International Journal of Dentistry and Oral Science (IJDOS)
ISSN: 2377-8075

Comparative Evaluation On The Cytotoxicity Of Moringa Oleifera Leaf Extract and Calcium Hydroxide On Periodontal Ligament Fibroblast Cells

Research Article

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Abstract

Introduction: Intracanal medicaments are placed between appointments to eliminate those microorganisms that persist after instrumentation. Herbal alternatives to chemical medicaments are being extensively researched owing to their low toxicity, reduced side effects, medicinal value and better patient tolerance. Moringa Oleifera is one such herb with promising antibacterial efficacy. Hence the present study was conducted to evaluate the cytotoxicity of Moringa Oleifera on periodontal ligament fibroblast cells.

Materials and Methods: The leaf extracts of Moringa Oleifera were obtained using the Soxhlet extraction method and its cytotoxicity on periodontal ligament fibroblast cells was tested using the MTT colorimetric assay. Statistical analysis of the result was performed using the ANOVA test.

Results: Percentage of cell viability with 25µg/ml of Moringa Oleifera was 94.8 %, with 50µg/ml of Moringa Oleifera it was 89.8%, with 75µg/ml of Moringa Oleifera it was 84.4 %, with 100µg/ml of Moringa Oleifera it was 76.8%. Calcium hydroxide in concentration of 5mg/ml had viability of 27.1%.

Conclusion: The results of the present study revealed that 25µg/ml concentration of Moringa Oleifera leaf extract was least cytotoxic and the toxicity increased with the increase in concentration of the extract. Moringa Oleifera leaf extract, it could be a potential herb that could be further investigated for its use as an intracanal medicament.

Keywords: Calcium Hydroxide; Cytotoxicity; Medicinal Herbs; Moringa Oleifera; Root Canal Medicaments.

Introduction

The aim of endodontic treatment is to eliminate bacteria from an infected canal and prevent reinfection. Irrigation is imperative in the process of eliminating bacteria from the canal [1]. However, for the elimination of the persisting microorganisms that survive the process of cleaning and shaping of canals, an intracanal medicament is placed between appointments [2]. Further, the use of an intracanal medicament in cases of infected canals has been advocated to reduce the inflammation of periapical tissue and pulp remnants, neutralize tissue debris and render the contents of the canal inert and dry persistently wet canals [2]. Calcium hydroxide, chlorhexidine and triple antibiotic paste are some of the intracanal medicaments that have been routinely used over the years [3]. The use of chemical medicaments is associated with the disadvantages of having cytotoxicity and side effects. Herbal products are

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Received: May 05, 2021
Accepted: May 28, 2021
Published: May 30, 2021

Citation: Rukhsaar Akbar Gulzar, Ajitha, Haripriya Subbaiyan. Comparative Evaluation On The Cytotoxicity Of Moringa Oleifera Leaf Extract and Calcium Hydroxide On Periodontal Ligament Fibroblast Cells. *Int J Dentistry Oral Sci.* 2021;08(05):2610-2614. **doi:** http://dx.doi.org/10.19070/2377-8075-21000511

being studied as an alternative to chemical compounds owing to their low toxicity, side effects and better patient tolerance [4, 5].

Herbal products have been used for centuries to treat and cure diseases [6]. Medicinal plants contain biologically active natural compounds such as alkaloids, flavonoids, coumarins, triterpens, phytoesters, tannins and vitamins. Neem, Tulsi, Triphala, Green tea, Turmeric are a few amongst the many herbs whose antibacterial efficacy has been proven in the past [7].

Moringa Oleifera which is commonly known as drumstick plant, is a herb that is native to India. Its extracts have been studied for anti-inflammatory, anti-fungal and anti-bacterial properties with promising results. Different parts of the plants such as leaves, roots, seeds, fruit, flower and unripe pods are responsible for these properties [8].

The extract from the leaves of Moringa Oleifera have been tested and proven for its antimicrobial activity against E. faecalis which is the persistent organism in cases of Endodontic failure [9]. An ideal medicament should have good antibacterial activity and at the same time it should be compatible with the surrounding tissues.

Previously our team has a rich experience in working on various research projects across multiple disciplines [10-24] Now the growing trend in this area motivated us to pursue this project.

In the present study the cytotoxicity of Moringa Oleifera has been tested on periodontal ligament fibroblast cells and it has been compared to the cytotoxicity of calcium hydroxide.

Materials and Methods

Extraction Methods

The leaves of the Moringa Oleifera plant were ground to powder after washing with distilled water and drying in shade. About 200g of the leaf powder was soaked separately in 400ml of 95% Ethanol and this was then allowed to stand for 7 days. Following Whatman No1 filter paper was used to filter out ethanol. The filtrate was placed in to the thimble of the Soxhlet extraction apparatus chamber. The sample was extracted at 4 cycles per hour for 12 hours. A rotary evaporator was used to remove the solvent after extraction, yielding the extracted compound. By redissolving the crude extracts in 10% dimethyl sulfoxide, the final concentration was maintained as 1mg/ml for bioassay analysis and fractionated into 100µg/ml, 75 µg/ml, 50 µg/ml and 25µg/ml concentrations needed for the bioassay.

Maintenance Of Cell Lines

Periodontal ligament (PDL) normal fibroblast cell lines were purchased from Nation Centre for Cell Science, Pune. The culturing of the PDL Cells was performed in the cell growth Dulbecco's Modified Eagle Medium (DMEM) which contained 10% fetal bovine serum, L-glutamine ,1% penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified CO2 (5%) chamber and 95% air. EDTA Trypsin at a concentration of 0.25% was used to detach the cells. Neutralization of the Trypsin was achieved using DMEM containing 10% Fetal Bovine Serum (FBS) and PSGF, and the cells were mechanically separated using a pipette. There were 96-well plastic culture plates filled with 200µl of medium in each well. To permit attachment of the cells to the plates, the plates were then incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air for 24h.

Cell Viability Assay

Cells were seeded on to 96-well plates at a concentration of 5x103 cells/well for cell viability assay, which was followed by the addition of Moringa Oleifera extract of various concentrations prepared in cell culture media.

A colorimetric assay known as MTT assay was performed. It measures the reduction of yellow 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The PDL cells were seeded at the density of 1 × 103cells/ml and were placed on in to well plates and were then treated with oil for 24 h. The cells were allowed to adhere for 24 hours, and using micropipette the growth medium (MEM) was removed. To remove dead cells and excess FBS, the monolayer of cells was washed twice with MEM with out FBS. As a negative control for assessment of cell viability, cell culture medium (DMEM) was used. In the respective wells, 1ml of medium (without FBS) containing different dilution of drugs were added; 200 µl of MTT (5mg/ml in PBS) were added to each well, and the cells were incubated for a further 6□7 hrs in 5% CO2 incubator. After removal of the medium, 1ml of DMSO was added to each well and the positive control (Allantoin (6mg/ml)) was tested. The effect of MOE (25-100µg/ml) on cell growth inhibition was assessed as percent cell viability, where vehicle□treated cells were taken as 100% viable. Addition of 50µl of propanol was done after the supernatant was removed and the plates were gently shaken to solubilize the formed formazan. The MTT enters the cells and passes into the mitochondria. With in the mitochondria it gets reduced to formazan which is insoluble and dark purple in colour. MTT reduction can occur only in active cells and hence

Table 1. Percentage of cell viability for Moringa Oleifera Extract (MOE) and calcium hydroxide.

Sr No	Treatment	Concentration (µg/ml)	Absorbance 570 nm	% of cell viability
1	PDL untreated cells	-2	0.424±0.03	100
2	MOE	25	0.402 ± 0.25	94.8
3	MOE	50	0.381 ± 0.22	89.8
4	MOE	75	0.358 ± 0.21	84.4
5	MOE	100	0.326 ± 0.19	76.8
6	Calcium Hydroxide (mg/ml)	5	0.115± 0.08	27.1

the number of viable cells is measured by the level of activity of the cells. The plates were placed on a shaker for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. From the values obtained, the percentage cytotoxicity (IC50 value) was calculated. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC50) of the test samples as the percentage survival of the cells was calculated according to the formula provided below:

Percentage of viable cell concentration was calculated thus:

Viability (%)=(Mean test Optical Density/Control Optical Density)×100

Statistical Analysis

The results obtained from the study were expressed as mean ± SD. One-way analysis of variance (ANOVA) and post hoc least-significant difference test was used to determine the statistical significance. Results were deemed statistically significant if the p value was less than 0.05.

Results

Moringa Oleifera extract (MOE) at a concentration of 25µg/ml showed the maximum cell viability and least cytotoxicity. As the concentration increased, the cytotoxicity also increased. Percentage of cell viability with 25µg/ml of Moringa Oleifera was 94.8%, with 50µg/ml of Moringa Oleifera it was 89.8%,with 75µg/ml of Moringa Oleifera it was 84.4 %, with 100µg/ml of Moringa Oleifera it was 76.8%. Calcium hydroxide in concentration of 5mg/ml had viability of 27.1%.Moringa Oleifera treated PDL cells showed statistically significant difference ($p<0.05$) when compared with negative control. Calcium hydroxide treated PDL cells showed statistically significant difference ($p<0.05$) as compared with Moringa Oleifera treated PDL cells.

Discussion

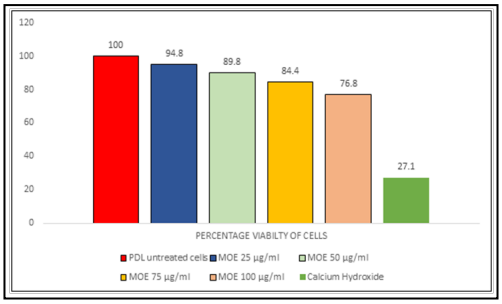
Our institution is passionate about high quality evidence based research and has excelled in various fields [14, 25-34].

Calcium Hydroxide was introduced by Hermann in 1920 and ever since it has widely been used as an intracanal medicament for treatment of cases with apical periodontitis [35]. Because cleaning and shaping alone is not a reliable procedure for complete elimination of bacteria, a canal must be medicated with an antibacterial agent after instrumentation [36]. When introduced in to the periapical region, Calcium Hydroxide appears to be well tolerated and is resorbed subsequently and it was found to be one of the least irritating root filling materials [37]. When used as an intracanal medicament during routine endodontic therapy, Calcium Hydroxide has been reported to have a detrimental effect on periodontal tissues [38]. An increased but not statistically significant inhibition of attached human gingival fibroblasts has been reported by the use of Calcium Hydroxide and hence it was proposed that when trying to regenerate or establish new attachment in tissues adjacent to endodontically involved teeth, calcium hydroxide should be avoided as an interim medicament [39]. Contrary to these findings, Hammarström et al. (1986) demonstrated that the healing of replanted monkey teeth with intact cementum was not affected by Calcium hydroxide and it was only temporarily affected in those undergoing cemental repair [40]. Similarly, Holland et al. (1998) observed that filling infected root canals filled with calcium hydroxide did not hinder the periodontal healing 6 months after experimental periodontal surgical injury in dogs [41].

The need to look for herbal alternatives in the modern era of dentistry is due to the fact that they have fewer side effects, better patient tolerance, and they are less expensive as well as renewable. Herbal extracts are rich in their medicinal properties which makes them an effective means of treating many diseases. Moringa Oleifera is one such alternative herbal species that belongs to the monogeneric family moringaceae and is native to sub-Himalayan regions of North West India. It possesses a broad spectrum of pharmacological activities. Almost every part of the Moringa Oleifera tree (leaves, roots, bark, fruit flowers, immature pods and seeds) has a high nutritional quotient which makes it a plant that is highly valued [42]. Previous studies have proven its anti-inflammatory and analgesic activity. The ethanolic extracts of Moringa Oleifera has been tested for its antimicrobial activity against species such as Escherichia Coli, Pseudomonas aeruginosa, Staphylococcus aureus and Salmonella typhi with positive results. The antibacterial property of Moringa Oleifera has been attributed to the presence of flavonoids, tannins, glycosides and terpenoids [43].

This is a novel study where the cytotoxicity of Moringa Oleifera leaf extract on periodontal ligament fibroblast cells has been evaluated. According to the present study 25µg/ml of Moringa Oleifera leaf extract showed the least cytotoxicity against periodontal ligament cells. As the concentration of the extract increased, its cytotoxicity also increased but its cytotoxicity was significantly less as compared to that of Calcium Hydroxide. There have been studies conducted to evaluate the wound healing potential

Figure 1. Graphical representation of the percentage viability of periodontal ligament (PDL) cells when treated with different concentrations of Moringa Oleifera Extract (MOE) and Calcium Hydroxide. X axis represents the different test drug groups and Y axis represents the percentage viability.



of Moringa Oleifera. A study conducted by Muhammad AA.at. al demonstrated that the aqueous fraction of M. oleifera significantly enhanced proliferation and viability as well as migration of human dermal fibroblast (HDF) cells [44]. In another study conducted by the ethyl acetate (EtOAc) fraction of Moringa Oleifera revealed a significant enhancement of cell proliferation and migration of diabetic human dermal fibroblast HDF-D cells [45].

Asare.at.al studied the potential toxicity of an aqueous leaf extract of M. oleifera in several different experimental systems. In one set of experiments, cytotoxicity of the extract was assessed on human peripheral blood mononuclear cells in vitro by exposing them to graded doses of the extract. Cytotoxicity occurred at 20 mg/kg which is a concentration that cannot be achieved by oral ingestion. In another set of experiments, rats were assessed for 14 days after giving them 1000 and 3000 mg/kg of the extract. The M. oleifera leaf extract was shown to be genotoxic at a dose of 3000mg/Kg, based on blood cell analysis. This is a dose that greatly exceeds commonly used doses. A dose of 1000 mg/kg did not produce genotoxicity when given to rats and was deemed safe. It was still a dose in excess of commonly used doses [46]. Awodele.at.al demonstrated the toxicity of an aqueous extract of M. oleifera leaves in mice and concluded that no significant effects were observed with respect to hematological or biochemical parameters or sperm quality with a high degree of safety was observed on oral administration [47]. Bakre.et.al demonstrated that the lethal dose of 50% of an orally administered ethanol extract of Moringa oleifera leaves in mice was greater than 6.4 g/kg [48]. Based on the results obtained from various animal studies, various preparations of M. oleifera leaves including aqueous extracts appear to be exceedingly safe at the doses and in the amounts commonly utilized [49].

Conclusion

With in the limitations of the present study it can be concluded that leaf extracts of Moringa Oleifera shows low cytotoxicity on the periodontal ligament fibroblast cells. This further opens perspectives for its use as an intracanal medicament owing to its good antibacterial properties and biocompatibility. Although the in vitro observations of the effectiveness and the cytotoxicity results of Moringa Oleifera extracts seem promising, further research may be required before conclusively recommending it as an intracanal medicament.

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