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Cytotoxicity Effects of Guava Leaf Extract on Fibroblast Cells- An Invitro Study

Running Title: Guava Leaf Extract On Fibroblast Cells

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Introduction

Root canal infections are polymicrobial in nature and as we know there are a number of mechanisms involved in eliminating these infectious microorganisms. From access opening to obturation,a constant used to eliminate the biofilm is endodontic irrigants. The aim of this study was to evaluate the effect of Sodium hypochlorite and Guava leaf extract on the L929 fibroblast cell line.

Materials and Method

The guava leaf powder was obtained and the extract was prepared. For cell viability assay, cells were seeded onto 96-well plates at a concentration of 5×103 cells/well, followed by the addition of test samples of various concentrations prepared in cell culture media. MTT assay was done which measures the reduction of yellow 3-(4,5dimethiazol-2-yl)-2, 5-diphenhydramine tetrazolium bromide(MTT) by mitochondrial succinate dehydrogenase. The effect of test samples (25-100µg/ml) on cell growth inhibition will be assessed as percentile viability, where vehicle-treated cells will be taken as 100% viable.

Results

Statistical analysis was done using One-way Anova and a post hoc test. $p<0.05$ was considered as significant. $P<0.001$ as compared with negative control. Guava leaf extract at different concentrations are $P<0.001$ as compared with sodium hypochlorite.

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Conclusion

Guava leaf extract had no or less cytotoxic effect based on its concentration. Hence it can be used as an alternative endodontic irrigant.

Keywords

Endodontic Irrigants, Fibroblast Cells, Root Canal Treatment,Guava Leaf , Natural Irrigant.

Introduction

Root canal treatment is the one which is carried out for pulpal diseases in dentistry. The main aim of root canal treatment is to remove bacteria and metabolite waste inside the root canal system, thereby preventing reinfection of the canal and healing process to take place.Irrigation is the important step of root canal treatment. Irrigation material serves as a debris solvent and makes the instrument movement easier during root canal preparation (Estrela et al., 2014; Kandaswamy & Venkateshbabu, 2010). The ideal root canal irrigation material should have low surface tension and low toxicity as well as should be smear layer-free, inexpensive, and easy to use(Dotto, Sarkis Onofre, Bacchi, & Rocha Pereira, 2020; Jaju & Jaju, 2011; 'Root Canal Irrigants in Horizon: A Review', 2016).

Chemomechanical preparation and three dimensional filling with an adequate seal are considered essential for long-term endodontic success(Martinho & Gomes, 2008; Sedgley, Lennan, & Clewell, 2004). Intracanal irrigants play an essential role in removal of smear layer and disinfection. Combined use of sodium hypochlorite (NaOCl) and ethylenediaminetetraacetic acid (EDTA) has been advocated as the gold standard for the above purpose(Johal, Baumgartner, & Marshall, 2007).

NaOCl (1%–5.25%) and EDTA (15%–17%) have been recommended for removal of organic and inorganic components of the smear layer, respectively. Although NaOCl is a potent antimicrobial irrigant, only higher concentrations are recommended in eradicating Enterococcus faecalis(Önçağ et al., 2003; Radcliffe et al., 2004). Currently, E. faecalis biofilm were found to be resistant to 5.25% NaOCl(Liu, Wei, Ling, Wang, & Huang, 2010). On the contrary, longer exposure times and higher concentrations are recommended for NaOCl to disinfect the canal system(Retamozo, Shabahang, Johnson, Aprecio, & Torabinejad, 2010). EDTA has limited or no antimicrobial efficacy and thus serves as an adjunct rinse only(Buck, Eleazer, & Staat, 1999; Yoshida, Shibata, Shinohara, Gomyo, & Sekine, 1995).

Most complications of the use of NaOCl appear to be the result of its accidental injection beyond the root apex which can cause violent tissue reactions characterized by pain, swelling, hemorrhage, and in some cases, development of secondary infection and paresthesia. A great deal of caution should therefore be exercised when using NaOCl during endodontic irrigation(Spencer, Ike, & Brennan, 2007; Verma, Goel, Bala, & Singh, 2012). Ehrich et al. suggested that a clinician should check, both clinically and radiographically for immature apices, root resorption, apical perforations, or any other conditions that may result in larger than normal volumes of irrigant being extruded from the root-canal system into the surrounding tissue(Ehrich, Brian, & Walker, 1993).

The most common bacteria found in root canals are Enterococcus faecalis. These bacteria are usually found in the failed root canal treatment. The prevalence of E. faecalis bacteria in root canal failure reached 38% (Rôças & Siqueira, 2012; Zoletti et al., 2011) The irrigation material that commonly used in root canal treatment is sodium hypochlorite, which has certain weaknesses, such as becoming toxic when in contact with periradicular tissue. Sodium hypochlorite is antimicrobial to bacteria, both Gram-positive and negative, spores, fungi, and viruses (Mack, 2014; Vidana, Sullivan, Billström, Ahlquist, & Lund, 2011). However, the optimal property of NaOCl to dissolve organic tissue is non-selective that means it is able to dissolve both necrotic and vital pulp remnants indistinguishably, especially at high concentrations.The in vitro studies showed that 0.5% sodium hypochlorite has a longer incubation time than other irrigation ingredients in eradicating E. faecalis (Borzini, Condò, De Dominicis, Casaglia, & Cerroni, 2016).

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Toxicity of irrigants has been evaluated on various cell lines depending on their growth potential, metabolic characteristics, and potential of DNA damage. Cell lines of target or non-target tissues have been utilized and the results obtained have been mixed; thus no definite data exists to confirm simultaneously on both cell lines(Hauman & Love, 2003; Ravinanthanan, Hegde, Shetty, & Kumari, 2018). Hence there is a need to check the potential of all endodontic irrigants for the toxic effects on the fibroblast cells.

Psidium guajava is a phytotherapic plant commonly known as Guava. It belongs to the Family Myrtaceae species(Ismail, Minhas, Khanum, & Sahana, 2012; Jang et al., 2014)**.** The leaves of the plant P. guajava Linn are reported to possess antioxidant, hepatoprotective, anti allergy, antimicrobial, antigenotoxic, antiplasmodial, cytotoxic, antispasmodic, cardioactive, anti cough , antidiabetic, anti inflammatory and antinociceptive activities(Jaiarj et al., 2000; Jayakumari et al., 2012 (April - June); Jose et al., 2016; Nayak et al., 2019; Ukwueze, Osadebe, & Okoye, 2015).Guava plants have been reported to contain phytochemicals active against some viruses, included HIV-1(Mao et al., 2010; Sriwilaijaroen et al., 2012). The leaves of guava are rich in flavonoids, particularly quercetin as the main active substance, as well as saponins, among others(Lozoya et al., 2002). The aim of this study was to evaluate the effect of Sodium hypochlorite and Guava leaf extract on the L929 fibroblast cell line.

Materials and Methods

Chemicals

The materials used for MTT test were 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 10% fetal bovine serum (FBS), 100 units/ml of fungizone, dimethyl sulfoxide (DMSO), human fibroblast cell lines (primary culture), Eagle's minimum essential medium (EMEM), kanamycin, and phosphate-buffered saline.

Plant Materials

The guava leaf was purchased from the local market, shade dried and made into fine powder for the extract preparation. The plant sample was authenticated by a Botanist from Central Drug Research Institute, Tamil Nadu.

Ethanolic Extract

About 100 gm of the powdered sample was mixed with 1000 ml of ethanol and kept for 48 hrs at room temperature to ensure maximum metabolite extraction. The extract obtained was filtered and concentrated. The extraction method is based on the solubility of the constituents of the sample in ethanol. The filtrate is placed into the thimble of the Soxhlet extraction apparatus chamber. The sample was extracted for 12 hours at 4 cycles per hour. After extraction, the solvent was removed by the means of a rotary evaporator, yielding the extracted compound. The final concentration was maintained as 100mg/ml by redissolving the crude extracts in 10% dimethyl sulfoxide for bioassay analysis.

Maintenance of Cell Lines

L929 fibroblast cell lines were purchased from NCCS Pune. The L929 Cells were cultured in a humidified atmosphere at 37 °C in the cell growth DMEM medium with 10% fetal bovine serum, L–glutamine ,1% penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified CO_2 (5%) chamber and 95% air. The cells were detached using 0.25% EDTA Trypsin. Neutralization of the Trypsin was achieved using DMEM containing 10% FBS and PSGF, and cells were mechanically separated using a pipette. There were 96-well plastic culture plates filled with 200µl of medium containing in each well. The plates were then incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air for 24 h to permit attachment of the cells to the plates.

Cytotoxicity test by MTT assay

The EMEM, Kanamycin, 1% pen strep, 10% FBS, and 100 units/ml of fungizone 100 µl was added as a control media to the 96 well microtiter plates. The microplates are filled with 100 µl of fibroblast cells with a density of 3×10^3 in EMEM, kanamycin, 1% pen strep, 10% FBS, and 100 units/ml of fungizone as negative control. The cells were permitted to adhere for 24 hours, and the growth medium using micropipette and the monolayer of cells was washed twice with MEM without FBS to remove dead cells and excess FBS.

1ml of medium (without FBS) containing different dilution of guava leaf extract (25-100µg/ml) were added in respective wells; 20 µl of MTT (5 mg/ml in PBS) were added to each well, and the cells incubated for a further 6-7 hrs in 5% CO_2 incubator. After removal of the medium, 1ml of DMSO was added to each well and the positive control

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(Sodium hypochlorite) was tested. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. 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. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC_{50}) of the test samples as the percentage survival of the cells was calculated.(Figure 1).

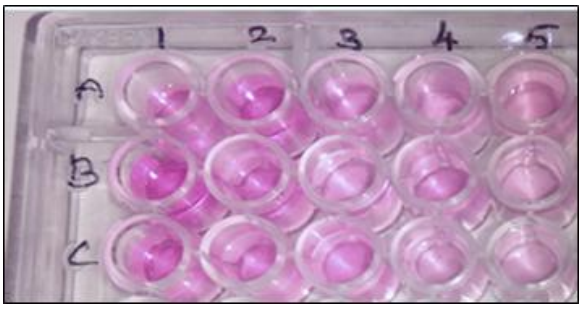

Figure 1- Microplate images showing the colour changes for different test groups. 1-L929 cells untreated negative control; 2-L929 cells treated with GE (25µg); 3- L929 cells treated with GE (50 µg) ; 4- L929 cells treated with GE (100 µg); 5- L929 cells treated with NaOCl.

Statistical analysis

Results were expressed as mean ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference test. $P<0.05$ was considered as significant.(Table 1).

S.N	Treatment	Conc (µg/ml)	Absorbanc 570nm	% of cell Viability
1	L929 untreated cells	-	0.512±0.0	100
2	Guava leaf extract	25	0.481 ± 0.08*[a]	93.9
3	Guava leaf extract	50	0.415 ± 0.03*[a]	81.0
5	Guava leaf extract	100	0.396 ± 0.09*[a]	77.3
6	NaOCl	3%	0.108± 0.08*	21.0

Table 1-Values are expressed as Mean±SD (n=3); *P<0.001, as compared with Negative control. [a]P<0.001, as compared with NaOCl. The LC_{50} of the Guava leaf extract is <100µg/ml

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Results

Cytotoxicity in cell culture is typically expressed as LC50. The LC50 of the guava leaf extract is <100ug/ml. P<0.001 as compared with negative control. Guava leaf extract at different concentrations are P<0.001 as compared with sodium hypochlorite.

Discussion

The present in-vitro study evaluated the cytotoxicity of guava leaf extract on the L929 fibroblast cell line. The results showed that the guava leaf extract at 25µg/ml concentration showed about 93.9% cell viability and even at 100µg/ml showed cell viability of about 77.3 % cell viability, Which is less toxic to L929 fibroblasts cells.

Guava leaf is rich in bioactive compounds and in order to utilize these compounds in the preparation of value added products, these are extracted using ethyl acetate. Seo et al, reported the extraction of essential compounds of guava using water, ethanol and methanol, and found the highest content of phenolic compounds in water extract(Seo et al., 2014). The ethyl acetate extract was found to be rich in sesquiterpenes and fatty acids. Sesquiterpenes such as cubenol, 6S-2,3,8,8-tetramethyl tricyclo[5.2.2.0(1,6)]undec-2-ene,benzene,(1,3,3-trimethyl nonyl) and 1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl-, (E) act as flavouring and fragrance agents. Apart from these, *cis*-à-bisabolene, an intermediate in the biosynthesis of hernandulcin which is a natural sweetener was also identified in the extract(Christianson, 2017). Another major compound identified in the extract was fatty acid esters which are found to be fragrance ingredients. Thus, the GC–MS analysis of guava leaf extract identified various beneficial compounds with enormous medicinal importance.

The essential oil of *P. guajava* is efficient in inhibiting the growth of both Gram-positive and Gram-negative bacteria at higher concentration(Nisha, Darshana, Madhu, & Bhupendra, 2011).The phenolic compound content of water extract was higher than pure ethanol and methanol extract. The phenolic compound content of hydroethanolic extracts was higher than water extracts. The antioxidant activity of those hydroethanolic extracts was higher than that of the water extracts and was significantly high in the 50% hydroethanolic extract that had the highest content of phenolic compounds(Seo et al., 2014).

Psidium guajava extract reduced the cell population in both in vitro and in vivo the extract accelerated wound healing and helps in faster healing of tissues (Porta, Fernandes, Bussadori, Marques, & Martins, 2010).The ethanolic and water extract of guava leaves possess antibacterial activity against L.acidophilus with 20% ethanolic extract being as efficacious as 0.2% chlorhexidine(Jain et al., 2014). Extracts rich in guava flavonoids have demonstrated their potential for preventing dental caries due to the growth inhibition of the oral flora(Thalikunnil, Ashok, & Sukesh, 2012). Ethanolic and water extracts of guava leaves possess antibacterial activity against L. *acidophilus* with 20% ethanolic extract being as efficacious as 0.2% chlorhexidine(Jain et al., 2014).

Cytotoxicity in cell culture is typically expressed as LC50, which means that the concentration of a given agent is lethal to 50% of the cells. Moreover, the most common way to describe cytotoxicity in cell culture is LC50, the concentration of a drug that kills half of the tested cells in culture (Zhang et al., 2007).

In this research, the cytotoxic effects of the psidium guajava leaf extract at the concentrations of 25µg/ml, 50µg/ml and 100µg/ml were examined on human gingival fibroblast using MTT assay in comparison with positive and negative control. The results showed that the psidium guajava leaf extract has no cytotoxic effect. Percentage cell viability at MIC (25µg/ml) was 93.9% , MIC (50µg/ml) was 81% and MIC (100µg/ml) was 77.3%. Psidium guajava leaf extract even at 100 % concentration was not cytotoxic and also it is capable of killing E. faecalis, so it has the potential to be further investigated as a root canal irrigation material. Further studies will be designed to investigate its biomedical applications with a detailed mechanism through appropriate experimental models.

Conclusion

Guava leaf extract had no or less cytotoxic effect based on its concentration. Comparatively most of the cells showed less cytotoxic effect when compared to sodium hypochlorite at different concentrations. Hence it can be used as an alternative endodontic irrigant for better prognosis and outcome of the root canal treatment.

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Conflicts of interest

There are no conflicts of interest.

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