Anti-Inflammatory Activity and Cytotoxic Effect of Orange and Grape Peel Herbal Formulation Mediated Silver Nanoparticles Based Mouth Rinse

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**Abstract:** Dental caries and periodontal disease are major oral health concerns associated with plaque biofilm. Current chemical-based antiplaque agents have drawbacks, necessitating safe alternatives. Inflammation from bacterial accumulation and oxidative stress contribute to periodontal diseases. Herbal mouthwashes show promise, but cytotoxicity concerns exist. Orange and grape peel extracts, rich in antioxidants, coupled with silver nanoparticles, are explored for potential anti-inflammatory and cytotoxic effects in a mouthrinse formulation.Orange and grape peel extracts were prepared by drying, grinding, and heating. Silver nitrate solution was synthesized with an 80:10:10 ratio. Anti-inflammatory activities were assessed using Bovine Serum Albumin and Egg Albumin Denaturation Assays, and a Membrane Stabilization Assay. Cytotoxicity was evaluated through the Brine Shrimp Lethality Assay.The antiinflammatory assays suggest that with the increase in concentration of the mouthrinse solution, it's activity also increases, that is the maximum percentage of inhibition is seen at 50 μg/mL concentration.For cytotoxicity Viable nauplii were treated with the Nanoparticle based mouthrinse solution of various concentrations and no significant mortality was found after 24 hrs of exposure,and extended exposure to 48 hours did induce high mortality where with increase in concentration of the mouthrinse, high mortality rate was found that is at 80 μg/mL concentration mortality rate is 40%.This herbal mouthrinse incorporating silver nanoparticles mediated from orange and grape peel extracts demonstrates notable anti-inflammatory activity and cytotoxic effects. This mouth rinse holds promise as an effective oral care solution, added with the anti-inflammatory and cytotoxic properties of these fruit peels and silver nanoparticles, offering a potential alternative for maintaining oral health and hygiene.

**Keywords**: Anti inflammatory, Cytotoxicity, Grape peel , Orange peel, Silver Nanoparticles

# Introduction

Dental caries and periodontal disease are the primary infectious conditions that significantly impact the oral cavity. A key element in these oral issues is the microbial community referred to as plaque biofilm, distinguished by an exopolysaccharide matrix that is closely associated with various oral diseases [(Zhang et al., 2016)](https://paperpile.com/c/qgp0ER/LPSp). This intricate structure not only serves as a nutrient source but also acts as a protective shield against detrimental environmental elements, such as antibiotics and disinfectants [(Chung et al., 2016; Laghari et al., 2023)](https://paperpile.com/c/qgp0ER/29q0+7xqD). Addressing plaque biofilm necessitates active biological treatments capable of penetrating the microbial biofilm. Despite considerable research efforts, the development of a potent antiplaque agent has faced challenges, including issues of toxicity and inefficacy [(*Anti-Inflammatory Potential of a Mouthwash Formulated Using Clove and Ginger Mediated by Zinc Oxide Nanoparticles: An In Vitro Study*, n.d.; Singh et al., 2020)](https://paperpile.com/c/qgp0ER/AErO+22ma). Currently available chemical-based antiplaque medications, containing substances like chlorhexidine, triclosan, and gluconate, exhibit various negative effects, such as allergic stomatitis and taste alterations associated with prolonged usage. This underscores the imperative need to explore safe, effective, and biocompatible alternatives [(Kanchi & Ahmed, 2018; Muthuswamy Pandian et al., 2022)](https://paperpile.com/c/qgp0ER/cTjQ+ZUeK) [(Sinduja et al., 2021)](https://paperpile.com/c/qgp0ER/itWi).

Scientific research indicates that bacterial accumulation on teeth can lead to inflammation of the gingiva. The activation of hyperresponsive polymorphonuclear leukocytes (PMNs) was a consequence of the innate immune system responding to the lipopolysaccharide and DNA of periodontal pathogens, resulting in increased production of reactive oxygen species (ROS) [(Bassani et al., 2023; Chokkattu et al., 2023)](https://paperpile.com/c/qgp0ER/bAsl+VTox). The deterioration of periodontal tissue leads to an overproduction of inflammatory mediators, lipid peroxides and oxidized proteins. These products can further stimulate macrophages, fibroblasts, and neutrophils, leading to more ROS production. Addressing oxidative stress, regulating inflammatory responses, modifying immunological reactions, and promoting tissue repair are crucial aspects of managing periodontal diseases [(Sczepanik et al., 2020; Solanki et al., 2023; Subramanian & Harikrishnan, 2023)](https://paperpile.com/c/qgp0ER/54ZH+JdgT+G3cX). The use of herbal mouthwash aids in the removal of bacteria, debris and irritants, contributing to reduced inflammation in the oral cavity. However, it is essential to be cautious about the cytotoxic effects of mouth rinses, which can vary based on their formulation and the concentration of active ingredients [(Cai et al., 2020)](https://paperpile.com/c/qgp0ER/GFaB).

Orange peels emerge as a rich source of phenolic acids and flavonoids, surpassing even the edible part in quantity. Research suggests that orange peel extracts, particularly due to glycosides like hesperidin and naringin, exhibit significant antioxidant activity. Key phenols present in orange peels, such as phlorin and coniferin demonstrate radical-scavenging capabilities, especially when consumed as orange peel molasses [(Adel et al., 2023; Shehata et al., 2021; Sreevarun et al., 2023)](https://paperpile.com/c/qgp0ER/UO0c+mKg4+ANUN). Grapes are known for their antineoplastic and antimutagenic attributes, additionally diminish allergic inflammation and LDL oxidation in humans. The functional components, such as phenolics and anthocyanins found in grape peels and seeds, possess antioxidant and radical-scavenging properties. Due to the potential mutagenicity of synthetic antioxidants, there's an increasing inclination towards natural antioxidants. The combination of extracts from orange and grape peels ensures the retention of phytochemicals inherent in both extracts [(Sabra et al., 2021; Wadhwani et al., 2022)](https://paperpile.com/c/qgp0ER/hQzY+ovXa).

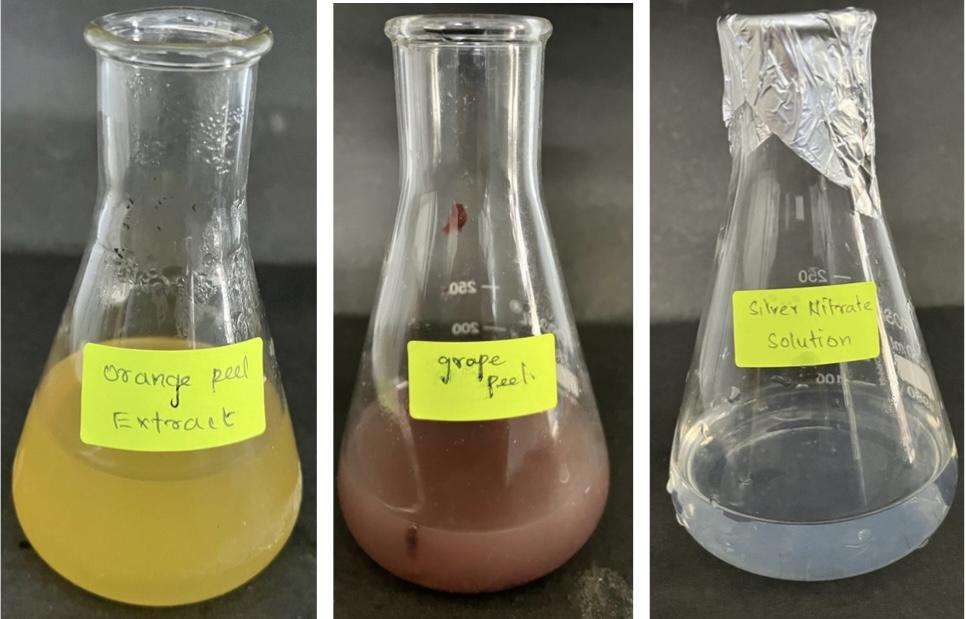
Nanoparticles represent an effective drug delivery system for delivering phytoconstituents from these peels to targeted cells. Biogenic synthesis of nanoparticles, utilizing reducing agents from plants, algae , fungi and microbes is considered more environmentally friendly. Silver nanoparticles (AgNPs) are notably significant in biomedical applications due to their therapeutic properties, encompassing antibacterial ,antiviral, antifungal, anti-inflammatory, antiangiogenic and anticancer effects. The stability and focused administration of nanodrugs contribute to their widespread use as therapeutic agents [(Imtiaz et al., 2021; Jain & Verma, 2022)](https://paperpile.com/c/qgp0ER/L4Nn+zMIl) [(Rajeshkumar & Bharath, 2017; Sinduja et al., 2021)](https://paperpile.com/c/qgp0ER/itWi+8a90). The incorporation of orange peel and grape peel extracts into a mouthwash formulation holds the potential to enhance its functional qualities. The combination of these extracts creates a synergistic effect, amplifying the anti-inflammatory action of the product [(Tharani et al., 2023)](https://paperpile.com/c/qgp0ER/FOpu). This research aims to investigate the anti-inflammatory and cytotoxic effects of a silver nanoparticle-based mouthrinse mediated by orange peel and grape peel extracts[(Mary et al., 2023; Marya et al., 2022)](https://paperpile.com/c/qgp0ER/Mwue+CCcw).

# Materials and methods

## Study setting

Orange and grape peels were collected, autoclaved for 24 hours, and subsequently utilized for the study.

## Preparation of Extracts



**Figure 1 :** Prepared Aqueous Orange and Grape Peel extract and Silver nitrate solution

On the subsequent day, 2 grams each of orange and grape peels were dried and ground using a mortar and pestle. Distilled water (100 ml) was added to the ground mixture, and the resulting concoction was filtered through a sterile cotton cloth. The filtered liquid was then subjected to a heating mantle, maintaining a temperature of 50 to 60 degrees, allowing it to condense until the extract reached a volume of 10 ml (Figure 2).

## Silver Nitrate Solution Preparation

To prepare the silver nitrate solution, one millimole of silver nitrate was measured using a micropipette and mixed with 10 ml of orange and grape peel extract in an 80:10:10 ratio. The entire solution was placed in a heating mantle and condensed down to a final volume of 10 ml.



Figure 2: Initial stage and Final stage of synthesis

## Anti-inflammatory activity

## Bovine serum albumin denaturation assay

Two assays, such as the Bovine Serum Albumin Denaturation Assay and the Egg Albumin Denaturation Assay, were used to examine the green silver nanoparticles that were synthesised for their anti-inflammatory properties. A. paniculata-mediated silver nanoparticles were combined with 0.45 mL of bovine serum albumin and 0.05 mL of various doses (10–50 g/mL). A pH adjustment of 6.3 was made. It was then held at room temperature for 10 minutes, followed by 30 minutes of incubation in a water bath at 55°C.The standard group utilised was diclofenac sodium. The samples were then spectrophotometrically analysed at 660 nm.

Percentage of protein denaturation was determined utilizing following equation,

% inhibition= Absorbance of control- Absorbance of sample×100

Absorbance of control

## Egg Albumin denaturation assay

2.8 mL of phosphate buffer and 0.2 mL of fresh egg albumin were used to perform the egg albumin denaturation experiment. The reaction mixture was supplemented with various amounts (10–50 g/mL) of A. paniculata mediated silver nanoparticles. A pH adjustment of 6.3 was made. It was then held at room temperature for 10 minutes, followed by 30 minutes of incubation in a water bath at 55°C. The standard group used was diclofenac sodium.The samples were then spectrophotometrically analysed at 660 nm.

Percentage of protein denaturation was determined utilizing following equation,

% inhibition= Absorbance of control- Absorbance of sample×100

Absorbance of control

## Membrane stabilization assay

A common method for assessing a compound's ability to stabilise membranes in vitro is the in vitro membrane stabilisation assay. By avoiding the cell membrane's disruption and the subsequent release of intracellular contents, this assay evaluates a substance's capacity to maintain the membrane's integrity. Human red blood cells (RBCs), phosphate-buffered saline (PBS), Tris-HCl buffer (50 mM, pH 7.4), various silver nanoparticle concentrations (10–50 g/mL), centrifuge tube, and UV–Vis spectrophotometer are among the components.

## Preparation of RBC suspension

Take fresh human blood samples in sterile tubes with anticoagulant.Separate the RBCs from other blood components by centrifuging the blood at a 1000 g rate for 10 minutes at room temperature. Take away the supernatant, then PBS wash the RBCs three times. To obtain a 10% (v/v) RBC suspension, resuspend the RBCs in Tris-HCl buffer.

## Assay procedure

Fill each centrifuge tube with 1mL of the RBC suspension using a pipette. Then, various silver nanoparticle concentrations were introduced to each tube.Gently stir, then let the tubes sit at 37 °C for 30 minutes. To pellet the RBCs, centrifuge the tubes at 1000 g for 10 minutes at room temperature. Using a UV-Vis spectrophotometer, determine the supernatant's absorbance at 540 nm.

Calculated the percentage inhibition of hemolysis using the following formula:

% inhibition = [(OD control – OD sample) / OD control. ] x 100

where the absorbance of the RBC suspension without the test chemical or compounds is called the OD control, and the absorbance of the RBC suspension with the test compound is called the OD sample.

## Cytotoxic activity

## Brine shrimp lethality Assay

200ml of distilled water was used to dissolve 2g of iodine-free salt. 10–12 ml of saline water were added to 6 well ELISA plates. Each well received 10 nauplii, which were added gradually (20 l, 40 l, 60 l, 80 l, and 100 l). The nanoparticles were then introduced in the appropriate concentrations. For 24 hours, the plates were incubated(Rafi et al., 2024).

The ELISA plates were examined after 24 hours to count the live nauplii that were present and to determine their number using the procedure below.

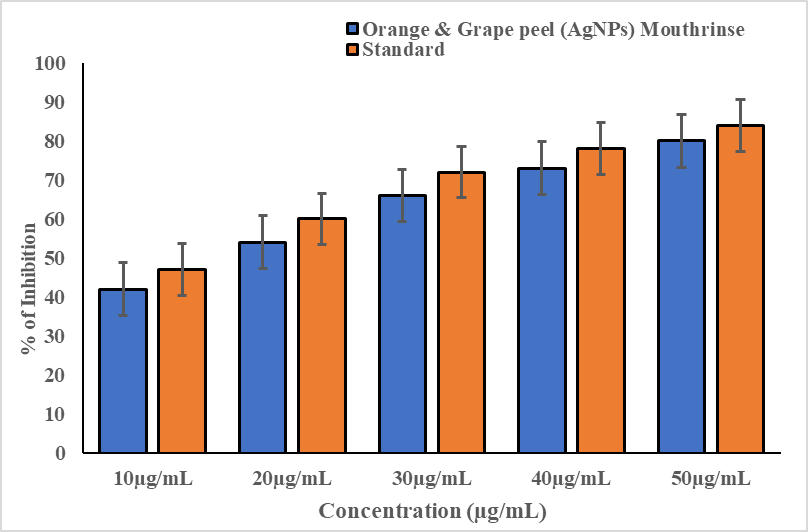
(%) mortality = number of dead nauplii**/**number of dead nauplii+number of live nauplii×100

(% of live nauplii)

# Results

## Anti-inflammatory attributes of Silver Nanoparticles

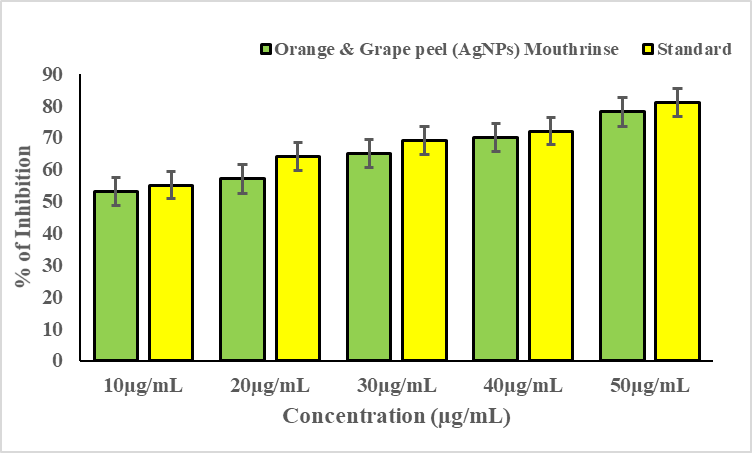
## Bovine Serum Albumin Assay



**Figure 3:** Bovine Serum Albumin Assay of Orange and grape peel extract mediated Silver Nanoparticles based mouthrinse in a solution at various concentrations

This concentration-dependent inhibition of protein-denaturation activity was observed for this Nanoparticle based mouthrinse. For protein denaturation assay, diclofenac sodium was used as a positive control (Tuluwengjiang et al., 2024). 10 to 50 μg/mL concentrations to represent the percentage inhibition of protein denaturation activity ranging between 42% to 80%, respectively. As shown in Graph 1, percent inhibitions attained by varying extract concentrations exhibited less activity compared to the standard agent and the maximum percent inhibition was observed by the highest extract concentration (50 μg/mL) as 80%.

## Egg Albumin Denaturation Assay

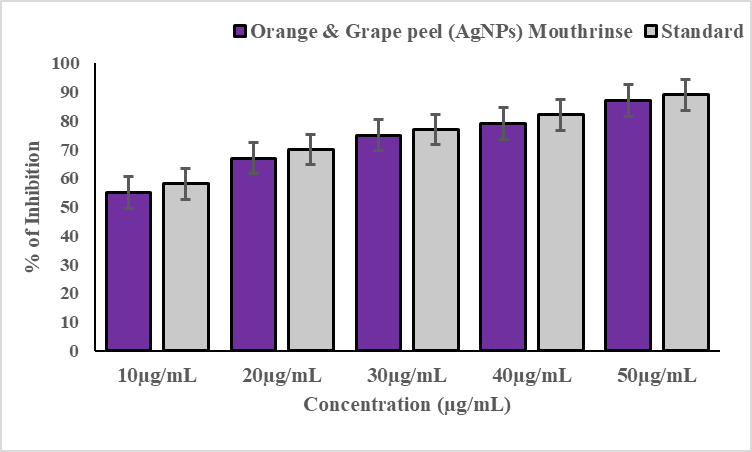


**Figure 4**: Egg Albumin Denaturation Assay of Orange and grape peel extract mediated Silver Nanoparticles based mouthrinse at various concentrations

The results provided seem to be related to an Egg Albumin Assay, specifically measuring the concentration-dependent inhibition percentage of an Orange & Grape peel (AgNPs) Mouthrinse added with dimethyl sulfoxide which induce free radicals to study its anti-inflammatory properties. The experiment was conducted with varying concentrations of Orange & Grape peel (AgNPs) Mouthrinse, ranging from 10µg/mL to 50µg/mL. The percentage of inhibition represents the ability of the mouthrinse to inhibit or reduce the activity of egg albumin. Egg albumin is often used in assays to simulate protein conditions. At a concentration of 10µg/mL, the inhibition percentage was 53%. At a concentration of 20µg/mL, the inhibition percentage increased to 57%. At 30µg/mL, the inhibition percentage further increased to 65%. At 40µg/mL, the inhibition percentage is 70%, and at 50µg/mL, it is 78%. A standard included for comparison is diclofenac sodium. The standard inhibition percentages are given at each concentration level.

At 10µg/mL, the standard inhibition is 55%, at 20µg/mL it's 64%, at 30µg/mL it's 69%, at 40µg/mL it's 72%, and at 50µg/mL it's 81%. At each concentration level, it seems that the mouthrinse performs slightly better than the standard, showing higher inhibition percentages. The concentration-dependent increase in inhibition suggests that as the concentration of the mouthrinse increases, its inhibitory effect on egg albumin also increases. The fact that the inhibition percentages of the mouthrinse is comparable to the standard containing diclofenac sodium indicates that the mouthrinse has a stronger inhibitory effect on egg albumin in this experimental setup.

## Membrane Stabilization Assay

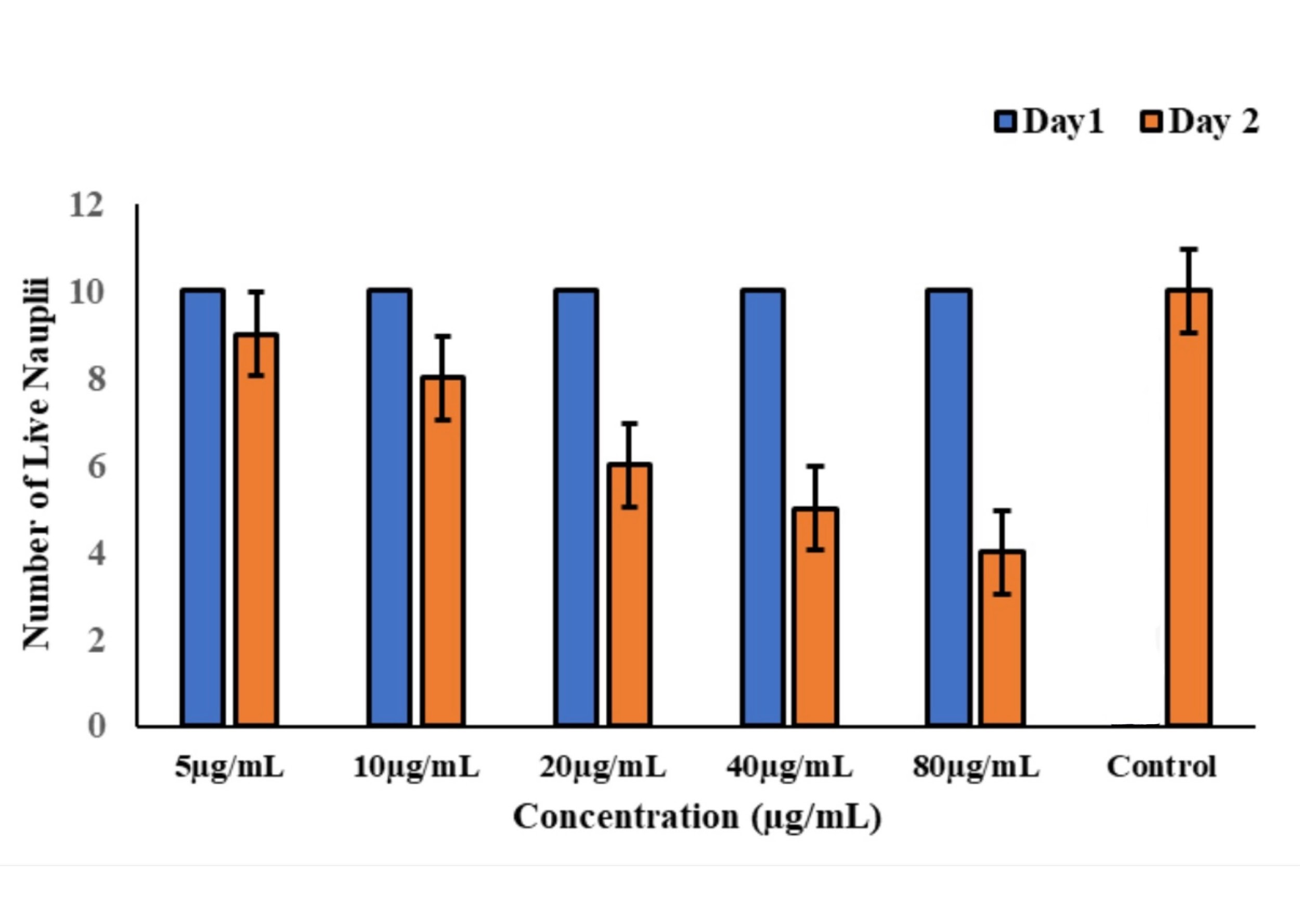


**Figure 5:** Membrane Stabilization Assay of Orange peel and grape peel extract mediated Silver Nanoparticles based mouthrinse at various concentrations.

The results shows that the percentage inhibition of orange and grape peel mediated silver nanoparticles based mouth rinse at different concentrations from 10µg/mL , 20µg/mL,30µg/mL, 40µg/mL and 50µg/mL are 55% , 67%, 75%,79% and 87% respectively as compared to Diclofenac sodium having percentage inhibition of 58%, 70%, 77%, 82% and 89% for the same concentrations respectively as shown in graph 3 .

## Cytotoxic effect

## Brine shrimp lethality Assay



**Figure 6:** Brine Shrimp Lethality Assay to study the cytotoxicity of Orange peel and grape peel extract mediated Silver Nanoparticles based mouthrinse at different concentrations of the mouthrinse .

The prepared mouthrinse solutions at varying concentrations (5, 10, 20, 40 & 80 µg/ ml) were added to the premarked vials of ELISA well plate containing 10 live brine shrimp nauplii in 5 ml stimulated seawater. The vials were inspected using a magnifying glass and the number of live viable nauplii in each vial was counted after 24 and 48 hours respectively. Diclofenac sodium is the positive control.The findings indicate that the viability of all nauplii was maintained after 24 hours at the specified concentration. However, a decline in the count is observed after 48 hours, with values of 9, 8, 6, 5 and 4 numbers corresponding to the increase in concentration respectively.

# Discussion

The herbal formulation-mediated silver nanoparticles may possess anti-inflammatory properties due to the presence of bioactive compounds in the herbs. These compounds could potentially inhibit the production of pro-inflammatory molecules and modulate the immune response, leading to a reduction in inflammation. However, it is important to note that the specific herbs and their concentrations in the formulation would determine the efficacy of the anti-inflammatory activity [(Chokkattu et al., 2022; Ramamurthy et al., 2022; Rocha et al., 2024)](https://paperpile.com/c/qgp0ER/FfCl+cNWF+tkmz).The increased percentage of inhibition in Egg albumin assay and bovine serum albumin assay with the increased concentration of the mouthwash signifies that with the increased levels of mouthwash, percentage of protein denaturation is also decreased but slightly lower than the control where Diclofenac sodium has been used.Also for Membrane stabilisation assay, which shows the substance’s ability to maintain the membrane integrity. With the increased concentration of this herbal mouthrinse, better membrane integrity was achieved. For cytotoxicity effect, Brine shrimp lethality assay was done where 10 viable nauplii for each of 5 groups with increased concentration of the mouthwash was added and the nauplii in the control group is added with diclofenac sodium after which the nauplii count was seen to be reduced with the increased concentration of this herbal mouth rinse which confirms its cytotoxicity. Previous research was done to study the cytotoxicity of silver nanoparticles synthesized from Leea macrophylla [(Merchant et al., 2022; Pandiyan et al., 2022; Sharmin et al., 2023)](https://paperpile.com/c/qgp0ER/toAN+xJVE+Sg2z),where the lowest dose of 20 μg/mL showed mortality of about 58.3 %. As concentration further spanned between 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL the mortality increased to 76.6 %, 86.6 %, 90 % and 93.3 % respectively after 24hrs, which concurs with this research results where the cytotoxicity is found to be increasing with the increase in mouth rinse concentrations.A Meta analysis study was done in which oral herbal products reduced gingivitis which suggests reducing plaque and calculus [(Chauhan et al., 2020)](https://paperpile.com/c/qgp0ER/kZLy). Herbs such as Neem, Pomegranate, Tulsi, Cranberries and Guava are used to reduce plaque and gingivitis whose organic compounds are used to reduce gingivitis and have no side effects compared to inorganic compounds [(Shukla & Iravani, 2018)](https://paperpile.com/c/qgp0ER/nsC3). A study was conducted to study the Phytochemical composition of the grape peel, where higher flavonoid content was found than other plant extracts. This gave an insight on the therapeutic properties of grape peel extract [(Ganapathy 2021; Vijayashree Priyadharsini, 2019)](https://paperpile.com/c/qgp0ER/BFtZ+aX1y).

Silver nanoparticles have shown promise in antimicrobial applications, their cytotoxic effects on human cells have also been a subject of concern [(Chiu et al., 2021; Poornima et al., 2021; Verma & Muthuswamy Pandian, 2021)](https://paperpile.com/c/qgp0ER/MIMA+oaeS+537C). High concentrations or prolonged exposure to silver nanoparticles may cause cellular damage and toxicity. Therefore, it is crucial to determine the optimal concentration of silver nanoparticles in the mouth rinse formulation that balances antimicrobial efficacy and toxicity, proper cell viability tests have to be done. This can kill bacterial endospores and it has effective pathogens and control microorganism. A previous by (IndumathyPandian.et.al,) on anti inflammatory and antioxidant activity of silver nanoparticle mediated from Ocimum tenuiflorum- and Stevia rebaudiana showed a percentage of inhibition at 10,20,30,40 and 50(µL) as 84.50%, 84.40%, 83.50%, 82.10% and its peak at the 50 µL concentration (92.6%) which is comparable to this study results where the peak is at 50µL as 80%.The specific herbal formulation used in the mouth rinse plays a significant role in determining its effectiveness and safety. Different herbal extracts may possess varying levels of anti-inflammatory activity and could interact differently with silver nanoparticles. It is important to identify and understand the active constituents of the herbal formulation and their potential synergistic effects with silver nanoparticles.[(Aparna et al., 2021)](https://paperpile.com/c/qgp0ER/a2ZM) Preliminary findings reveal promising anti-inflammatory effects, suggesting the potential efficacy of the herbal formulation. However, careful consideration is given to cytotoxicity data to guarantee the safety profile of the product for oral use. The outcomes of this study contribute valuable insights into the development of natural, nanoparticle-based oral care products, offering a safer alternative to traditional mouthwashes.The limitations of our present study was done in the in vitro condition in small sample size further research must or can be done in large sample size to provide better results. Much more assays need to be checked for antiinflammatory and cytotoxicity .

# Conclusion

With the presence of therapeutic phytocompounds in orange and grape peel, addition of their extracts enhances the functional properties of the mouth rinse. The addition of functional properties of both the extracts results synergistic effect by enhancing the properties of the product, which was seen in this research where anti-inflammatory and cytotoxic activities of this herbal rinse was studied, thus developing innovative herbal medicine with the potential to produce physiological benefits which is effective against various oral, periodontal diseases. Our present study was done in invitro condition were anti-inflammatory and cytotoxic activities of Orange and grape peel extract mediated silver nanoparticle is studied. Further research targeting animal models in vivo conditions that would substantially confirms these therapeutic properties.

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