained at a concentration of 12.5% although the viability at a concentration of 12.5% was still lower than control cells.

The cytotoxicity evaluation of root canal sealer materials when using human cell lines is currently being developed to determine the biocompatibility of new materials. In this study, using the immersion of sisal nanofiber as a root canal sealer within 24 hours with serial concentrations (12.5%, 25%, 50% and 100%) as treatment. Incubation of fibroblast cells NIH-3T3 with supernatant sisal nanofiber as treatment for 24 hours. The timing of 24 hours was based on a previous study by Ehsani et al.[14] who compared two resin-based sealers (2Seal and AH Plus) against two osteosarcoma-like (fibroblast-like) cell lines, namely Saos-2 and MG-63. The results showed that both materials had a cytotoxic effect on both types of cell lines with an incubation time of 24 or 72 hours. Incubation time for 24 hours compared to 72 hours showed a higher cytotoxic effect on 2 seal material, although there was no significant difference in the Saos-2 cell line. The results also showed that 72 hours on AH Plus was more toxic, it was indicated that AH Plus released more toxic substances after 72 hours.

Epoxy resin sealer material contains bismuth oxide, methanamine, silver, Titanium dioxide. Methenamine is a material used for resin sealer polymerization, during the polymerization process, methenamine will release

formaldehyde. This siler releases formaldehyde up to 48 hours after mixing. Formaldehyde is known to have strong bactericidal properties but is toxic to tissues. Another opinion expressed Ingle et al.[15] that the formaldehyde produced during the polymerization of the epoxy resin sealer will continue to decrease until it disappears after 48 hours so that the bacteriocidal properties of the sealer will work well at the first 48 hours and are also toxic to the tissue at the beginning of its application, so that the intrusion of the sealer enters the periapical tissue should be avoided.

The cytotoxicity effect in this study may be because the sisal nanofiber material in the root canal filling material (AH Plus) contains an epoxy resin that can cause cytotoxicity, especially at dilute concentrations. Epoxy resin contained in root canal filling material (AH Plus) is mutagen and can cause damage to cellular DNA chains. The release of small amounts of formaldehyde content (3.9 ppm) can occur with the use of AH Plus material. Another mechanism of cytotoxicity in root canal filling materials (AH Plus) is thought to be in addition to the release of formaldehyde as well as the combination of release of amines and epoxy resins.[16,17,18]

The lowest cell viability of fibroblasts NIH3T3 was obtained in the undiluted supernatant of sisal nanofiber in root canal sealer (100% concentration) while the highest cell viability was obtained after serial concentration of 12.5%. It is indicated that formaldehyde and other volatile materials still have an effect on the culture media through warm incubation.[20] The decrease in cytotoxicity at low concentrations was indicated by high cell viability, possibly after serial concentrations of sisal nanofiber material in the root canal sealer was cross-reactions to serum proteins in cell culture media resulted in a decrease in toxicity.[19]

The root canal sealer material must be biocompatible, because the root canal sealer material can communicate directly with the periapical tissue and its accessories.[20] The addition of sisal nanofiber as a filler in the sealer is expected to increase biocompatibility. This can be seen from the viability of NIH-3T3 fibroblast cells after treatment in the form of sisal nanofiber immersion media in root canals with a concentration of 100% showing a value of 69.45%. In a previous study conducted by Cotti et al.[21] stated that the confluence and cytotoxicity of L929 mouse fibroblast cells after exposure to an epoxy resin root canal sealer (AH Plus) was about 20% with an incubation time of 24 hours.

This study supported previous results that the content of sisal nanofiber as a root canal filling material for epoxy resin did not change the wettability of the epoxy resin-based sealer.[11] In that study, it was stated that the optimal filler content was determined based on the wettability characteristics. The results of previous studies also stated that sisal nanofiber as a canal filling material showed a contact angle of less than 90$^{o}$ and the consistency of the sealer paste could break when lifted 1.5-2.5 cm from the glass plate. According to the opinion of Torabinejad et al.[22] that a retractable sealer up to 1.5-2.5 cm will provide good sealing properties, especially properties related to stability, sealing ability, and minimizing toxicity. Toxicity was lower than the treatment group (immersion media of sisal nanofiber as filler in root canal sealers) at 100% concentration and serial concentration when compared to previous studies. This may the component of sisal nanofiber did not change the wettability of the epoxy resin-based sealer.

This study may answer that the addition of sisal nanofiber as a filler in the root canal sealers could increase the biocompatibility of the material. These results can be seen from the increase in cell viability along with a decrease the concentration. In this study, 1% sisal nanofiber was used as a filler for root canal sealers. This result supported the previous research conducted by Queiroz et al.[23] that the use of sisal lignocellulosic biomass in the form of hydrogel is not toxic to fibroblast cell culture so that it can be used as a biocompatible material. The results of this study indicated cell viability between 60-90% which is included in the low category. According to the opinion of Barrioni et al.[24] that cell viability of less than 30% indicates high cytotoxicity, 30-60% moderate cytotoxicity, 60-90% high cytotoxicity and above 90% non-toxic. The results of Setiawatie et al. also mentioned that fibroblast cell viability more than 90% after application of Nigella sativa toothpaste showed that the material was not toxic.25

## Results

Results of this study also indicated that the addition of sisal nanofibers could reduce the cytotoxicity of sealers made of epoxy resin when

compared to previous studies that sealers made of epoxy resin without the addition of sisal material cause a decrease in confluency and cell viability about 20%.[21] The active ingredients of sisal nanofiber may play a role in increasing biocompatibility include hydroxyl groups and phenolic methoxy found in the chemical structure of lignin. These ingredients are biologically active and have excellent antioxidant activity. [26,27]

## Conclusion

The results of this study concluded that the addition of sisal nanofiber material (Agave Sisalana) as filler material in the root canal sealer significantly affected the confluency and viability of NIH-3T3 fibroblast cells, which was marked by a decrease in cell cytotoxicity. The highest cell viability was obtained at a concentration of 12.5% from the immersion of sisal nanofiber as filler material in the root canal sealer with an incubation time of 24 hours.

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## Declaration of Interest

The authors report no conflict of interest.

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