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An Invitro Cytotoxic Analysis of Citrus Sinensis (Sweet Orange) as Root Canal Irrigant on Fibroblast Cells

Running Title : An Invitro Cytotoxic Analysis Of Citrus Sinensis On Fibroblast cells

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Abstract

Background: Resistant microbes always represent a challenge in the treatments of various well-known infections and urges the need for substances with potent antimicrobial properties. Citrus sinensis is known for its medicinal value. Many medicinal properties of orange peel extract, such as against colic, upset stomach, cancer, diuretic, immuno enhancing, and fight viral and bacterial infections. This study aims to evaluate the cytotoxicity of citrus sinensis against fibroblasts.

Methodology : Aqueous and ethanol extracts prepared from peel of Citrus sinensis were screened for in vitro cytotoxic activity against L9292 cells using MTT cell viability assay. For cell viability assay, the microplates filled

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with 100 µl of L929 Cells and growth medium using micropipette, read on an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. Each experiment was carried out in triplicate and the IC50 of the test samples as the percentage survival of the cells was calculated.

Results: CSE did not adversely affect the fibroblasts even up to 50% concentration showing a nontoxic effect even till 200 µg/ml dose in comparison with CHX on these cells.

Conclusion: Citrus sinensis peels extract demonstrated less cytotoxic activity warranting further in vivo clinical studies to determine the exact dosages as root canal irrigant and its effectiveness in practical situations.

Keywords Citrus Sinensis, Sweet orange, Orange peel, Cell viability assay, Cytotoxicity, Fibroblast cells.

Introduction

Orange, the tasty, juicy fruit, belonging to the family Rutaceae is botanically known as Citrus sinensis. Citrus sinensis is one of the most important and widely grown fruits, with total global production reported to be around 120 million tons. Orange trees are widely cultivated in tropical and subtropical climates for its tasty juice and medicinal value.(Hotwani, Baliga and Sharma, 2014; Chemat and Strube, 2016) .

Major medicinal properties of orange peel extract are immuno – enhancing, stomachic, tonic to digestive system, immune system and proven against colic, upset stomach, cancer. It is mainly used to treat and prevent vitamin deficiencies, colds, flu, and scurvy and help to fight viral and bacterial infections.The fruit usually contains a sweet pulp and several to numerous seeds within.The fruit pulp is typically formed of eleven segments of juice filled with flavor that goes from sour to sweet.The fruit is perennial and it has adapted to a variety of climates. (Akdemir Evrendilek, 2015; Mehmood *et al.*, 2015; Chemat and Strube, 2016)

Citrus sinensis is consumed all over the world as an excellent source of vitamin C. Citrus sinensis is a rich source of secondary metabolites which contribute to the pharmacological activities attributed to this plant. Several types of chemical compounds have been identified in fruits, peel, leaves, juice and roots of C. sinensis, which include the following groups: flavonoids, steroids hydroxy amides, alkanes and fatty acids ,coumarins, peptides, carbohydrates, carbamates and alkylamines, carotenoids ,volatile compounds and nutritional elements such as potassium, magnesium, calcium and sodium.(Satthanakul *et al.*, 2015; Shimada *et al.*, 2015)

Antibacterial effects of orange peel have also been demonstrated in the literature. Mehmood et al. (2015) showed potent antibacterial activity (against Enteric pathogens) of extract from Orange peels (4). Orange peel extract was also found to be effective against Klebsiella pneumonia by Akdemir (2015).(Mistry *et al.*, 2014; Nayak, Metgud and Bolmal, 2014)

Natural products have been and will be important sources of new pharmaceutical compounds. Recently, there has been a renewed interest in natural product research due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas.(Varaldo, 2002; Harrewijn, van Oosten and Piron, 2012) In this sense, considering the health benefits of C. sinensis it presents excellent options for treating or helping in a disease due to its bioactive compounds. Previously our team has a rich experience in working on various research projects across multiple disciplines (Soh and Narayanan, 2013; Campeau *et al.*, 2014; Christabel, 2015; Thamaraiselvan *et al.*, 2015; Christabel *et al.*, 2016; Kumar and S, 2016; Ramesh *et al.*, 2016; Thangaraj *et al.*, 2016; Govindaraju and Gurunathan, 2017; Kumar and Rahman, 2017; Sridharan, Ramani and Patankar, 2017; 'Fluoride, fluoridated toothpaste efficacy and its safety in children - review', 2018; Ezhilarasan, Apoorva and Ashok Vardhan, 2019; Mehta *et al.*, 2019; Ponnulakshmi *et al.*, 2019) Now the growing trend in this area motivated us to pursue this. The aim of this study is to analyse the cytotoxicity of Citrus Sinensis on the fibroblast cells.

Methodology

Oranges (Citrus sinensis) were purchased from the local market and orange peels were obtained. The peels were carefully washed under running tap water followed by sterile distilled water. These were air dried at room temperature (30°C) for two days, pulverized to a fine powder using a sterilized mixer grinder and stored in air-tight bottles. Two different solvents namely etha- nol (hot and cold) and water (hot and cold) were used for extraction to obtain a total of 4 extracts. For the purpose of extraction, a 10 g amount of the pulverized peel was separately soaked in 100 ml of ethanol (96%) and cold sterile distilled water for 24h. Also the same amount (i.e. 10 g) of pulverized peel was immersed in 100ml of hot sterile distilled water (100°C) and allowed to stand for 30 min on a water bath

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with occasional shaking and kept undisturbed for 24 h. Each preparation was filtered through a sterilized Whatman No.1 filter paper and the filtered extract was concentrated under vacuum below 40°C using Heidolph, VE-11 rota evaporator. The dried extract thus obtained was exposed to UV rays for 24h and checked for sterility on nutrient agar plates and stored in labelled sterile bottles in a freezer at 4°C until further use.

Chemicals

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

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 CO2 (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 200 µl 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.

Cell viability by MTT assay

For cell viability assay, the microplates filled with 100 µl of L929 Cells with a density of 1×105 as negative control. The cells were permitted to adhere for 24 hours, and the growth medium using micropipette and the monolayer of cells washed twice with MEM without FBS to remove dead cells and excess FBS. About 1ml of medium (without FBS) containing different dilution of Citrus sinensis extract (25, 50, 100, 200 µg/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% CO2 incubator. After removal of the medium, 1ml of DMSO was added to each well and 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) (MINDRAY90) reader at 570 nm. Each experiment was carried out in triplicate and the IC50 of the test samples as the percentage survival of the cells was calculated.

Statistical analysis

Results were expressed as mean ± S.E.M. Statistical significance was determined by one- way analysis of variance (ANOVA) and post hoc least-significant difference test by SPSS software (version 22.0). P values less than 0.05 were considered significant.

Results

Table 1 :The MTT assay absorbance of L929 cells after the treatment with Citrus sinensis extract

S.no	Treatment	Concentration (µg/ml)	Mean ± SEM
1	L929 untreated cells	-	0.507 ± 0.03
2		25	0.495 ± 1.10*
3		50	0.487 ± 1.15*
4		100	0.459 ± 0.24*
5		200	0.302 ± 0.13*
6	CHX	0.2%	0.396 ± 0.01*

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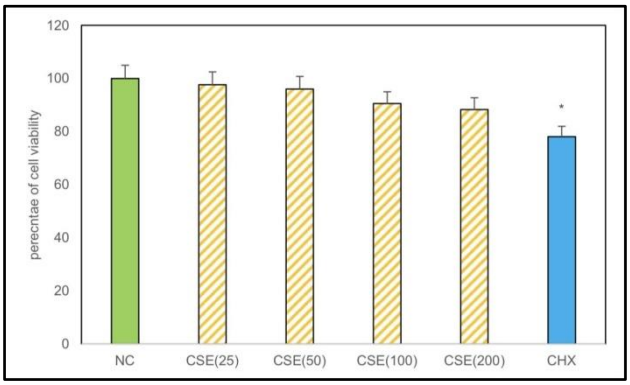
7	Saline	0.9%	0.499 ± 0.26*

Values are expressed as Mean ± SEM (n=3); *P<0.001, as compared with Negative control. aP<0.001, as compared with CHX. The IC50 of the extract is more than 200µg/ml

Graph 1 Showing the cell viability in different groups



Inference : Values are expressed as Mean ± SEM (n=3); *P<0.05 statistically significant as compared with Negative control. However, CSE did not adversely affect the fibroblasts even up to 50% concentration showing a nontoxic effect even till 200 µg/ml dose in comparison with CHX on these cells.

Discussion

The toxic effects of materials used for endodontic therapy are of particular concern, because damage or irritation could cause degeneration of the periapical tissue and delayed wound healing.In in vivo tests such as implantation and usage tests have an advantage in that they allow complex interaction between the host and the material to be examined.(Cowan, 1999; Ghannoum and Rice, 1999)(Escudero-López *et al.*, 2013, 2016) In vitro tests such as cell culture enable experimental factors and variables to be controlled which often is a significant problem when performing experiments in-vivo. These in vitro model assays are increasingly being used for initial screening of new dental materials intended for clinical use .(Blatter and Reich, 2003; 'Compounds found in Herbal products', 2009; Seo and Efferth, 2017)

A variety of test systems are available to determine the cytotoxicity of dental materials in cultured mammalian cell populations. Permeability assays monitor the integrity of cell membranes by the inclusion or exclusion of vital dyes or by the release of radiolabeled chromium. Replication assays indirectly assess the ability of cells to proliferate by measuring the incorporation of nucleotide analogues that have been radiolabeled or are detectable by immunoassay during DNA synthesis.(Ryu *et al.*, 2013; Gilbert and Friedrich, 2017) Changes in the cellular cytoskeleton or at the cell surface are observed by morphological studies. Finally, functional assays typically evaluate the cell's ability to provide the energy necessary for anabolic activities, or the end products of such activities. The assay used in the present study used the tetrazolium salt MTT to measure mitochondrial dehydrogenase activity. It is a plate yellow substrate that produces a dark blue formazan product when cleaved by active mitochondria. The decision to use a particular test system should be based on its consonance with the chemical nature of the material being tested. For example, if a material is not likely to cause a change in the permeability of cell membranes, a permeability assay is less apt to determine cytotoxicity in a valid manner. (Schmalz and Bindslev, 2008; Küçük, Yıldırım and Çetiner, 2021; Nashaat, Sabry and Hassan, 2021)

After mixing materials, in order to achieve effective dilutions for performing the tests, a serial dilution method was used, which is applied for evaluation of dose- response effect in material toxicity studies and was due to Keiser's method. Thus, according to quality and quantity assessment in this investigation, it possesses the privilege that what was observed in optical microscope qualitatively was also evaluated quantitatively using MTT assay test. While most of other studies were only based on whether quantitative or qualitative assessment, the histological investigations of Christopher et al. on tissue response of dog's periapical(Pavan *et al.*, 2015), Torabinejad et al. on tissue response of monkey's periapical incisor,(Torabinejad *et al.*, 1985) Zhu et al. on osteoblast cell response in

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contact with retrofill compounds,(Arbez and Libouban, 2017) all were qualitative. While, the studies of, Ossorio et al. evaluating MTT assay (Molaae *et al.*, 2017)and crystal violet assay, Torabinejad et al. based on two techniques of Agar over lay and Radiochromium Release method and Keisser with MTT assay technique, represent quantitative assessment of materials' cytotoxicity.(Plumb, no date)(Breitinger *et al.*, 2021)

The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oils and flavonoids. These compounds are known to be biologically active and therefore aid the antimicrobial activities of the plants..(Družić *et al.*, 2016; Kumar, Prem Kumar and Oprian, 2017)(Grace and Fetsch, 2018; Mukherjee *et al.*, 2021) These secondary metabolites exert antimicrobial activity through different mechanisms. Tannin as observed in citrus sinensis peel extract have been found to form irreversible complexes with proline rich protein resulting in the inhibition of cell protein synthesis.

Our institution is passionate about high quality evidence based research and has excelled in various fields ((Pc, Marimuthu and Devadoss, 2018; Ramesh *et al.*, 2018; Vijayashree Priyadharsini, Smiline Girija and Paramasivam, 2018; Ezhilarasan, Apoorva and Ashok Vardhan, 2019; Ramadurai *et al.*, 2019; Sridharan *et al.*, 2019; Vijayashree Priyadharsini, 2019; Chandrasekar *et al.*, 2020; Mathew *et al.*, 2020; R *et al.*, 2020; Samuel, 2021)

Clinical significance

The peels of fruits of Citrus sinensis which are generally treated as wastes can serve as an effective and economical antimicrobial agent as they are available for no cost, and have no side effects. In future, in vivo clinical studies should be conducted to conform in vitro results and for the assessment of safety and efficacy by incorporating these plant extracts into dental products such as mouth rinses and toothpastes.

Conclusion

Citrus sinensis peels extract demonstrated less cytotoxic activity warranting further in vivo clinical studies to determine the exact dosages as root canal irrigant and its effectiveness in practical situations.

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Conflicts of interest

There are no conflicts of interest.

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