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Cytotoxic activity of *Calendula officinalis* extract on *3T3* mouse fibroblast cell line using MTT assay- An *in vitro* study

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Abstract

Calendula officinalis (CO) is often known as the Pot marigold. It is a common topical therapy in homoeopathic medicine. It contains quercetin, carotenoids, lutein, lycopene, rutin, ubiquinone, xanthophylls, and other antioxidants. It has anti-inflammatory, anti-microbial, anti-oxidant and analgesic properties. The pulp and periapical tissues are in contact with intracanal medications employed during root canal therapy. For any novel material to be used in clinical scenarios, the material's cytotoxicity must be assessed. Hence this study aimed to evaluate the cytotoxic effects of a novel herbal intracanal medication on 3T3 mouse fibroblasts using the Methyl Thiazolyl Tetrazolium test (MTT assay) (3-4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide). The CO aqueous extract was utilized for the cytotoxicity test. The 3T3 fibroblast cells were treated by CO in different concentrations (0.1, 0.2, 0.5, 1, 2.5, 5 and 10 mg/ml) for 48-hour incubation period. Cell viability was determined with MTT assay. CO showed no cytotoxicity when used in less concentration and at highest concentration of 10% showed little cytotoxicity. At optimal concentration, the CO medication showed low cytotoxic effects, indicating that it is safe to use.

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Introduction

The fundamental goal of endodontic therapy is to eliminate pathogens from the root canal system. The root canal system's anatomical complexity may harbor leftover bacteria that withstand biomechanical preparation and result in recurrent periapical infections. The selection of disinfecting agents is critical in achieving a successful long-term endodontic therapy. Intracanal medicaments play a major role in disinfection of root canal system by neutralising endotoxins, preventing microbes from entering through saliva, reducing pain and inflammation, and fostering an environment that promotes hard tissue healing (Kandaswamy *et al.*, 2010).

Intracanal medications, which are utilised in endodontic therapy, come into close contact with living pulpal and periapical tissue cells, promoting both periapical tissue healing and repair (Zapata, 2022).

Recent trend towards herbal agents with antibacterial effects due to the hazardous side effects of synthetic medications and the growing number of antibiotic-resistant strains has gained popularity in the medical field (Vaou *et al.*, 2021).

Calendula officinalis (CO), frequently referred to as 'Pot Marigold', belongs to the Asteraceae family. The "Food and Drug Administration" (FDA) and the "Flavours and Extracts Manufacturers Association" (FEMA) have recognized the safety of plant parts and dried flowers used as spices. CO exerts several therapeutic effects such as antibacterial, antifungal, antiviral, anti-HIV, antioxidant, anti-inflammatory, analgesic, hepato-protective, cardio protective, gastro protective and wound healing properties (Nagaraj *et al.*, 2022).

CO can be utilized as an intracanal medicament for long-standing periapical lesions due to its higher resistance to root dentin fracture when compared to calcium hydroxide (Nagaraj *et al.*, 2020). Yalgi *et al.* demonstrated the antibacterial activity of CO as a root canal irrigant against *Streptococcus mutans*. Because of its antibacterial qualities, shown excellent results in eradicating microbes and can be utilized as an alternative disinfecting agent (Yalgi *et al.*, 2020). Though this plant has been extensively studied, very few studies have examined the cytotoxic effects of CO in dentistry.

The biocompatibility of a material used for the replacement or filling of biological tissue like teeth always had high concern for patients within the health care disciplines. For any material to be used for clinical scenario the cytotoxicity evaluation of the material has to be tested (Shahi *et al.*, 2019). Hence, this in *v*itro study was done to evaluate the cytotoxic effect of CO in 3T3 mouse fibroblast cell lines.

Materials and methods

CO medicament preparation

CO petals in dried form were procured (Organic Bioherbs, USA). An Erlen Mayer flask (EM) was filled with 20 g of powdered flower petals and 100 ml of sterile water. For complete elucidation of the active ingredients to dissolve in the solvent, the mouth of the EM flask was closed with aluminium foil and placed in a reciprocating shaker with constant agitation at 150 rpm for a duration of 24 hours (Shekawat, 2013). The extract was then filtered in a micro-fluid filtering device using muslin cloth and Whatman No. 1 filter paper. The final extract was lyophilised for 24 hours at -40°C using a lyophilizer to make it as powder form (Martin Christ, Germany) (Thakur *et al.*, 2011).

1 g of this powder was dissolved in 5 ml of distilled water and volume completed to 10 ml to obtain a stock solution with concentration of 10%. The different concentrations (0.1, 0.2, 0.5, 1, 2.5, 5 and 10 mg/ml) were prepared from these stock solutions for carrying out the cytotoxic activity (Yaseen, 2020).

Cytotoxicity of CO using 3T3 cell lines by MTT assay

Preparation of cell suspension

A subculture of 3T3 cells in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. 25 ml of DMEM

with 10% fetal calf serum (FCS) was added to the disaggregated cells in the flask. The cells were suspended in the medium by gentle passage with the pipette and the cells were homogenized (Aishwarya Devi *et al.*, 2017).

Seeding of cells

One ml of the homogenized cell suspension was added to each well of the 24 well culture plates, along with different concentration of aqueous CO extract (0.1, 0.2, 0.5, 1, 2.5, 5 and 10 mg/ml) and incubated at 37°C in a humidified CO_2 incubator with 5% CO_2. The cells were observed after 48 hrs incubation under an inverted tissue culture microscope. With 80% confluence of cells cytotoxicty assay was performed (Aishwarya Devi *et al.*, 2017).

Cytotoxicity assay

The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48 h incubation, the wells were added with MTT solution and left for 3 hrs in room temperature. The contents were removed from the wells using sterile pipette and 100µl sodium dodecyl sulphate (SDS) in Dimethyl sulfoxide (DMSO) were added to dissolve the formazan crystals. The absorbance values of the solution were read in micro plate reader (Lark LIPR-9608), at 540 nm (Mosman *et al.*, 1983).

Results and discussion

The results revealed that after 48 hours incubation, CO showed no cytotoxic activity in less concentrations and at highest concentration of 10% showed almost 70% viable cells (Fig. 1). 3T3 cell lines were triggered significantly with the increasing of sample concentration and it was observed in results (Table 1).

Table 1. *In vitro* cytotoxicity effect of CO against 3T3 mouse fibroblast cell lines

Sample concentration (µl/mL)	% Cell viability 3T3 cells Calendula extract
0	100.00
0.125	99.33
0.25	98.45
0.5	97.37
1	94.41
2.5	92.59
5	81.75
10	68.96

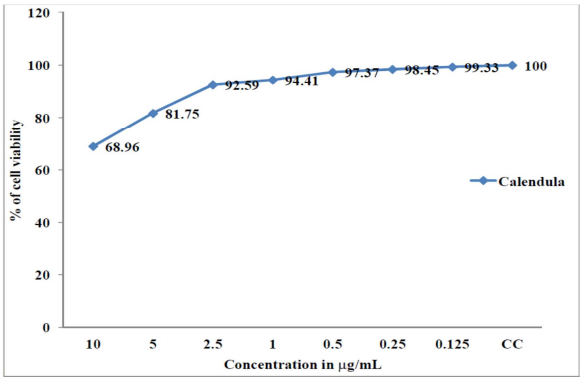

Fig. 1. Graph showing the cytotoxic activity of CO on 3T3 mouse fibroblast cell lines

Discussion

Biomechanical preparation alone is insufficient to completely eradicate bacterial toxins. Antimicrobial medications, such as irrigants and intracanal medicaments, are required to disinfect the root canal system (Bystrom *et al.*, 1981). An intracanal medication should ideally have antibacterial activity that exceeds cytotoxicity; while these medications may eradicate pathogens, they may also harm periapical tissues (Karapınar-Kazandag, 2011).

According to the literature, antimicrobial medications and irrigants used in endodontic therapy have a significant impact on stem cell survival in the root canal environment, hence it's critical to assess their cytotoxicity profile (Trevino *et al.*, 2011).

Potential sources of novel antimicrobial compounds, particularly those that target bacterial infections, include herbal remedies. They also exhibit hydrophobic properties. As a result, they are able to break down the lipids in the mitochondria and the

bacterial cell wall, which will ultimately lead to the destruction of bacterial architecture. CO, which has strong antibacterial, anti-inflammatory, antioxidant, analgesic, and wound-healing effects (Muley *et al.*, 2009). The antimicrobial effects of CO using various extracts against both Gram-positive and Gram-negative organisms, and discovered that aqueous extracts were more efficient against all the microbes (Mathur *et al.*, 2011). Hence, aqueous extract of CO was used in this study.

MTT is one of the most extensively used colorimetric assays for evaluating the cell viability and cytotoxicity of many medications at various doses. The assay uses colorimetry to measure the amount of viable cells in a sample by reducing the yellow tetrazolium dye MTT (3-{4, 5-dimethylthiazol–yl}-2, 5-diphenyltetrazolium bromide) to a violet formazan product that can be measured spectrophotometrically at 540 nm. The more purple the colour, the more viable cells are in the sample (Van de Loosdrecht *et al.*, 1994).

For cytotoxic evaluation of CO, an immortalized 3T3 fibroblast cell line was employed because it is a well-characterized cell model and has been previously used to assess the cytotoxic effects of dental materials (Wataha *et al.*, 1993). Fibroblasts are biologically similar to pulp and periapical cells, hence cell type is a significant element in cytotoxicity study. They are rapid and simple to develop, and they are numerous in pulp and periapical tissues that are sensitive to the effects of intracanal medications and their byproducts. Fibroblasts play an important part in wound healing and repair because they create a substantial amount of collagen (Ghivari *et al.*, 2022).

The results revealed that cell viability reduces as the concentration of CO increases but this change is not significant. The highest concentration of CO showed upto 70% cell viability. The highest concentration of CO used in this investigation was 10% since research demonstrated that 10% of CO dramatically decreased inflammation and promoted the healing of wounds in wistar rats (Ghodoosi *et al.*, 2022).

In this study increased concentration of CO at 10 % showed little cytotoxicity over the 3T3 cell lines and it is in accordance with the previous study who evaluated cell viability using (25, 50, 100) mg/ml concentrations of the extract (Jalill *et al.*, 2014).

Future scope involves evaluating the cytotoxicity of methanolic and ethanolic CO extracts on human cell lines at greater concentrations in order to more properly extrapolate the findings of this in vitro investigation to clinical situations.

Conclusion

Within the limitations of this in vitro investigation, it may be concluded that CO had a cytotoxic impact at higher doses and was safe to use in clinical settings at smaller doses. CO can be utilized as an effective alternative intracanal medicament due to its low toxicity, bactericidal action, low side effects, low cost, and ease of availability. However, this conclusion is more optimistic and should be verified by long-term evaluation with in vitro models and randomized control trials before being applied to the clinical context.

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