Research Article

# Comparative Evaluation on Neutralizing Effect of Reactive Oxygen Species of Limonene (Orange Peel Extract) Mixed with Calcium Hydroxide and Chlorhexidine - An *In Vitro* Study

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## Abstract

**Background:** *Citrus sinensis* is one of the most important and widely known fruits known for its medicinal value. Many medicinal properties of orange peel extract are known to fight viral and bacterial infections. ROS are oxygen-containing, chemically reactive molecules. ROS can kill bacteria but it also destroys the adjacent infected host tissues. The aim of the study was to determine the neutralizing effect of limonene (orange peel extract) on Reactive Oxygen Species (ROS) generated by the mixture when used as an intracanal medicament. **Methods:** Aqueous and ethanol extracts prepared from peel of *Citrus sinensis* were screened for neutralizing effect of limonene (orange peel extract) on Reactive Oxygen Species (ROS) generated by the mixture when used as an intracanal medicament. The groups were analyzed for ROS formation using the mass spectrometer (JEOL GC MATE II) immediately after preparation. **Results:** The peak value of 3345.6 m/z denotes ROS formation. Group C shows a higher peak value than other groups. **Conclusion:** The peak value of 3390.8 m/z was probably originated by the production of reactive compounds. Thus decreased ROS formation was noted in orange peel aqueous extract warranting further *in vivo* clinical studies to determine the exact dosages and its effectiveness in practical situations.

**Keywords:** *Citrus sinensis*; Sweet orange; Mass spectrometer; Orange peel; Reactive oxygenspecies; Reactive compounds

## 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. [1-3]

Various medicinal properties of orange peel extract helps to treat and prevent vitamin deficiencies, colds, flu, and scurvy and help to fight viral and bacterial infections. [4,5]

ROS are oxygen-containing, chemically reactive molecules. The high reactivity is mainly due to the presence of unpaired valence shell electrons. During normal metabolism of oxygen, ROS is formed as a byproduct and plays an important role in cell signaling and homeostasis. [6-9]

However, during oxidative stress, ROS levels increase intensely and result in significant damage to cell structures.

These species are cytotoxic and have been implicated in the etiology of a wide array of human diseases. Thus ROS can kill bacteria but it also destroys the adjacent infected host tissues. [10]

To counteract the ROS formation, ROS scavengers/ antioxidants are of prime importance for preventing and controlling human diseases. Antioxidants are necessary for the destruction of these free radicals (ROS) by reacting with oxygen and thereby preventing the harmful effects caused by oxygen radicals. Limonene in *Citrus sinensis* are potent



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bioactive antioxidants naturally occurring in orange peel extracts, respectively and act as ROS scavengers. They are antibacterial, antiallergic, and inhibit platelet aggregation and capillary permeability; these effects contribute to the potent antioxidant ability. Limonene occurs commonly as the D-or (R)-enantiomer, but racemizes to dipentene at 300°C. When warmed with mineral acid, limonene isomerizes to the conjugated diene α-terpinene (which can also easily be converted to p-cymene). [11] Previously our team has a rich experience in working on various research projects across multiple disciplines. [12-20] Now the growing trend in this area motivated us to pursue this project.

The aim of the study was to determine the neutralizing effect of limonene (orange peel extract) on Reactive Oxygen Species (ROS) generated by the mixture when used as an intracanal medicament.

## Materials and Methods

### Materials

- 2% CHX
- Calcium hydroxide
- Limonene (orange peel extract)

### Groups

- Group A: 2% CHX gluconate (control group)
- Group B: A mixture of 125 mg of $Ca(OH)_2$ with 2% CHX gluconate solution.
- Group C: was a mixture of 125 mg of $Ca(OH)_2$ with 1 mL of 2% CHX gluconate solution and dichloromethane (orange peel extract).

### 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 ethanol (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 24 hrs. Also the same amount (*i.e.*, 10 g) of pulverized peel was immersed in 100 ml of hot sterile distilled water (100°C) and allowed to stand for 30 min on a water bath with occasional shaking and kept undisturbed for 24 hrs. 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 rotaevaporator. The dried extract thus obtained was exposed to UV rays for 24 h and checked for sterility on nutrient agar plates and stored in labeled sterile bottles in a freezer at 4°C until further use [Figure 1].

**Figure 1:** *Citrus sinensis* extract.

### Mass spectrometer

Full scan mass spectra were measured between m/z 150 and 2000 μ in positive ion mode and negative ion mode for other compounds. High purity nitrogen was used as nebulizer gas at 27.5 psi, 350°C and at a flow rate of 8 L/min. Fragmentation amplitude of 1.00 V (MS/MS). The helium is used as the collision gas. In the mass spectrometer, the collision energy used was between 0 eV and 10 eV. Peaks were collected using a peak width at 5% height, 1 second, a noise elimination of 6, and an intensity threshold of 70. Data were aligned with a mass tolerance of 0.04 Da and a retention time window of 0.2 minute. All spectra were aligned and normalized to an external standard [Figure 2].

**Figure 2:** Mass spectrometer.

### Statistical analysis

The Mass Lynx software version 4.1 was used to calculate accurate masses. All spectra were aligned and normalized to an external standard. Assignment of metabolites contributing to the observed variance was performed using the Chem Spider Database.

## Results

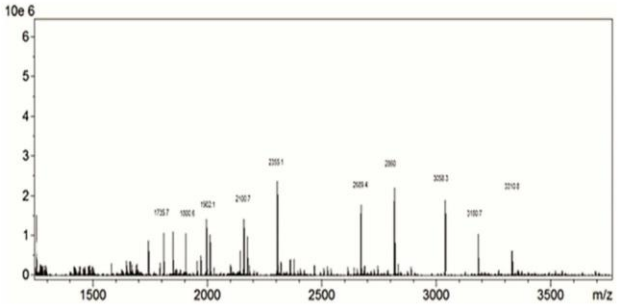
The peak value of 3390.8 m/z was probably originated by the production of reactive compounds. The decreased ROS

formation was noted in orange peel aqueous extract [Table 1, Graph 1].

**Table 1: Comparison of ROS formation between the groups.**

| Comparison | p value |
|---|---|
| Group A *vs.* Group B | (p>0.05)* |
| Group A *vs.* Group C | (p<0.05)** |
| Group B *vs.* Group C | (p<0.05)** |

*: Not significant; **: Statistically significant.




**Graph 1:** X-axis: mass-to-charge (m/z); Y-axis: Intensity. According to the database peak value of 3345.6 m/z denotes ROS formation.

## Discussion

ROS such as superoxide radical, hydrogen peroxide, singlet oxygen, and hydroxyl radical are small, short- lived and highly reactive molecules formed by incomplete one-electron reduction of oxygen. They are cytotoxic and have been implicated in various diseases like diabetes and neurodegenerative diseases, and influence cellular processes such as proliferation, apoptosis, and senescence, responsible for cancer development. [21,22] Generally, harmful effects of ROS on the cell most often include damage of DNA, oxidation of polyunsaturated fatty acids in lipids, oxidation of amino acids in proteins, and oxidative inactivation of specific enzymes by oxidation of cofactors. [23-26] However, ROS in lesser quantities are shown to be bactericidal and can enhance cell proliferative activity and information signaling. ROS inactivate bacteria and their proteins and contribute to the microbicidal activity of phagocytes, regulation of signal transduction, and gene expression, and cause oxidative damage to nucleic acids, proteins, and lipids. [26-29] 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. [29-31] 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. [32-35]

Waris and Ahsan have reported that elevated levels of ROS and down regulation of ROS scavengers and antioxidant enzymes are associated with various human diseases including different types of cancer. In normal conditions, a dynamic equilibrium exists between ROS activity and defense capacity of antioxidants. [36-39] The shift in equilibrium in favor of ROS activity results in oxidative stress. This might happen either due to an increase in ROS production or a decrease in defense capacity of antioxidants. Antioxidants are substances which considerably delay or inhibit oxidation of the oxidizable substrate at lower concentrations. [40-42] Our institution is passionate about high quality evidence based research and has excelled in various fields. [43-50]

According to the results of this study, the peak value of 3345.6 m/z denotes ROS formation. The peak of 3345.6 m/z was probably originated by the production of reactive compounds (ROS), as a result of the high concentration of hydroxyl ions (alkaline environment) in the presence of CHX. The maximum production of ROS was observed in group B which is reflected in its higher peak value. When compared with group A (CHX), group B ($Ca(OH)_2$+CHX) shows a higher ROS formation. This is probably due to the formation of hydroxyl ion which is the dissociation product of $Ca(OH)_2$ at an alkaline pH. Group C (limonene+CHX+$Ca(OH)_2$) showed significant decrease in ROS when compared with group B and group A. The reduction in ROS is attributed to the inherent antioxidant properties found in group C.

Hence, strength of the study noted were 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 confirm *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

The combination of $Ca(OH)_2$ and CHX generates excessive amounts of ROS which is detrimental to the host tissues. Limonene (*Citrus snensis* extract) reduces the ROS significantly, shows more antioxidant property when compared with other groups. Addition of natural antioxidants to the $Ca(OH)_2$-CHX mixture increases the antibacterial efficacy and

also decreases damage to the host tissue by lowering ROS formation.

### Clinical Significance

The peels of fruits of *Citrus sinensis* which are generally treated as wastes can serve as an effective and economical antimicrobial agent can be used as potent root canal medicament as it shows good antioxidant property.

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