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Original Article

In vitro Insights: Investigating the Cytotoxic Properties of *Curcuma caesia* Roxb. and *Aloe vera in situ* Gel

Ankita Bohra, Thirupambaram Nataraja Sundaram Uma Maheswari*

Department of Oral Medicine and Radiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, INDIA.

ABSTRACT

Background: Chewing areca nuts is associated with the development of Oral Submucous Fibrosis (OSF), an oral disorder with malignant potential. While OSF has only recently begun to appear in Europe and North America, it remains highly prevalent in Southeast Asia. The use of chewing tobacco further increases the risk of malignant transformation in OSF patients. This study aimed to formulate and characterise an *in situ* gel solution containing *Curcuma caesia* Roxb. and *Aloe vera* with calcium carbonate nanoparticles and to evaluate their antimicrobial activity and cytotoxicity. **Materials and Methods:** An *in situ* gel was synthesised using *Curcuma caesia* Roxb., *Aloe vera*, and calcium carbonate nanoparticles. The antimicrobial properties were assessed against *Streptococcus mutans, Staphylococcus aureus*, and *Candida albicans*, while cytotoxicity was evaluated via a brine shrimp lethality assay. **Results:** The *in situ* gel demonstrated significant diffusion zones against *Staphylococcus aureus, Streptococcus mutans*, and *Candida albicans*, indicating strong antimicrobial and antifungal efficacy. Zeta potential analysis further supported these findings. At the highest concentration tested, the gel exhibited moderate cytotoxicity. **Conclusion:** The combination of herbal formulations displayed robust antibacterial and antifungal activities as well as notable cytotoxic effects. Its promising therapeutic applications, including potential antitumor and antiangiogenic properties, warrant further investigation.

Keywords: Aloe, Anti-inflammatory, Curcuma, Fibrosis, Non-steroidal, Neoplasms.

Correspondence:
Dr. Umamaheswari Thirupambaram Nataraja Sundaram
Professor and Head of Academics, Department of Oral Medicine and Radiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, INDIA.
Email: umamaheswaritn@saveetha.com
Scopus ID: 25960432200
ORCID: 0000-0002-2366-2336

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INTRODUCTION

Cytotoxicity assays involve assessing cell death before and after administration of the material causing it; this consists of observing cells or organisms, destruction, or death. The various methods available include ultraviolet spectrophotometry and cell morphological change observation assays; with more modern techniques, animals and aquatic biota can be used for cytotoxicity analysis (Anuradha *et al*., 2017). Curcumin content in *Curcuma caesia* Roxb. is known to exhibit increased antioxidant content and is a potent tumour cell scavenger (Dosoky *et al*., 2018). Many studies have shown the presence of long-term cytotoxicity in curcumin-containing compounds that cause anti-cancer effects (Horst, 2022). It contains numerous essential phytochemicals, such as essential oils with camphor, ar-turmerone, (Z)-ocimene, ar-curcumene, and 1,8-cineole. It has anti-cancerous, anti-inflammatory, antioxidant, and cytotoxic properties, showing promising results against breast, skin, rectal, cervical, and hepatobiliary cancer. The main compounds present in *Curcuma caesia* Roxb. are flavonoids and polyphenols (Pulido *et al*., 2016). Cell-based studies have shown antibacterial properties, ranging from mild to moderate levels, specifically against immune cells in the body, such as macrophages and leukocytes. However, none of the modern studies have reported severe toxicity (Poornachitra 2023). However, antimicrobial and antifungal activities are significant, and the background of cytotoxicity cannot be ignored. The report suggests using an aqueous herbal extract solution, which causes cytotoxicity within hours of usage. This study assessed the cytotoxic effect of an herbal formulation- nanoparticle-based aqueous solution extracts containing *Curcuma caesia* Roxb., *Aloe vera*, and calcium carbonate-laden nanoparticles.

MATERIALS AND METHODS

Kali haldi, the rhizome of *Curcuma caesia* Roxb., is known for its anti-inflammatory, antioxidant, antimicrobial, and anti-mutagenic properties. Its combination with *Aloe vera* and calcium carbonate nanoparticles has a synergistic effect on the underlying fibrous tissues. The Institutional Ethical Committee of Saveetha Dental College and Hospital approved the study with an approval number of 1617/21/235. This study aims to formulate *Curcuma caesia* Roxb. and *Aloe vera* calcium carbonate *in situ* gel nanoparticles to assess the therapeutic effects in managing oral

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submucous fibrosis. Making an *in situ* gel with a mixture of all three components holds a central position in the accuracy of the therapeutic dose. Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Thiobarbituric Acid (TBA) and 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP) were obtained. *Curcuma caesia* rhizomes were obtained from the experimental farm of the Indian Institute of Spices Research at Calicut. A portion of fresh rhizomes was washed, air-dried, and stored in the freezer until the extraction, while the remaining rhizomes were cut into small pieces and sun-dried for 48 hr. Both fresh and dried rhizomes were subjected to extraction. *Aloe vera* leaves (six big and healthy) were weighed, washed, and cut in the middle. The gel was separated by scratching with a spoon, and the pulp was cut into small pieces (514 g) and homogenised with a Phosphate-Buffered Saline solution (PBS; pH 7; 600 mL) using a blender. The extract was kept at 4°C overnight and then filtered through muslin cloth, and the filtration was centrifuged at 20000 rpm for 30 min at 2°C in a refrigerated centrifuge. The green pellet was discarded, and the clear yellow supernatant was taken and lyophilised. Thus, 10 g of *Aloe vera* leaf pulp extract was obtained.

Preparation of plant extracts

The fresh and dried forms of *C. caesia* and *Aloe vera* leaf rhizomes (each 0.50 g) were sequentially extracted. Herbal extracts containing *Curcuma caesia* Roxb. and *Aloe vera*, weighing 0.50 g each (Figure 1), were mixed with 100 mL of distilled water. The extracts are boiled at 50°C for 1 hr, which is combined with 0.016 g/mm of calcium carbonate nanoparticles, which are thoroughly stirred, where a noticeable colour change of the fluid is seen (Figure 2); the liquid is then centrifuged at 8000 rpm, post which the concentrated fluid (pellets) is separated from the supernatant fluid. This herbal formulation is subjected to cytotoxicity testing using the brine shrimp lethality assay. The brine shrimp were administered to the 12 well ELISA plates chosen, and 10 nauplii were slowly added to each well, taken in 5 dose levels: 5 µL, 10 µL, 20 µL, 40 µL, 80 µL, and the control group. (Graph 1) The live brine shrimp are administered and observed for about 24 hr, and the survival level in each well of the ELISA plates is noted.

The number of nauplii, alive or dead, is calculated by the formula mentioned.

$$\text{Number of dead nauplii} / \text{Number of dead nauplii} + \text{Number of live nauplii} \times 100$$

RESULTS

The results are categorised under different concentrations and compared with a control group. Antioxidant and anti-inflammatory DPPH assay, EA, H_2O_2 assay, and BSA assay were performed using ascorbic acid at varying concentrations of 10 µL, 20 µL, 30 µL, 40 µL, and 50 µL (Graph 1). µL (Graph 1). The antimicrobial property was determined for *Curcuma caesia* Roxb., *Aloe vera*, and calcium carbonate nanoparticles by testing with a control antibiotic, ciprofloxacin (Figure 2). The stability of the prepared *in situ* solution was determined using zeta potential analysis using each ingredient for water-in-oil emulsion and oil-in-water emulsion, respectively. The value range above, from +30 mV to -30 mV, shows a moderately stable colloidal solution. Results obtained for the zeta potential of *Curcuma caesia* Roxb. for water-in-oil and oil-in-water emulsions were -36.1 and +34.1, respectively (Graphs 2 and 3). The zeta potential of *Aloe vera* for water-in-oil and oil-in-water emulsions was -32.36 and +36.2, respectively. Both the results show a stable colloidal solution formulation. The particle distribution graph shows an increase in the volume of the nano-emulsion of *Curcuma caesia* Roxb., the particle size distribution also increases to 120 nm when the volume reaches 100%. Afterwards, it gradually decreases to 180 nm. The particle distribution. The graph shows that with the increase in the volume of the nano-emulsion of *Aloe vera*, the particle size distribution also increases to 338 nm when the volume reaches 100%. Afterward, it gradually decreases to 500 nm (Graphs 4 and 5). The cytotoxic potential was determined using different sets of concentrations of *in situ* gel. Results were scrutinised and calculated from the first day onwards. On Day One, at all the concentrations of 5 µL, 10 µL, 20 µL, 40 µL, and 80 µL, and in the control group, the number of nauplii alive was 10. There was no harm to the viability of living beings. The second day shows that at a concentration of 5 µL, nine nauplii were alive, and one nauplius was dead. On a similar day, 8 nauplii survived at 10 µL concentration, and two nauplii were dead; at 20 µL concentration, seven nauplii were alive, and three were dead; at 40 µL concentration, six nauplii survived, and four died; and at 80 µL concentration, only four nauplii were alive, and in the control, all 10 nauplii survived without any harm (Graph 6).

The *in situ* gel combination's cytotoxic nature was evident in this assay. This ratio shows that the *Curcuma caesia* and *Aloe vera* solution gel with calcium carbonate nanoparticles shows potent cytotoxic attributes. The potential loss of nauplii with an increment in the drug concentration hints towards the drug combination's cellular destructive potential (Figure 3). This study showed that at the highest dose of about 80 µL, only four shrimp survived, but at the starting or lowest dose of 5 µL, about nine shrimp out of 10 survived the 24-hr window period. Even at lower dosages, the *in situ* gel was compelling enough to cause cytotoxic damage to the internal tissue of the living creature; while considering the highest dosage of 80 µL, four shrimps were still alive, resulting in 60% cytotoxic potential and not 100% life-threatening.

DISCUSSION

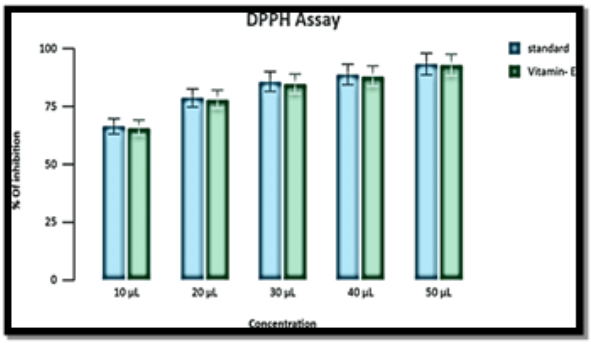
In the current study, the cytotoxic effect at the highest dose showed the presence of survival of 4 nauplii shrimps, indicating less toxicity even at the highest dosage of 80 µL. Research conducted

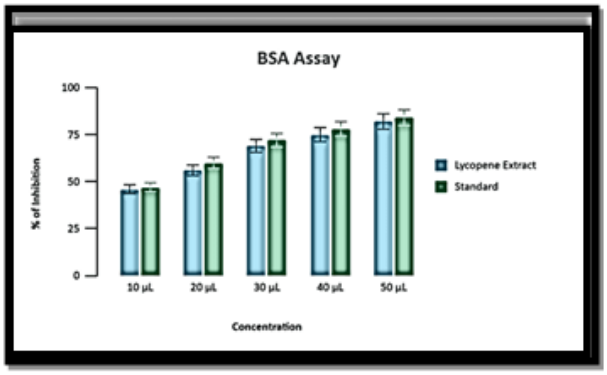
by Verma *et al*., (2018) using neem herbal formulation with silver nanoparticles demonstrated increased cytotoxicity only at concentrations above 75%, while chlorhexidine mouthwash exhibited more cytotoxicity at a concentration of 10%. In this study, maximum cytotoxicity was observed only at close to 100% or the maximum administered dosage (80 µL), which is similar. Anti-inflammatory and antioxidant potentials were evaluated using DPPH, EA, BSA, and H_2O_2 assays. At a concentration of 50 µL, the results indicated promising antioxidant and anti-inflammatory potential of the *in situ* gel (Verma *et al*., 2018). The zeta potential of the *in situ* gel was assessed and found to be stable for both water-in-oil and oil-in-water emulsion preparations. This contributes to the overall effectiveness, reliability, and stability of the formulation and the durability of the drug under standard conditions. Particle distribution graphs were plotted, demonstrating the stability of nano-emulsion size and drug durability. *In situ* gel-forming polymeric formulations offer several advantages, such as sustained and prolonged action in comparison to conventional drug delivery systems. The formation of gels depends on factors like temperature modulation, pH change, presence of ions, and ultraviolet irradiation, from which the drug gets released in a sustained and controlled manner. Various biodegradable polymers used for the formulation of *in situ* gels include gellan gum, alginic acid, xyloglucan, pectin, chitosan, poly (dl-lactic acid), poly (DL-lactide-co-glycolide), and polycaprolactone. Mainly, *in situ* gels are administered by oral, ocular, rectal, vaginal, injectable, and intraperitoneal routes. The *in situ* gel-forming polymeric formulations offer several advantages, like sustained and prolonged action in comparison to conventional drug delivery systems (Wu C, 2014).

Comparative studies have shown that *in situ* gel delivery systems have several advantages over ointments, the most notable being the ability to provide regular and continuous drug delivery with no impact on visual clarity (Harsh 2023). Bioavailability, penetration, duration, and maximum medication efficacy are all improved by this mechanism. An optimal temperature-sensitive *in situ* gelling solution must have a phase change temperature greater than ambient temperature (25°C) to be able to be readily delivered to the eye; hence, it was fabricated at 35°C, which is the precorneal temperature (Sharuniveda 2024). In a pH-sensitive gelling system, a gel develops immediately when the bio-stimuli come into contact with it. An *in situ* gelling system with ionic strength-triggered medication can also perhaps be used in optical drug-delivery mechanisms (Maheswari 2022). Overall, *in situ* gel preparation offers several advantages over other drug delivery systems, including enhanced bioavailability, lower cytotoxicity, potent antimicrobial and antifungal properties, stability, and durability. These advantages make *in situ* gel a promising area for further research and development (Eswaramoorthy 2024).

In this study, cytotoxic analysis was conducted to evaluate the lethality of brine shrimp nauplii, which is a common approach for assessing cellular viability and cytotoxic effects. *Curcuma caesia* Roxb. is known for its rich anti-inflammatory properties and offers several benefits, including stress relief, immune support, enhanced stamina, and promotion of healthy metabolism and natural immunomodulation (Evanjelin, 2023). Key chemical constituents of Kali Haldi include camphor, 1,8-cineole, ar-curcumene, β-elemene, borneol, bornyl acetate, and α-terpineol as significant phytochemicals. *Aloe vera* contains mannoproteins with amino acids known for their

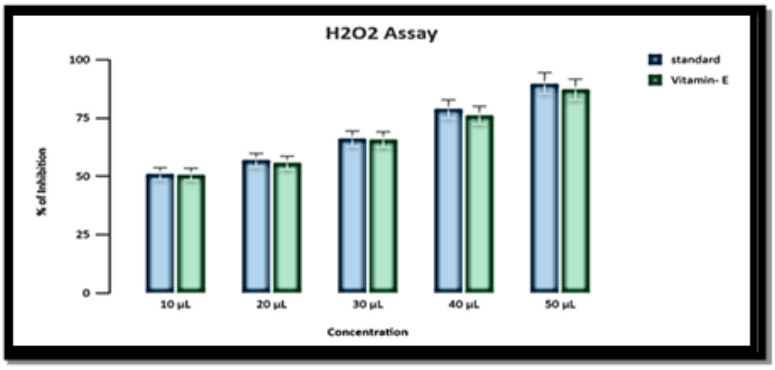
Figure 1: *In situ* Gel preparation.



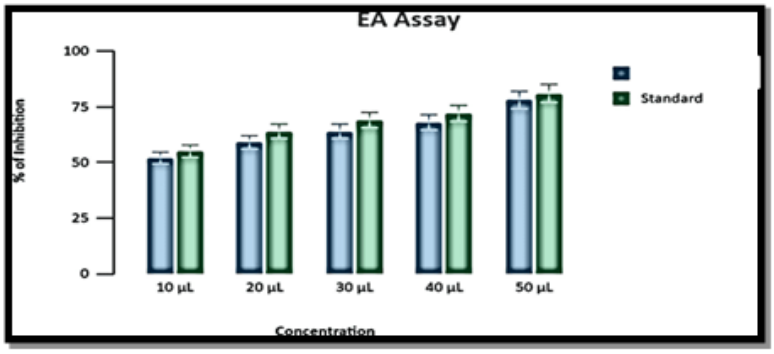


Scavenging activity (%) on DPPH radicals of Combination *In situ* Gel & used ascorbic acid as a standard in 5 different concentrations

Anti-inflammatory activity (%) on BSA of Combination *in situ* Gel & used ascorbic acid as a standard in 5 different concentrations



Scavenging activity (%) on H2O2 radicals of Combination *in situ* Gel, using ascorbic acid as a standard in 5 different concentrations.

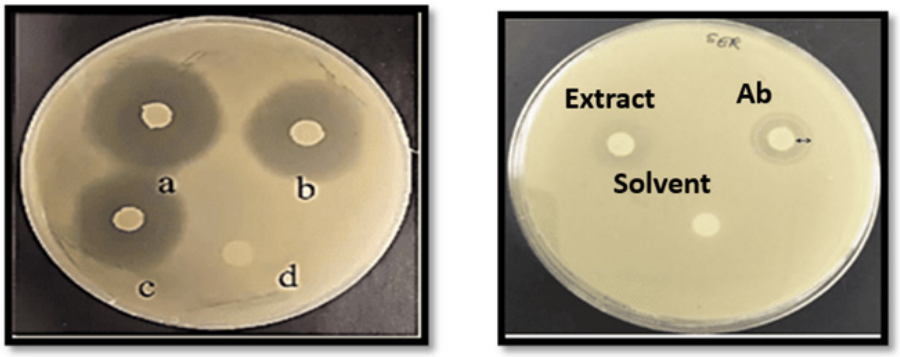


Anti-inflammatory activity (%) on EA of Combination *in situ* Gel, using ascorbic acid as a standard in 5 different concentrations.

Graph 1: Antioxidant Properties and Anti-inflammatory Properties of Black turmeric and *Aloe vera* & Calcium Carbonate NPs.

wound-healing properties (Bohra., 2021). The polysaccharides in the leaf gel have been shown to promote wound healing and possess anti-inflammatory, immunomodulatory, antioxidant, and gastroprotective properties. *Aloe vera* sterols exhibit a strong capability to inhibit inflammation like cortisone, but without any adverse side effects. Additionally, turmeric is recognised for its potent anti-inflammatory effects, improved blood circulation, and anti-mutagenic properties (Poornachitra, 2023).

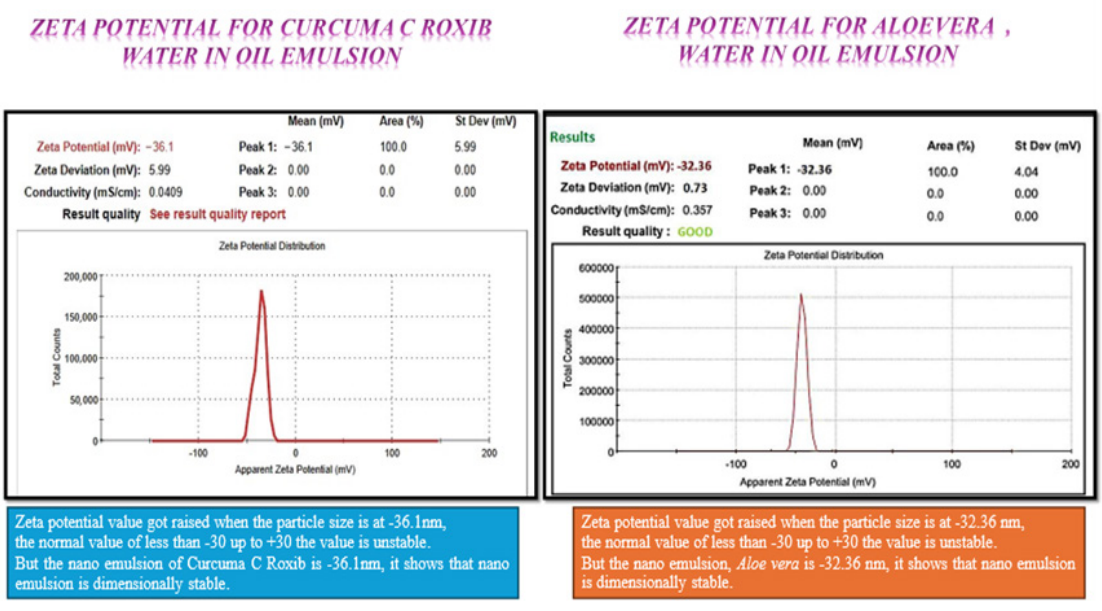
Das *et al*., demonstrated that the extract and synthesised Gold Nanoparticles (GNPs) exhibited no cytotoxic effects on HeLa and L929 cell lines. However, both the extract and GNPs showed promising activity against breast cancer cell lines, evidenced by a dose-dependent decrease in cell viability and inhibition of cell growth. This study investigates the potential of herbal extracts from *Curcuma caesia* Roxb. and *Aloe vera*, combined with calcium carbonate nanoparticles, to target potentially malignant



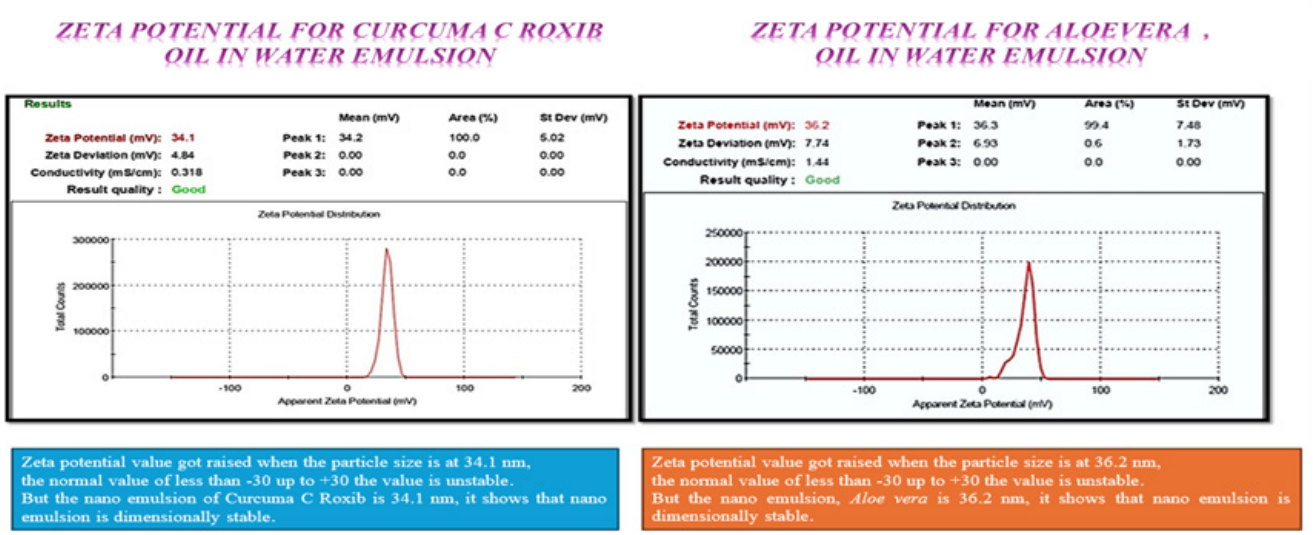
Antimicrobial activity of Curcuma C. Roxib Aloe vera and Calcium carbonate NPs against S. aureus and compared with Antibiotic.

Figure 2: Anti-microbial *in vitro* studies for *Curcuma caesia* Roxb., *Aloe vera*, and Calcium carbonate nanoparticles.

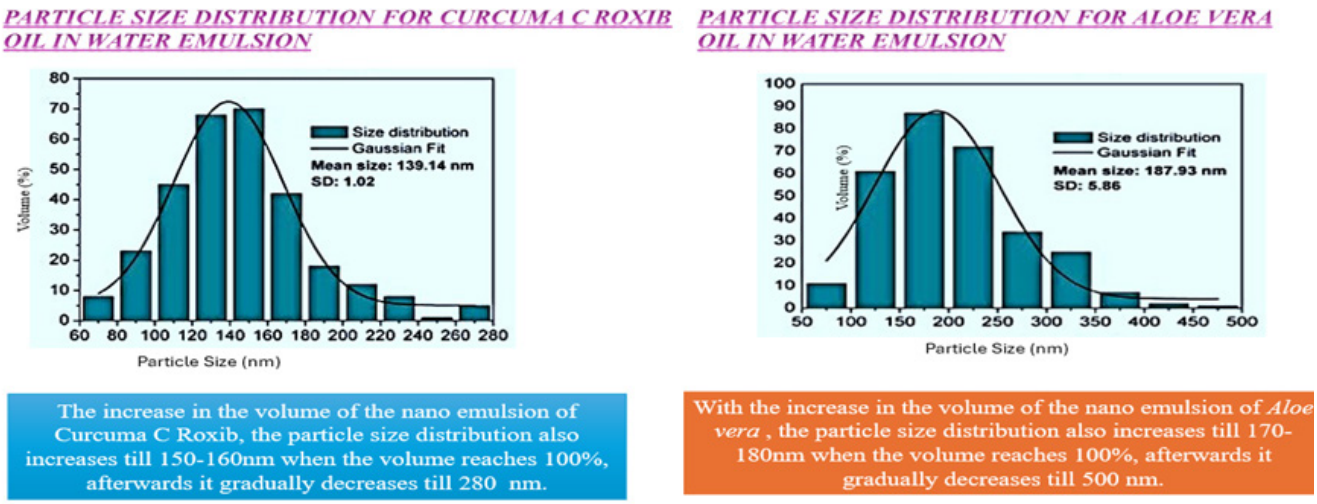
Figure 3: *In situ* gel used in different concentrations and compared with a control group.



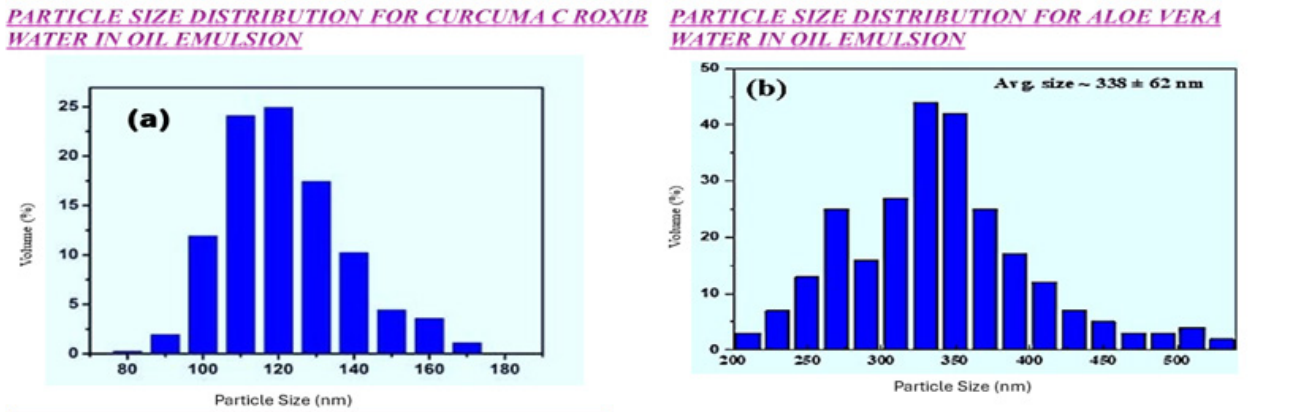
Graph 2: Zeta Potential Water in Oil Emulsion.



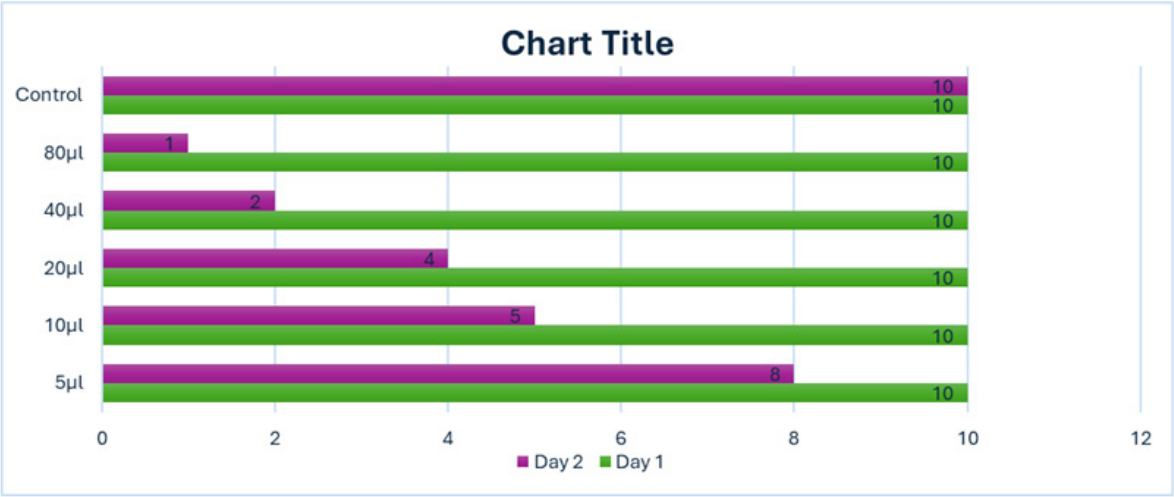
Graph 3: Zeta potential Oil in Water Emulsion.



Graph 4: Particle distribution Graph (Water in Oil Emulsion).



Graph 5: Particle distribution graph (Oil in water Emulsion).



Graph 6: Represents the percentage of inhibition seen in the various concentrations of Curcuma caesia Roxb., Aloe vera, and Calcium carbonate NPs.

dysplastic cells while protecting normal cells from the cytotoxic effects of anticancer drugs (Herendija *et al*., 2023). An *in situ* gel was formed, and the evaluation of *in vitro* cytotoxic activity revealed that Kali Haldi and *Aloe vera* demonstrated notable cytotoxic potential, with calcium carbonate particles exerting a synergistic effect in this combination (Abhay *et al*., 2023). These results highlight the potential of herbal formulation in providing a multi-faceted approach to managing oral submucous fibrosis and other related conditions (Fatt *et al*., 2021). The combination of *Curcuma caesia* Roxb. and *Aloe vera* with calcium carbonate nanoparticles enhances the bioavailability and effectiveness of the drug, making it a promising area for further research and development.

CONCLUSION

The conclusion of this study demonstrated that combining calcium carbonate nanoparticles with an *in situ* gel herbal formulation containing *Curcuma caesia* Roxb. (Kali haldi) and *Aloe vera* extracts exhibits significantly lower cytotoxicity compared to the herbal extracts alone without the inclusion of nanoparticles. Notably, this combination enhances the bioavailability and effectiveness of the drug with increasing concentration. Further research involving a larger sample size is recommended to explore additional outcomes and develop better therapeutic alternatives for potentially malignant oral disorders, such as oral submucous fibrosis. Initiating clinical trials to assess the efficacy and safety of the *in situ* gel formulation in human subjects. This will provide more concrete evidence of its therapeutic applications and potential side effects. Securing adequate funding and resources to conduct the trial is often a significant hurdle. This includes financial support, personnel, and facilities. Monitoring and managing potential adverse effects in participants are crucial for ensuring safety and efficacy. This requires continuous oversight and prompt response to any issues.

ACKNOWLEDGEMENT

The researchers would like to extend their gratitude to Saveetha University of Medical Sciences for providing research support for this study.

ABBREVIATIONS

OSF: Oral Submucous Fibrosis; **GNPs:** Gold Nanoparticles; **BHA:** Butylated Hydroxyanisole; **BHT:** Butylated Hydroxytoluene; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **TBA:** Thio Barbituric Acid; **ABAP:** 2,2'-Azobis-(2-amidinopropane) Dihydrochloride; **EA:** Egg Albumin Assay.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

LIST OF CONTRIBUTORS

Dr. Ankita Bohra- data selection, methodology, and interpretation.

Dr. T.N. Uma Maheswari- critical screening and manuscript writing.

ETHICAL APPROVAL

The Institutional Ethical Committee of Saveetha Dental College and Hospital approved the study with an approval number of 1617/21/235.

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