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Antibacterial, Anti inflammatory and Antioxidant Effects of *Punica granatum, Phyllanthus emblica and Citrus aurantifolia* Extracts in a Mouthwash Formulation: Implications for Oral Health

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ABSTRACT:
AIM: To assess the anti-inflammatory, antioxidant, anti bacterial properties of the formulated mouthwash.
MATERIALS AND METHODS: In this study, the formulated mouth wash formulation was subjected to anti-inflammatory, anti oxidant, antimicrobial and cytotoxicity testing
RESULTS: Highest anti-inflammatory and antioxidant properties were noted at 50 µl concentration when compared to the control. The antimicrobial activity of the kelp mouth rinse had the highest zone of inhibition seen in 50 µL concentration. The cytotoxic activity was found to be better at all concentrations studied.
CONCLUSION: The formulated mouth wash exhibited good anti-inflammatory, antioxidant and antibacterial activities.

KEYWORDS: Pomegranate extract, Lime, Amla, Mouthwash, Oral health.

Introduction:

The global trend for living and health has shown an increased propensity towards natural resources, and Indian society has always incorporated the use of herbal and natural products in daily life since ancient times (Mukherjee, 2019). Allopathic medicine only came into use in the late 19th century, and it was used more commonly than herbal medicine, nevertheless the recent trend is towards incorporation of herbal and natural products to traditional allopathic medicinal products, and recent researches have yielded results in its favor (Bodeker & Ong, 2005). Various previous researches have found that use of plant and plant based products promote oral health. Pomegranate, Amla and Lime extracts have been reviewed in Ayurveda and have been proven to have antibacterial action (Saniya et al., 2023). Pomegranate (*Punica granatum*) is packed with antioxidants that help fight against oral bacteria. Pomegranate extract assists in reducing plaque formation, gingivitis, contributing to overall oral health (Alami et al., 2023). Amla (*Phyllanthus emblica*) , also known as Indian gooseberry, is a potent source of vitamin C, which prevents the accumulation of free radicals. Amla and Lime (*Citrus aurantifolia*)are commonly consumed fruits known to be rich in many nutrients (*Website,* n.d.). Even though they are commercially available in many forms, the medicinal value and the therapeutic potentials have not been investigated scientifically (El Fihry et al., 2023; Mahajan et al., 2023). They have also been used extensively in Ayurvedic preparations to treat diverse infectious diseases since ancient times. Amla's anti-inflammatory properties can help reduce gingival swelling and promote a healthier oral environment. Lime has natural antibacterial properties that can help combat oral bacteria, contributing to improved oral hygiene while maintaining a healthy pH balance in the mouth, which is essential for overall oral health (Ting, 2014),(Amani et al., 2024).

Previous studies on Punica granatum have found that it has potent antioxidant potentials, along with high anti inflammatory and anti carcinogenic effects, due to its high concentration of polyphenols (Ahmed et al., 2023). It has been suggested that pomegranates can treat risk factors of various diseases such as hyperglycemia, hypertension, oxidative stress to name a few. It was found that the juice obtained from pomegranates can reduce oxidative stress, lipid periodisation and free radicals (Alami et al., 2023). Pomegranates also possess higher concentrations of tannins, predominantly Ellagitannins such as Punicalagin and Punicalin, which get metabolized into ellagic acid, a type of hydroxybenzoic acid, which possesses high antioxidant activity (El

Fihry et al., 2023). *Citrus aurantifolia* has been used extensively in olden times to treat and alleviate flu like symptoms, ear pain, headache, stomach ache, and it has also been found to be an appetite stimulant (Vazhacharickal et al., 2017). Extracts from *Phyllanthus emblica* contain various phytoconstituents, a high number of polyphenols such as Gallic acid, ellagic acid, tannins, amino acids, flavonoids. It has been used to treat various symptoms such as inflammation, cancer, neurological disorders, hypertension (Patel et al., 2020).

Pomegranate, Lime and Amla are indigenous traditional medicines of India, and these plants are common in India. Regardless of the fact that enormous investigations have been carried out to explore the antimicrobial property of these plant extracts, the effects of these combined extracts need to be evaluated. Hence, the aim of this study is to assess the anti-inflammatory, antioxidant and antibacterial properties of mouthwash containing extracts containing pomegranate seeds, lime and amla.

Materials and Methods:

Preparation of the extract:

In 100 ml of distilled water. (20gms of pomegranate extract, 2gms of amla and 3 ml of lime extract) were added along with glycerin, ethanol and cetylpyridum chloride. The extract was mixed and was allowed to spin at 5000 rpm for 6 hours and filtered prior to analysis.

1. **Antioxidant test**

DPPH ASSAY

The DPPH free radical is a stable organic nitrogen radical characterized by its deep purple color. Upon reaction with an antioxidant, the color of the DPPH solution shifts from purple to yellow, indicating the formation of the corresponding hydrazine. The antioxidant's reducing capacity against DPPH is assessed by observing the decrease in absorbance within the 515–528 nm range. The outcomes are reported as IC50 values or as the percentage of DPPH scavenging at a set antioxidant concentration for each sample.

Preparation of DPPH Solution:

To prepare the DPPH solution, 7.89 mg of DPPH was measured using a chemical balance and dissolved in 100 ml of 99.5% ethanol. This solution was then stored in the dark for 2 hours.

DPPH Assay Procedure:

In the assay, 1,000 µl of DPPH solution was combined with 800 µl of Tris-HCl buffer (pH 7.4) in a test tube. Subsequently, 200 µl of the test sample solution was added and mixed quickly. The mixture was left at room temperature for 30 minutes, after which the absorbance at 517 nm was recorded. As a blank, a mixture of 1,200 µl of ethanol and 800 µl of Tris-HCl buffer (pH 7.4) was used. The inhibition ratio (%) was calculated using the formula:

Inhibition ratio (%) = (A1 − A2) × 100 / A1,

where A1 is the absorbance with ethanol instead of the test sample, and A2 is the absorbance with the test sample.

2. Anti inflammatory test:

Inhibition of protein denaturation

The reaction mixture included test extracts at varying concentrations and a 1% aqueous solution of bovine serum albumin. The pH was adjusted using a small amount of 1N HCl. Diclofenac sodium was used as the reference drug. Samples were incubated at 37°C for 20 minutes and then heated to 57°C for 30 minutes. After cooling, turbidity was measured spectrophotometrically at 660 nm. The experiment was conducted in triplicate.

Percentage Inhibition = (A of Control – A of Sample)/A of Control x100

3. Antibacterial Test - Minimal Inhibitory Concentration (MIC):

The MIC test was performed using Mueller Hinton Agar (MHA), an ideal medium for routine susceptibility tests due to its reproducibility, and minimal content of sulfonamide, trimethoprim, and tetracycline inhibitors, facilitating satisfactory growth of most bacterial pathogens.

- Prepare Muller-Hinton broth or PBS1x.
- Pipette 50 µl of MHB/PBS into wells.
- Add 50 µl of the sample into the wells.
- Introduce 10 µl of bacterial samples (E. coli, S. aureus, and S. mutans) into separate wells.
- Use an antibiotic as a control in separate wells.
- Incubate for 48 hours at 37°C.

- Measure the optical density at 600 nm for all wells, including controls and sample-containing wells.

4. Surface characteristics using SEM and FTIR analysis:

The surface characteristics of the formulated mouthwash were analyzed using Field Emission Scanning Electron Microscopy (FE-SEM) with elemental Dispersive X ray analysis (EDX) (JSM-IT800 NANO SEM) SEM analysis was done to assess the morphology of the constituents of the mouthwash. FT-IR (Alpha II Bruker Model with wavelength of 4000 to 500 cm^{-1}) was used to characterize the organic and inorganic constituents of the samples, thereby providing the information on their molecular structure. The class of the functional groups can be determined based on the presence of peaks at specific wavenumber.

RESULTS:

The formulated mouthwash had better anti-inflammatory properties at 50µl concentration and was also found almost equal to the standard Vitamin E at lower concentrations. (Graph 1). The anti-inflammatory potential was 86% at 50µl (p value =0.026) (Table 1). Antioxidant activity was also found to be good when the solution was formulated at 50µl concentration and showed properties equal to the gold standard drug of choice diclofenac sodium. (Graph 2). The highest antioxidant potential of 88.6% was at 50µl, and was comparable to the standard at lower concentrations. (P value =0.039) (Table 2). The antibacterial activity of the formulated mouth rinse had the highest zone of inhibition seen in 50 µL concentration for S. aureus and E.coli. (Figure 3&4). FT-IR shows presence of the OH, carboxyl and amine groups in the formulation. (Figure 5).

DISCUSSION:

Pomegranate (Punica granatum) seeds have been deemed as “an ancient seed for modern cure”, because of its anti inflammatory, anti microbial, anti fungal and anti mutagenic properties. The active components, including polyphenolic flavonoids (e.g., punicalagins and ellagic acid), are believed to prevent gingivitis by reducing the oxidative stress in the oral cavity (Ciftci et al., 2023). The present study highlights the potential of *Punica granatum, Phyllanthus emblica,* and *Citrus aurantifolia* extracts as key components in a mouthwash formulation, given their notable

antibacterial, anti-inflammatory, and antioxidant properties. These natural extracts offer a promising alternative to synthetic chemicals commonly used in oral health products, addressing growing consumer demand for safer and more sustainable health solutions.

Our findings indicate that the extracts of *P. granatum, P. emblica, and C. aurantifolia* exhibit significant antibacterial activity against common oral pathogens such as *Streptococcus mutans* and *Escherichia coli. P. granatum,* known for its high polyphenol content, has demonstrated substantial inhibitory effects on bacterial growth, aligning with previous studies that have highlighted its broad-spectrum antimicrobial properties. (Samreen et al., 2024)Similarly, *P. emblica*, rich in ascorbic acid and tannins, has shown effective bacteriostatic and bactericidal activities. *C. aurantifolia*, containing citric acid and flavonoids, also contributes to the antimicrobial action, thereby reinforcing the mouthwash's ability to reduce bacterial load and prevent dental caries and periodontal diseases.(Mahapatra et al., 2024)

The anti-inflammatory properties of these extracts are crucial for managing gingival inflammation and preventing periodontal diseases. *P. granatum* has been documented to inhibit the production of inflammatory mediators such as prostaglandins and interleukins, thereby reducing gingival inflammation.(*JCDR - Evaluation of Anti-Inflammatory, Antioxidant and Antimicrobial Activity of Pomegranate Peel Extract: An In-Vitro Study*, n.d.) *P. emblica*'s high vitamin C content and bioactive compounds help modulate the inflammatory response by scavenging free radicals and inhibiting pro-inflammatory cytokines.(Bhavana et al., 2023) The incorporation of *C. aurantifolia* further enhances this effect due to its ability to downregulate the expression of inflammatory markers. (Amorim et al., 2016)These combined anti-inflammatory actions contribute to maintaining healthy gingival tissue and preventing chronic inflammation.

Oxidative stress plays a significant role in the pathogenesis of oral diseases. The antioxidant properties of *P. granatum, P. emblica,* and *C. aurantifolia* extracts are instrumental in neutralizing reactive oxygen species (ROS) and protecting oral tissues from oxidative damage. *P. granatum* is particularly effective due to its high content of ellagitannins and punicalagins, which have strong antioxidant activities.(Gan et al., 2022) *P. emblica*'s efficacy is attributed to its high levels of vitamin C and polyphenols, which provide substantial free radical scavenging abilities (Renuka et al., 2024). *C. aurantifolia*, with its rich flavonoid content, also contributes to reducing oxidative stress. (Ugwuoke et al., 2023). Together, these extracts enhance the mouthwash's

ability to protect against oxidative damage, promote oral health, and potentially reduce the risk of oral cancers.

The integration of *P. granatum, P. emblica,* and *C. aurantifolia* extracts into a mouthwash formulation offers a multi-faceted approach to oral health management. By combining antibacterial, anti-inflammatory, and antioxidant activities, this natural formulation addresses several key aspects of oral hygiene. The findings suggest that such a mouthwash can effectively reduce microbial load, control inflammation, and protect against oxidative damage, making it a valuable addition to daily oral care routines. Moreover, the use of natural extracts aligns with the increasing preference for organic and eco-friendly health products, potentially enhancing user compliance and satisfaction.

Previous study conducted by Menezes SM et al, 2006 showed that pomegranate extract was more effective against the adherence of biofilm and they suggested that this phytotherapeutic agent might be used in the control of adherence of different microorganisms in the oral cavity. (Menezes et al., 2006). In a study by de Nigris *et al*., they compared the influence of pomegranate fruit extract with pomegranate juice on nitric oxide and arterial function in obese Zucker rats. They have demonstrated that both pomegranate fruit extract and juice significantly reduced the vascular inflammatory markers expression, thrombospondin, and cytokine TGFP 1. Increased plasma nitrite and nitrate were observed with administration of either pomegranate fruit or juice.(de Nigris et al., 2007)

Previous studies suggest that lime peel extract contains flavonoids that inhibits S. mutans formation by inhibiting the glucosyltransferase activity and thereby preventing biofilm formation. Lime extract showed an elevated amount of limonene and linalool, which have potent anti-inflammatory, antioxidant, anti-stress. The antioxidant activity of the extract prevents tissue damage caused by oxidation of reactive oxygen species. (Fazmiya et al., 2022). The antioxidant activity of the extract was attributed to the presence of cineol, pinene, and limonene. The antioxidant activity in the extract used in the present study was in accordance with the previous results.

Previously, it was found that *P. granatum* can be useful in the treatment of type 2 diabetes mellitus. The extracted nanoparticle had a maximum inhibition of 79.28% and 76.17% against α-amylase and α-glucosidase respectively at the highest concentration of 160 µg/ml and was comparable to the standard Acarbose control. (Royapuram et al., 2023)In the present study, the

combined triple action of Pomegranate, Amla and lime extracts has an enhanced synergistic combined effect against S.aureus and E.coli.

CONCLUSION:

In conclusion, the study demonstrates that the mouthwash formulation containing P. granatum, P. emblica, and C. aurantifolia extracts provides comprehensive oral health benefits. These extracts' synergistic effects offer a potent combination of antibacterial, anti-inflammatory, and antioxidant properties, which are essential for maintaining oral hygiene and preventing oral diseases. Further clinical studies are warranted to confirm these findings and explore the long-term benefits and safety of this natural mouthwash formulation in diverse populations.

SCOPE FOR FUTURE RESEARCH:

Further clinical trials can be conducted using the formulated mouthwash and patient compliance along with clinical antibacterial effects of the mouthwash can be assessed in terms of reduced DMFS/dmfs.

AUTHOR CONTRIBUTION:

Author 1(Dr. Deeksheetha. P) took part in conceptualisation, data acquisition, drafting and revising of the article. Author 2 (Dr. Bargavi. P) took part in the design, data analysis, data interpretation and drafting of the article. Author 3 (Dr. Pratibha Ramani) took part in the conceptualisation, design and final approval of the manuscript.

CONFLICT OF INTEREST: None declared

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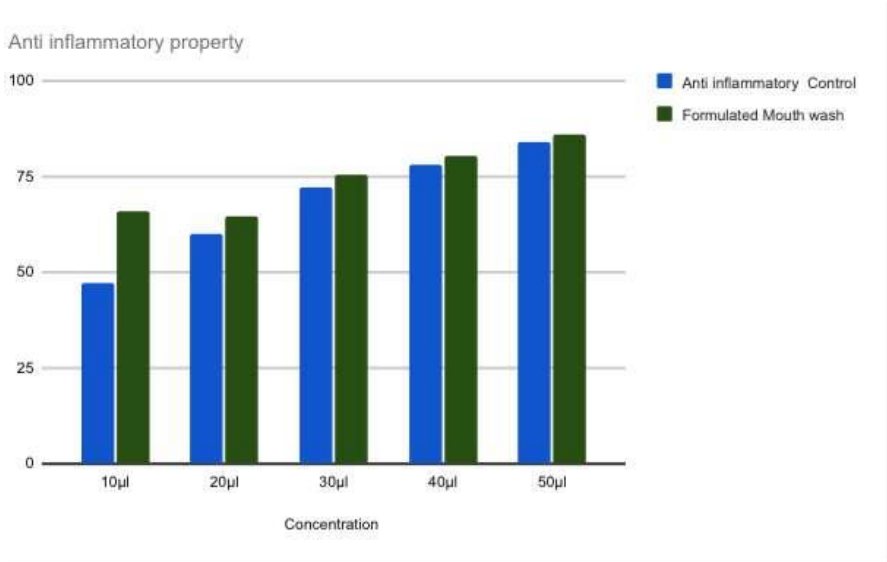
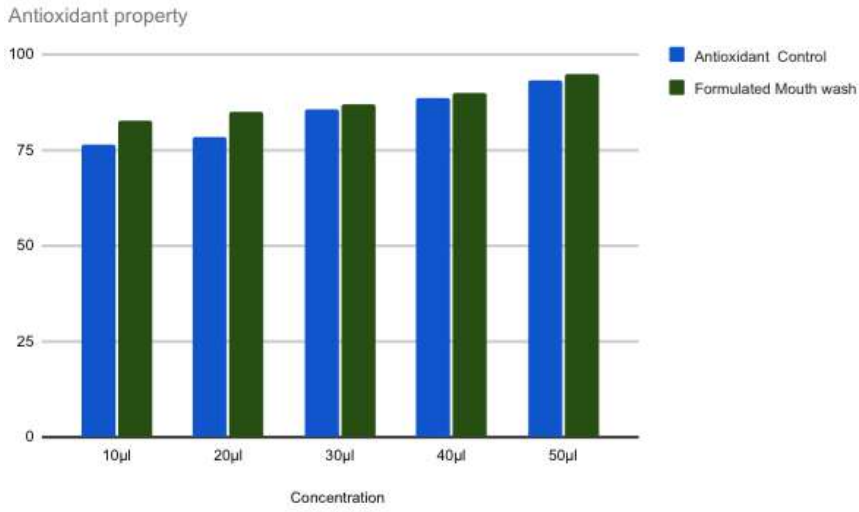
FIGURES:



Graph 1: Showing anti inflammatory properties of formulated mouthwash and standard control



Graph 2: Showing antioxidant properties of formulated mouthwash and standard control

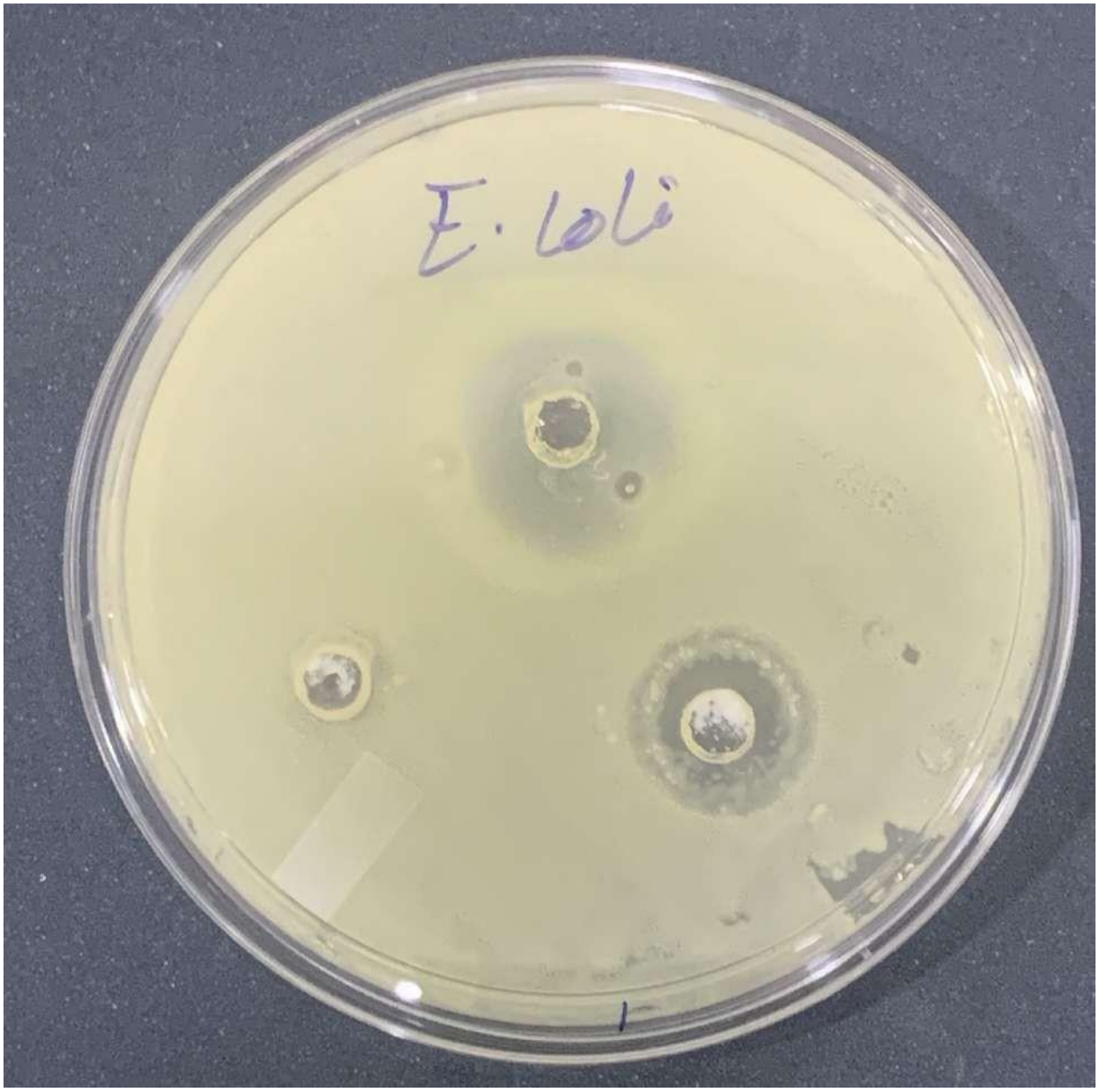


Figure 1: Zone of inhibition of formulated mouthwash against E. coli

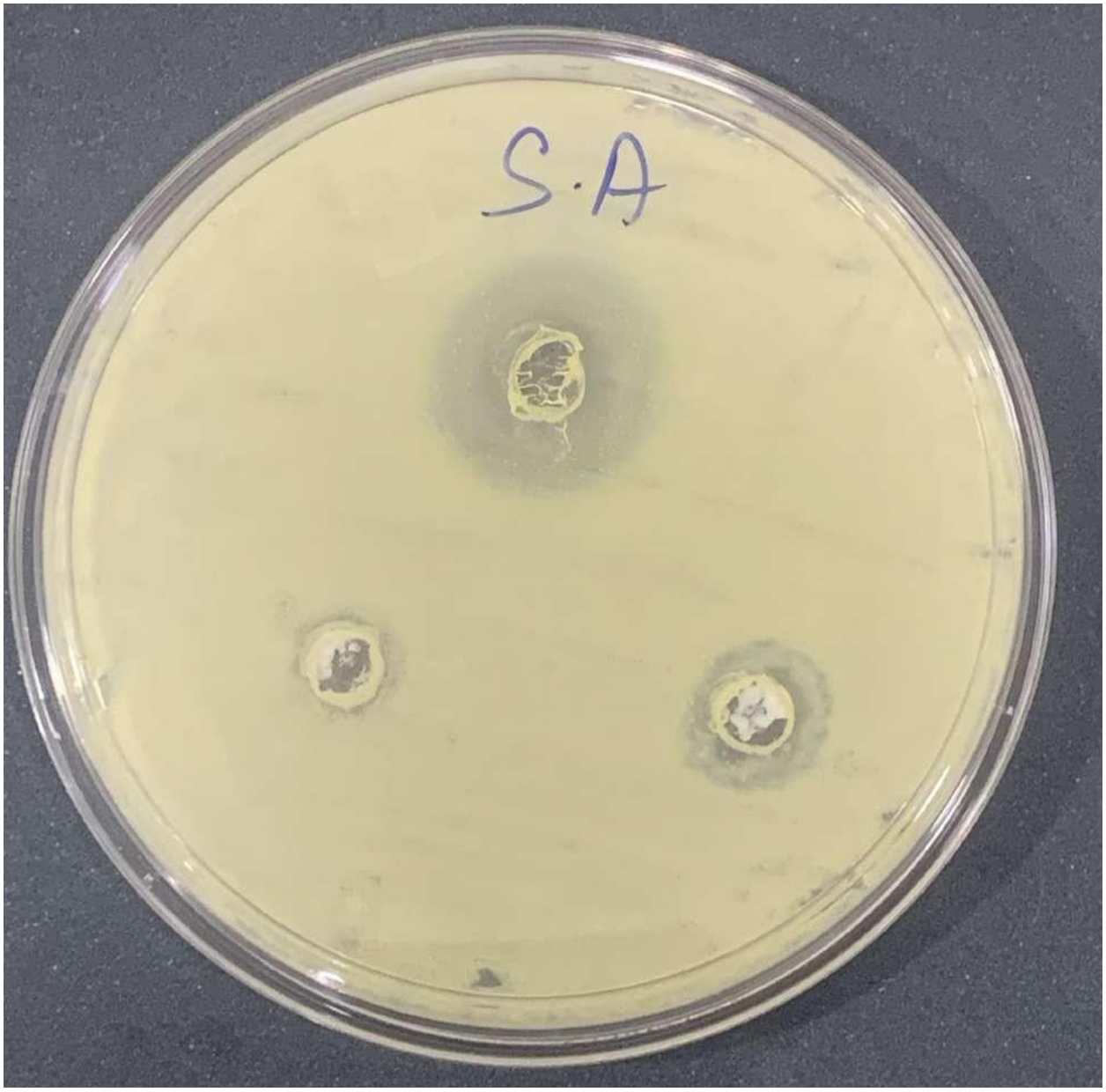


Figure 2: Zone of inhibition of formulated mouthwash against S. aureus.

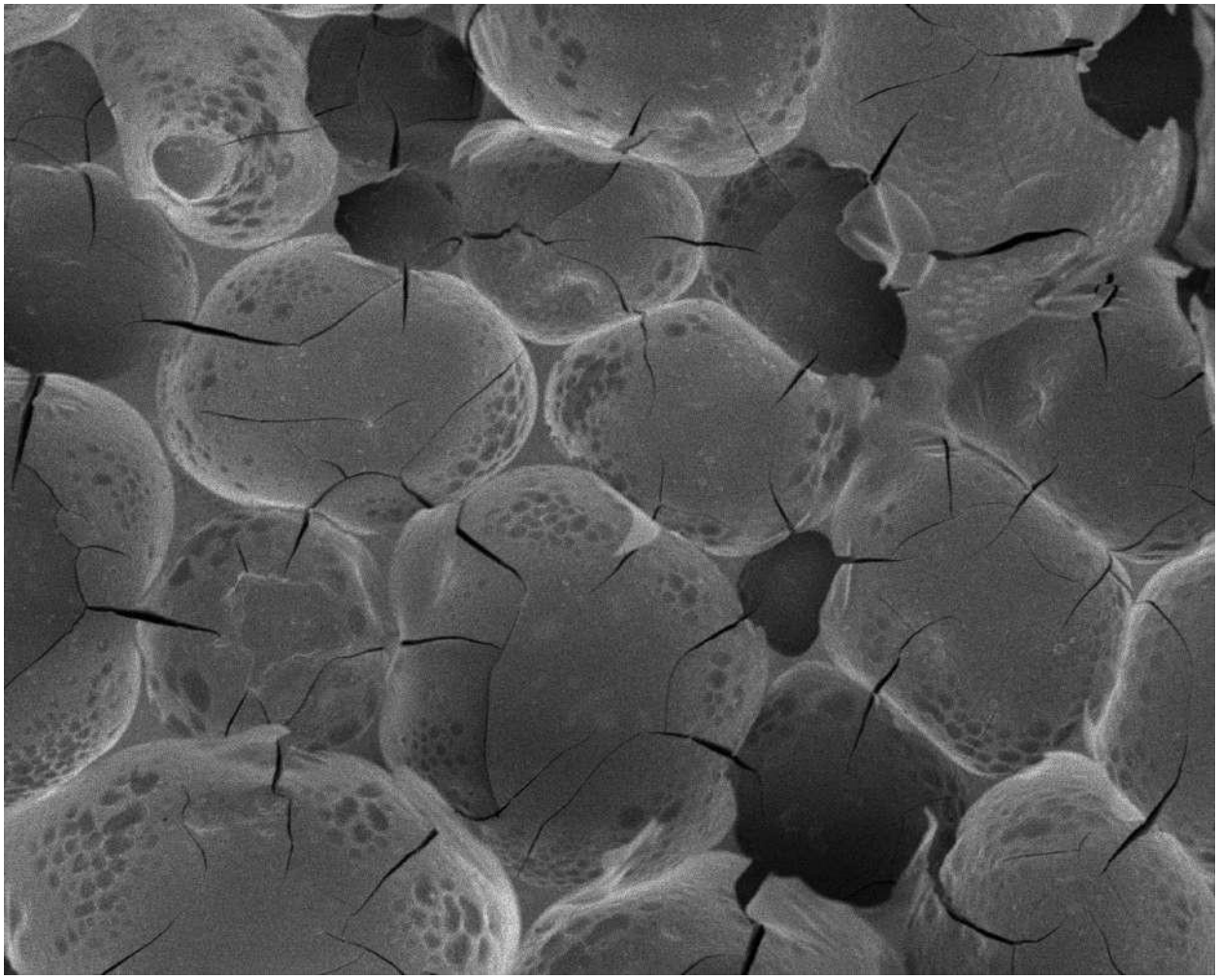


Figure 3: SEM showing uniform morphology

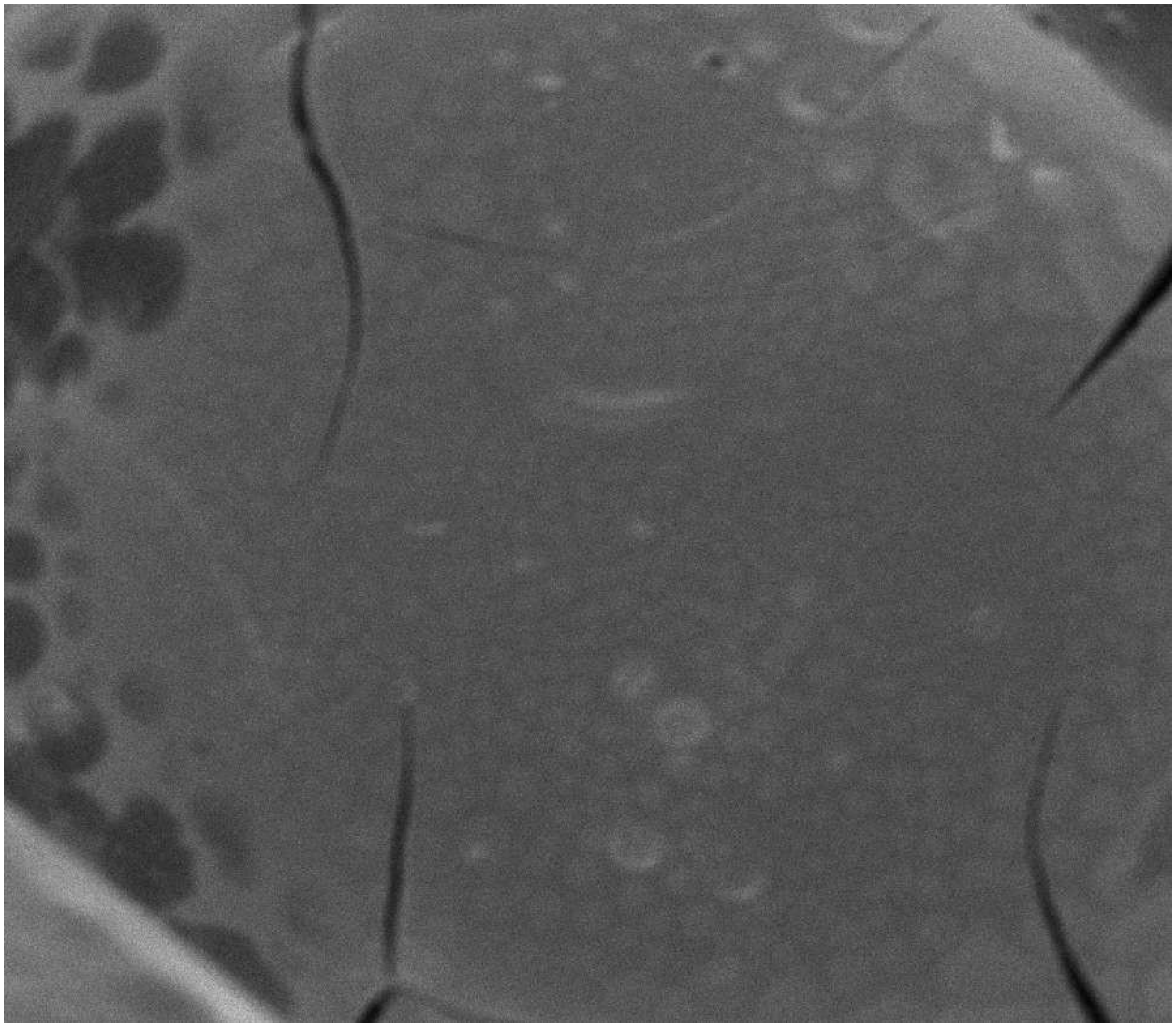


Figure 4: SEM showing even distribution of nano particles.

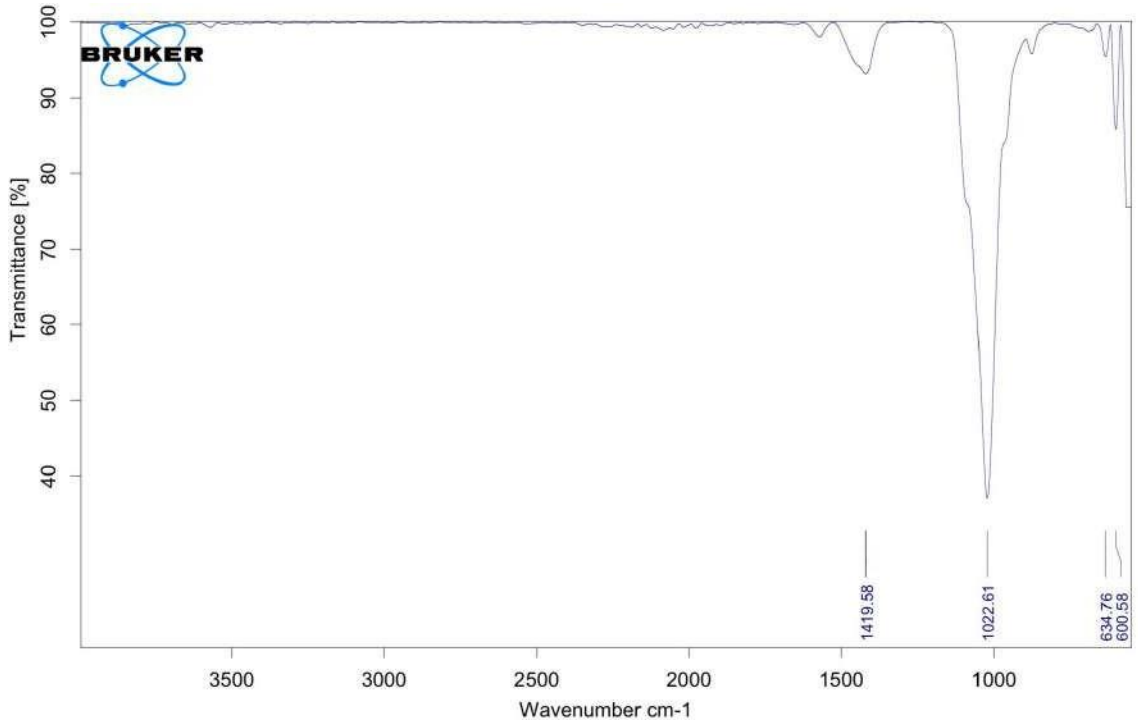


Figure 5: FTIR analysis of the formulated mouthwash

TABLES:

Anti inflammatory properties		
Control	**Marigold extract**	**Sig.**
10 µl	10 µl	0.004
20 µl	20 µl	0.032
30 µl	30 µl	0.012
40 µl	40 µl	0.043
50 µl	50 µl	0.026

Table 1: Paired t test Anti inflammatory properties

Antioxidant properties		
Control	**Marigold extract**	**Sig.**
10 µl	10 µl	0.000
20 µl	20 µl	0.043
30 µl	30 µl	0.047
40 µl	40 µl	0.008
50 µl	50 µl	0.039

Table 2: Paired t test antioxidant properties