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www.commprac.com
ISSN 1462 2815

ANTI-INFLAMMATORY AND CYTOTOXIC EFFECT OF MOUTHWASH PREPARED USING *ZIZIPHUS OENOPILA* MEDIATED SILVER NANOPARTICLES

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DOI: 10.5281/zenodo.12743210

Abstract

Oral inflammatory diseases are prevalent and pose significant health challenges, with current treatments often facing limitations. *Ziziphus oenoplia* (Jackal jujube), a plant known for its medicinal properties, combined with silver nanoparticles (AgNPs), which possess antimicrobial and anti-inflammatory effects, offers a novel therapeutic approach. This study aims to evaluate the anti-inflammatory and cytotoxic effects of a formulated *Ziziphus oenoplia* -silver nanoparticle (ZO-AgNPs) mouthwash to assess its efficacy in combating oral inflammatory diseases. The Z. oenoplia extract and AgNPs were prepared, and the anti-inflammatory activity was assessed using Bovine Serum Albumin (BSA) and Egg Albumin denaturation assays, alongside a membrane stabilization assay. Cytotoxic effects were evaluated through the brine shrimp lethality assay. The results demonstrated that the anti-inflammatory activity of the ZO-AgNPs mouthwash was comparable to a standard mouthwash, with increased membrane stabilization activity observed at higher concentrations. Additionally, the ZO-AgNPs mouthwash exhibited a dose-dependent cytotoxic effect on nauplii. These findings suggest that the ZO-AgNPs mouthwash holds promise for oral health applications, though further research is necessary before clinical implementation.

Keywords: *Ziziphus Oenoplia* , Silver Nanoparticles, Anti-Inflammatory, Cytotoxicity, Oral Inflammatory Diseases, Mouthwash, BSA Assay, Membrane Stabilization, Brine Shrimp Lethality.

1. INTRODUCTION

Oral inflammatory diseases are prevalent and serious health issues affecting millions of people globally. These conditions, such as gingivitis and periodontitis, are characterized by inflammation of the gums and surrounding structures, leading to the loss of both soft and hard tissues that support the teeth(Ambika, Manojkumar et al. 2019, Sczepanik, Grossi et al. 2020). This inflammation not only causes discomfort and pain but can also result in significant dental issues, including tooth loss(Scannapieco and Cantos 2016, Tayyeb, Priya et al. 2024). Traditional treatment methods primarily focus on mechanical removal of plaque and chemical interventions delivered via mouthwashes or dentifrices. However, these conventional approaches often have limitations and can sometimes result in adverse effects, necessitating the exploration of new, innovative treatment strategies(Mays, Sarmadi et al. 2012, Chockalingam, Sasanka et al. 2020). In recent years, there has been growing interest in harnessing the potential of herbal products and nanotechnology for managing oral diseases. One such promising herbal resource is *Ziziphus oenoplia* , commonly known as Jackal jujube. This plant, a member of the Rhamnaceae family, is a flowering shrub or small tree that thrives in tropical and subtropical regions of Asia and Australasia, including the southern deciduous forests of India. Known also as small-fruited jujube and wild jujube, *Ziziphus oenoplia* has a long history of use in traditional medicine for its various healing properties. *Ziziphus oenoplia* has been used traditionally to treat a range of ailments. In the Raigad district of Maharashtra, India, both the leaves and

berries are consumed as food, while a decoction of the root bark is applied to fresh wounds to promote healing. The fruits are also used to alleviate stomach aches(Grover, Kapoor et al. 2016, Sundaram and Saravanan 2022). Modern research has identified that *Ziziphus oenoplia* is rich in bioactive compounds, including alkaloids, flavonoids, phenolics, and terpenoids. These compounds contribute significantly to the plant's medicinal properties, with studies highlighting its anti-inflammatory, anticholinergic, and antibacterial activities. Of particular interest are the cyclopeptide alkaloids, especially ziziphine, which are believed to play a crucial role in its therapeutic effects. However, to fully validate these medicinal claims, further pharmacological research is necessary to isolate and understand the effective compounds(Kishore, Priya et al. 2020, Ravikumar, Marunganathan et al. 2024). Parallel to the exploration of herbal remedies, nanotechnology has emerged as a transformative field, particularly with the development of silver nanoparticles (AgNPs). AgNPs are distinguished by their unique physico-chemical properties and extensive potential across various applications. Typically ranging in size from 1 to 100 nanometers, these nanoparticles possess a significantly larger surface area relative to bulk silver. This increased surface area enhances their physical, chemical, and biological functionalities, making AgNPs highly attractive for a range of uses. One of the most notable properties of AgNPs is their potent antimicrobial activity. They are highly effective against a wide spectrum of bacteria, fungi, and viruses. This antimicrobial property is attributed to the ability of AgNPs to interact with microbial cell walls and membranes, disrupting cellular processes and ultimately leading to cell death(Rieshy, PRIYA et al. 2020). Although the precise mechanisms underlying their antimicrobial effects are still being investigated, it is widely believed that the release of silver ions and the generation of reactive oxygen species are crucial factors. Due to these properties, AgNPs are being explored for various healthcare applications, including wound dressings, catheters, and drug delivery systems.In addition to their antimicrobial properties, AgNPs also exhibit anti-inflammatory effects. Inflammation is a natural defense mechanism of the body, essential for healing injuries and combating infections. However, chronic inflammation can lead to a range of diseases, including arthritis and heart disease. Anti-inflammatory agents work by combating this defense mechanism, reducing inflammation and pain. These agents include medications such as nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, as well as lifestyle modifications such as diets rich in anti-inflammatory foods, regular exercise, and adequate sleep(Ponmanickam, Gowsalya et al. 2022). This study aims to combine the therapeutic properties of *Ziziphus oenoplia* with the unique benefits of silver nanoparticles to develop a novel mouthwash formulation. The objective is to evaluate the anti-inflammatory and cytotoxic effects of this *Ziziphus oenoplia* -mediated silver nanoparticle (ZO-AgNPs) mouthwash, assessing its potential efficacy in combating oral inflammatory diseases. The research involves preparing the Z. oenoplia extract and AgNPs, and then testing the anti-inflammatory activity using Bovine Serum Albumin (BSA) and Egg Albumin denaturation assays, alongside a membrane stabilization assay. The cytotoxic effects are evaluated through the brine shrimp lethality assay(Gupta, Verma et al. 2019, Sneka and Santhakumar 2021). The anticipated outcome is that the ZO-AgNPs mouthwash will exhibit significant anti-inflammatory and antimicrobial properties, offering a promising new approach for the management of oral inflammatory diseases. However, extensive research and clinical trials will be necessary to confirm these findings and ensure the safety and efficacy of this innovative treatment.

2. MATERIALS AND METHOD

Wash the *Ziziphus oenoplia* leaves thoroughly with clean water to remove any dirt, pat them dry with a clean cloth. Grind the leaves into a coarse powder using a grinder. In a container, add the powdered leaves to distilled water. Filter the extract using filter paper or cheesecloth to remove the plant material. Collect the filtrate, which is the aqueous extract of *Ziziphus oenoplia* (figure 1).

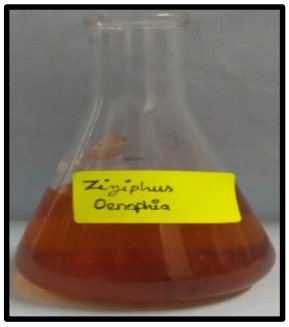


Figure 1 **Figure 2** **Figure 3** **Figure 4**

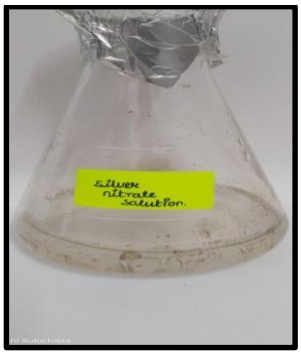
Figure 1: leaf powder of *z.oenopila*

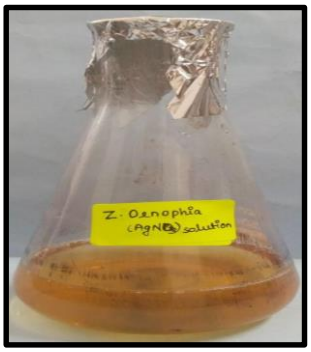
Figure 2: dissolved in 100ml of distilled water

Figure 3: filtration of extract

Figure 4: Extract of *z.oenopila*

Prepare a solution of silver nitrate (AgNO3) in distilled water. Slowly add the silver nitrate solution to the prepared *Ziziphus oenoplia* extract under constant stirring. Monitor the reaction by observing the color change of the solution. The formation of silver nanoparticles often results in a color change, typically yellow or brown (figure 2).





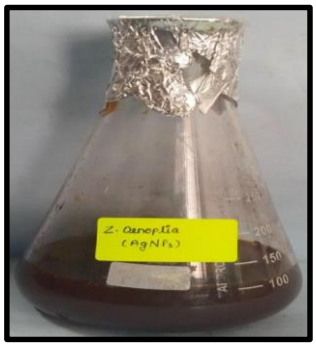


Figure 5 **Figure 6** **Figure 7**

Figure 5: silver nitrate solution

Figure 6: *z.oenopila* extract mixed silver nitrate solution

Figure 7: *z.oenopila* mediated silver nitrate solution

2.1 Anti-inflammatory activity

Bovine serum albumin denaturation assay

The green synthesized silver nanoparticles were tested for their anti-inflammatory activity using two assays: Bovine serum albumin denaturation assay. 0.45 mL of bovine serum albumin was mixed with 0.05 mL of different concentrations (10, 20,

30,40, 50 µg/mL) of *Z.oenopila* mediated silver nanoparticles. The pH was adjusted to 6.3. Then it was kept at room temperature for 10 minutes followed by incubation in a water bath at 55°C in a water bath for 30 min. Diclofenac sodium was used as the standard group while dimethyl sulphoxide was used as control. Then, the samples were measured spectrophotometrically at 660nm(Jin, Lamster et al. 2016).

The percentage of protein denaturation was determined utilizing the following equation,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

2.2 Egg Albumin denaturation assay

To perform the egg albumin denaturation assay, 0.2 mL of fresh egg albumin was mixed with 2.8 mL of 1X phosphate buffer. Different concentrations (10, 20, 30 ,40, 50 µg/mL) of *Z.oenopila*-mediated silver nanoparticles were added to the reaction mixture. The pH was adjusted to 6.3. Then it was kept at room temperature for 10 minutes followed by incubation in a water bath at 55°C in a water bath for 30 min. Diclofenac sodium was used as the standard group while dimethyl sulphoxide was used as control. Then, the samples were measured spectrophotometrically at 660nm(Chaiya, Senarat et al. 2022).

The percentage of protein denaturation was determined utilizing the following equation,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

2.3 Membrane stabilization assay:

The in vitro membrane stabilization assay is a widely used technique for evaluating the membrane stabilizing properties of natural and synthetic compounds. This assay measures the ability of a compound to stabilize the cell membrane by preventing its disruption and subsequent release of intracellular contents(Ranasinghe, Ranasinghe et al. 2012). The materials include Human red blood cells (RBCs), Phosphate-buffered saline (PBS), Tris-HCl buffer (50 mM, pH 7.4), Different concentrations of silver nanoparticles (10, 20, 30 ,40, 50 µg/mL), Centrifuge tube, UV-Vis spectrophotometer.

Assay procedure:

Pipette 1 mL of the RBC suspension into each centrifuge tube. Then different concentrations of silver nanoparticles (10, 20, 30 ,40, 50 µg/mL) were added to each tube. Mix gently and incubate the tubes at 37°C for 30 minutes. Centrifuge the tubes at 2500 RPM for 5 minutes at room temperature to pellet the RBCs. Measure the absorbance of the supernatant at 560 nm using a UV-Vis spectrophotometer.

Calculate the percentage inhibition of haemolysis using the following formula:

$$\% \text{ inhibition} = [(\text{OD control} - \text{OD sample}) / \text{OD control}] \times 100$$

Where OD control is the absorbance of the RBC suspension without the test compound(s) and OD sample is the absorbance of the RBC suspension with the test.

2.4 Cytotoxic effect

BRINE SHRIMP LETHALITY ASSAY:

Saltwater preparation:

In a study to evaluate the cytotoxic effects of nanoparticles, 2 grams of iodine-free salt were weighed and dissolved in 200 ml of distilled water. Six well ELISA plates were prepared, each filled with 10-12 ml of saline water. Ten brine shrimp nauplii were carefully added to each well, with varying concentrations of nanoparticles set at 5, 10, 20, 40, and 80 µg/mL. The nanoparticles were added in accordance with these concentration levels, and the plates were then incubated for 24 hours. Following the incubation period, the number of live nauplii in each well was recorded. The percentage of dead nauplii was calculated using the formula: (Number of dead nauplii / (Number of dead nauplii + Number of live nauplii)) × 100. This method allowed for a clear assessment of the cytotoxic effects of the nanoparticles on the brine shrimp nauplii(Olowa and Nuñeza 2013).

3. RESULTS

3.1 Anti-inflammatory activity

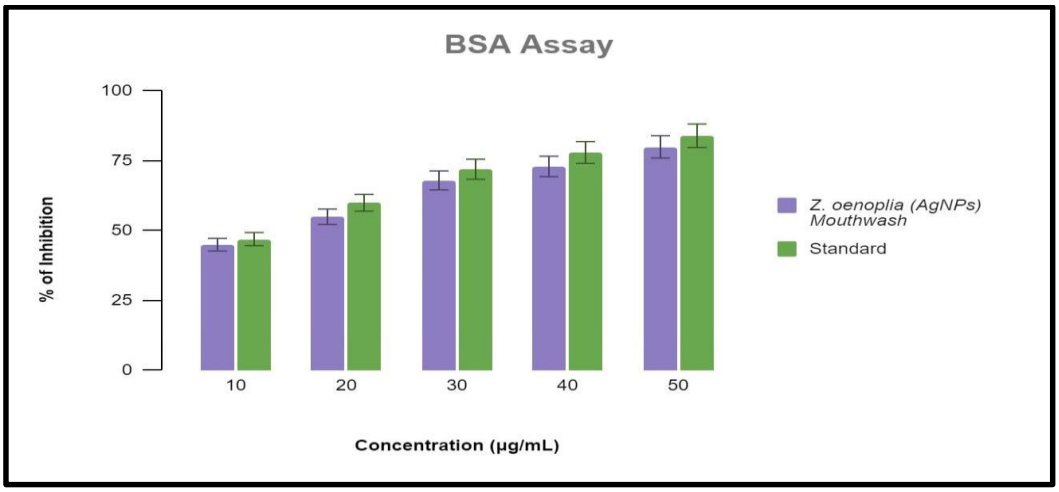


Figure 8 : Shows the percentage of inhibition of *Z. oenoplia* (AgNPs) mouthwash at various concentrations.

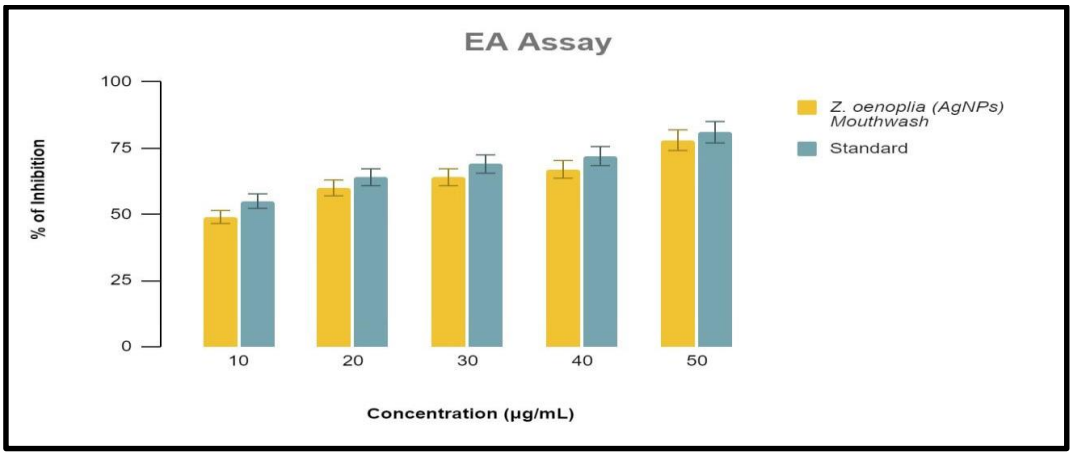


Figure 9: depicts the percentage of inhibition of *Z.oenopila* mouthwash against standard mouthwash, at various concentrations.

The results showed that the inhibition percentage was almost equal to that of the standard mouthwash (Figure 8&9). Compared to the commercial mouthwash, it was highest at 20µg/mL, followed by 30µg/mL and lowest at 50µg/mL. However, there was no significant difference between the two mouthwashes.

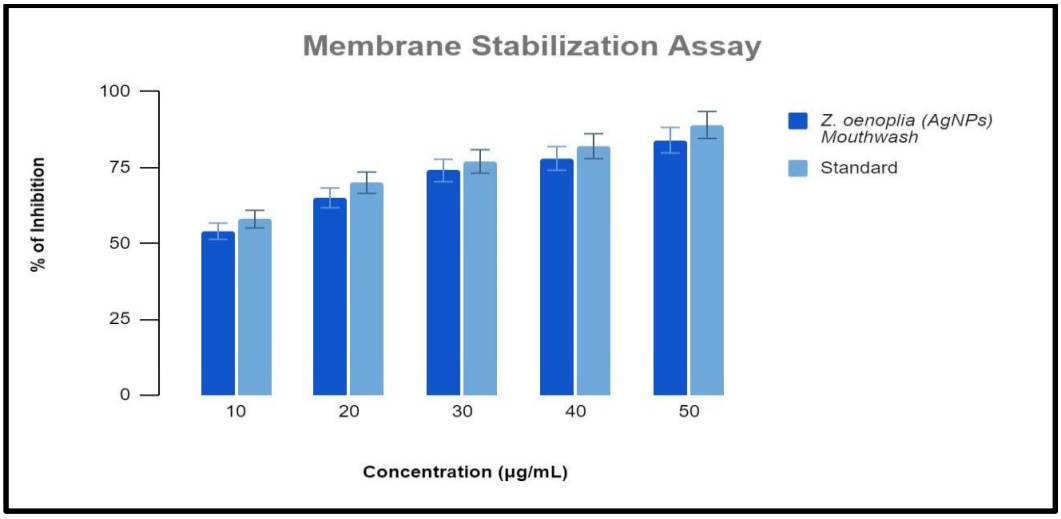


Figure 10: Shows the percentage of increase in the concentration of Z.oenopila in mouthwash and standard mouthwash.

The results showed that the percent inhibition of membrane stabilization increases as the concentration of the substance increases. At a concentration of 50 µg/mL, Z. oenoplia (AgNPs) has the greatest percent inhibition of membrane stabilization, followed by mouthwash and then the standard.

3.2 Cytotoxic effect

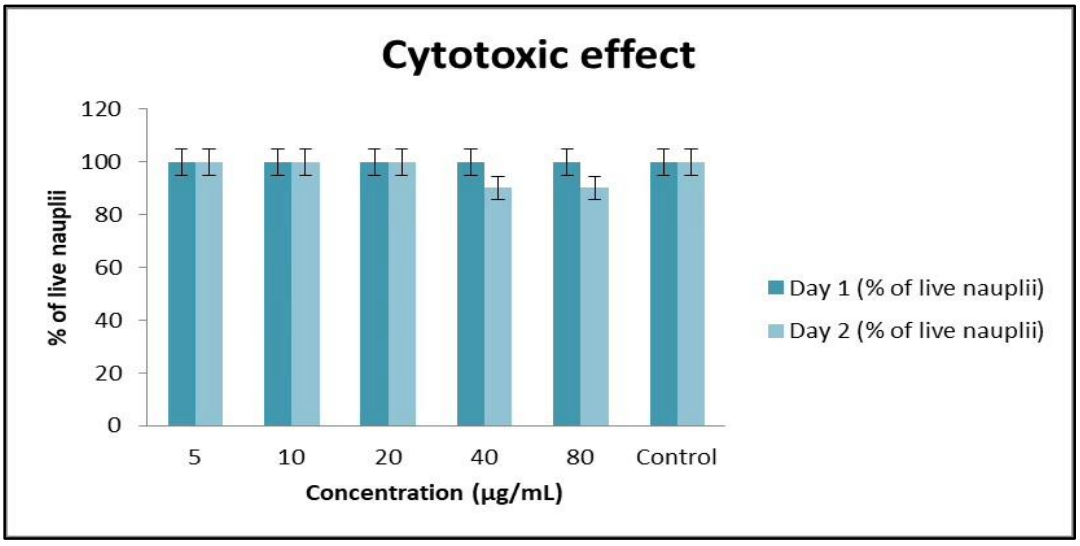


Figure 11: shows percentage of live nauplii over increased concentration.

The results show that the compound has a cytotoxic effect on nauplii. At the level of 5µg/mL, there was 100% survival of nauplii, followed by 10µg/mL and 20µg/mL . However, at the level of 40µg/mL and 80µg/mL the survival percentage decreased(Figure 11). Therefore, the novel compound is cytotoxic to nauplii and the effect is dose dependent and increases over time.

4. DISCUSSION

The study aimed to explore the anti-inflammatory and cytotoxic effects of a novel mouthwash formulation made from Ziziphus oenoplia-mediated silver nanoparticles (ZO-AgNPs)(Umapathy, Pan et al. 2024). The findings indicate that the inhibition rate of ZO-AgNPs was comparable to that of a standard mouthwash. Notably, the inhibitory rate of membrane stabilization increased with the concentration of the nanoparticles, peaking at 50 µg/mL. This suggests a dose-dependent effect, which is crucial for determining the optimal concentration for therapeutic use. The cytotoxic effects of ZO-AgNPs were evaluated using brine shrimp nauplii. The results showed 100% survival at 5 µg/mL, with a gradual decrease in survival rates at higher concentrations (10 µg/mL and 20 µg/mL)(Roshan, Jothipriya et al. 2020). At 40 µg/mL and 80 µg/mL, survival rates were significantly lower, indicating a dose- and time-dependent cytotoxic effect. This highlights the importance of careful dosing to avoid potential toxicity. Ziziphus oenoplia has been documented in various studies for its medicinal properties. Its anti-inflammatory and cytotoxic effects are attributed to its rich composition of bioactive compounds such as saponins, flavonoids, and alkaloids. These compounds are known to possess significant pharmacological activities, including antioxidant, anti-inflammatory, and antibacterial properties(Nasim, Rajeshkumar et al. 2021, Kamath, Nasim et al. 2022). The presence of these bioactive compounds likely contributes to the observed effects in the current study. Silver nanoparticles, on the other hand, have garnered attention for their versatile biological activities, particularly their antimicrobial and anti-inflammatory properties. The small size of AgNPs provides a large surface area, enhancing their interaction with microbial cell walls and inflammatory pathways. This unique property makes them effective agents in combating oral pathogens and inflammation. The current study's findings align with previous research, demonstrating the efficacy of AgNPs in reducing inflammation and exhibiting antimicrobial activity. The combination of Ziziphus oenoplia and AgNPs in a mouthwash formulation presents a promising approach to oral health care. The study observed that the anti-inflammatory effects of the ZO-AgNP mouthwash were comparable to those of a standard chlorhexidine (CHX) mouthwash, which is commonly used but known for side effects such as taste alteration and staining of teeth(Anbarasu, Vinitha et al. 2024, Raj, Martin et al. 2024). The natural origin of Ziziphus oenoplia, coupled with the eco-friendly synthesis of AgNPs, offers an advantage over traditional mouthwashes that often contain harsh chemicals. Moreover, the study underscores the potential of using plant extracts in nanotechnology. The synthesis of nanoparticles using plant extracts is an eco-friendly and sustainable approach, avoiding the hazardous waste associated with conventional chemical methods. This green synthesis not only reduces environmental impact but also leverages the medicinal properties of plants to enhance the biological activity of nanoparticles(Manickam, Giridharan et al. 2022, Nasim, Jabin et al. 2022).

While the current study did not observe significant cytotoxicity at the tested concentrations, it is crucial to consider the potential cytotoxic effects of silver nanoparticles. Other studies have reported adverse effects at higher concentrations, emphasizing the need for thorough evaluation of safe and effective dosages. The observed dose-dependent effects in the current study highlight the importance of optimizing the concentration of ZO-AgNPs to balance efficacy and safety.The uniqueness of this study lies in its novel approach to combining Ziziphus oenoplia and silver nanoparticles in a mouthwash formulation(Duraisamy, Ganapathy et al. 2021).

Previous studies have not explored this specific combination, making this research a valuable contribution to the field of oral health care. The promising results suggest that ZO-AgNP mouthwash could be a more acceptable and natural alternative to conventional mouthwashes, particularly for individuals seeking herbal remedies. In conclusion, the study demonstrates the potential of Ziziphus oenoplia-mediated silver nanoparticles as a novel mouthwash formulation with significant anti-inflammatory and cytotoxic effects. While the findings are promising, further research is necessary to fully understand the efficacy, safety, and optimal dosing of ZO-AgNPs. This study paves the way for future investigations into plant-based nanotechnology applications in oral health care, offering a natural and effective alternative to conventional treatments(Duraisamy, Ganapathy et al. 2021).

5. CONCLUSION

Ziziphus oenoplia-mediated silver nanoparticles (ZO-AgNPs) mouthwash has demonstrated significant anti-inflammatory and cytotoxic effects, indicating its potential in managing oral health by reducing inflammation and targeting harmful cells. However, further research is required to confirm its efficacy against specific oral conditions and to assess potential risks. Future studies should include clinical trials to evaluate safety, investigations into the molecular mechanisms of action, long-term impact assessments, and the development of standardized protocols for the green synthesis of AgNPs to ensure consistency and reproducibility in therapeutic applications.

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