"COMPARATIVE EVALUATION OF ANTIBACTERIAL, ANTI-INFLAMMATORY, ANTI-OXIDATIVE EFFICACY OF TISSUE CONDITIONER INCORPORATED WITH *PUNICA GRANATUM* EXTRACT IN DIFFERENT CONCENTRATIONS: AN IN-VITRO STUDY"

BY

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LIST OF ABBREVIATIONS USED IN THE STUDY

ABBREVATIONS	FULL FORMS
TCs	Tissue conditioners
Staph. aureus	Staphylococcus aureus
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
PPE	Pomegranate Peel Extract
C. albicans	Candida albicans
DPPH	2,2-diphenyl-1-picrylhydrazyl
BHI Agar	Brain heart infusion agar
DMEM	Dulbecco's modified eagle medium
DCFDA	7'-dichlorofluorescein diacetate (DCFDA)
MIC	Minimum Inhibitory Concentrations
MBC	Minimum Bactericidal Concentrations
MTT assay	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay
ELISA	Enzyme Linked Immunosorbent Assay
DMSO	Dimethyl sulfoxide
OD	Optical density
µg/ml	Microgram per millilitre
LPS	Lipopolysaccharide

ROS	Reactive oxygen species
DCF	2',7'-dichlorofluorescein
SA	*Staphylococcus aureus* bacteria
CFU	Colony forming units
ANOVA	Analysis of Variance
VIT C	Vitamin C

ABSTRACT

STATEMENT OF PROBLEM:

Obturators are widely used in prosthetic rehabilitation for maxillofacial defects. To lower the danger of contamination and infection, surgical obturators are first positioned after surgery. As healing advances, these are eventually replaced by temporary and permanent prosthesis. Infection, which is frequently caused due to pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa & Proteus mirabilis & Enterobacter spp*., can interfere with this process of healing. NSAIDs & corticosteroids are common anti-inflammatory drugs, however long-term use of these drugs can cause systemic toxicity. Tissue conditioners might exacerbate bacterial colonisation, which jeopardises recovery even more. Tissue conditioners are used for cushioning surgical obturators & protect healing tissues. Herbal remedies with established antibacterial and anti-inflammatory qualities have been suggested as an alternative to this issue. Rich in bioactive substances like tannins, flavonoids, & polyphenols, Punica granatum, has strong anti-inflammatory, antibacterial, and antioxidant properties. The purpose of this in vitro investigation is to assess the effectiveness of tissue conditioner supplemented with various concentrations of Punica granatum extract.

AIM:

To evaluate and compare the antimicrobial, anti-inflammatory and anti-oxidative efficacy of tissue conditioner incorporated with *Punica granatum* in different concentrations.

MATERIALS AND METHODS:

Pomegranate peel was shade-dried, powdered, and extracted with 70% ethanol, then concentrated using a rotary evaporator. Cytotoxicity, MBC, and MIC values were determined. Extract in concentrations of 30, 60, & 90 µg/mLwas added to tissue conditioner to prepare disc samples, which were then moulded in test discs with MIC, MBC, and cytotoxicity values in mind. Using S. aureus & colony count, the direct contact test was used to evaluate the antimicrobial efficacy. To measure nitric oxide inhibition, anti-inflammatory activity was evaluated upon LPS-stimulated RAW 264.7 cells with the Griess assay. GraphPad Prism was utilised to determine the IC50. Using the DCFDA test, which measures intracellular ROS by fluorescence intensity, antioxidant activity was determined.

RESULTS:

Based on MIC (7 µg/mL), MBC (15 µg/mL), and cytotoxicity (100 µg/mL) values, PPE was incorporated into soft liner discs at 30, 60, and 90 µg/mL. Anti-inflammatory activity, assessed by NO_2 inhibition, was highest at 30 and 60 µg/mL; 90 µg/mL matched NSAID effects. Anti-oxidative activity, measured via ROS inhibition, declined with concentration, with 90 µg/mL comparable to Vitamin C. Antibacterial efficacy, determined by CFU count, improved with increasing PPE concentration. The 90 µg/mL group showed CFU inhibition similar to Chloramphenicol. One-way ANOVA revealed significant differences across all groups ($p < 0.0001$). Tukey's post hoc test confirmed these differences. Overall, 90 µg/mL PPE showed strong anti-inflammatory, anti-oxidative, and antibacterial effects.

CONCLUSIONS

Given this study's limitations, PPE-incorporated tissue conditioners demonstrated potential biological activity. The determined concentrations (30, 60, and 90 μg/mL) appeared below the level of cytotoxicity threshold (100 μg/mL). As concentration of PPE is increased, CFU counts dramatically decreased, and PM90 (log CFU 4.23) exhibited antibacterial action comparable to that of chloramphenicol (log CFU 0). The anti-inflammatory effects of NO_2 inhibition were dose-dependent, with PM90 (54.71%) being on comparable levels with NSAID control (52.72%). PM90 (15.11%) was comparable vitamin C (12.91%), indicating antioxidant benefits associated with ROS suppression.

KEY WORDS: Maxillofacial prosthetics, Immediate surgical obturator, Tissue conditioner, Pomegranate peel extract, Antibacterial, Anti-inflammatory, Antioxidative

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INTRODUCTION

Maxillofacial deformities can be congenital, acquired, and developmental. Absence of hard palate in part or in full palate leads to impaired functioning such as deglutition, as well as articulation and airflow during speaking. Maxillo-facial prosthodontics is a branch of prosthetic dentistry which primarily specialises in rehabilitating congenital and acquired head and neck defects.[1]After surgery, patients require prosthetic rehabilitation to restore speech, mastication, swallowing, nutrition, or facial appearance. Maxillary defects, which arise from surgical treatments including the removal of cancer, cysts, or pathological disorders such osteomyelitis, create considerable problems in restoring oral aesthetics as well as function.

An obturator is a maxillofacial prosthesis used to close and maintain the integrity of altered oral and nasal compartments caused by such deformities. It is used to close, conceal or preserve the nasal and oral compartments caused by a congenital, acquired, or developmental process, defects such as cleft palate, cancer, palatal osteoradionecrosis.[2] It aids in maintaining the continuity of the orbit from the oral cavity, the maxillary sinus, and the hard palate from the nasal cavity. Obturators are classified as surgical, intermediate/interim, or definitive based on their treatment stage and healing procedure. Interim obturators are used throughout the post-surgical healing phase to keep function and aesthetics intact while facilitating tissue adaptation.

Definitive obturators are used after complete healing to assure long-term rehabilitation of function and aesthetics.

An immediate surgical obturator is placed during or just after surgery to replace all or part of the maxillary bones and adjoining alveolar tissues [3]. It offers a clean, stable, and anatomically precise scaffold to support the surgical dressing, promoting healing and shortening the length of hospital stay [4]. Because the patient can use the surgical obturator until the interim obturator is processed, it has a significant impact on their psychological well-being [5,6,7]. Speech articulation and other oral functions are significantly improved by immediate obturator restoration of the maxillectomy defect[8,9]. Also, it significantly improves patients' ability to carry out daily tasks, which enhances their overall quality of life.

Wound healing following such surgical procedures is a complex process involving a sequence of precisely coordinated biochemical & cellular actions. Haemostasis & inflammation, proliferation & remodelling are the distinct & predetermined stages of healing. Any disruption of these systems caused by a multitude of factors may result in slow or incomplete healing.

Postoperative wound infections usually involve potentially dangerous bacteria, including aerobic and anaerobic bacteria. Aerobic bacteria like the *Staphylococcus aureus* bacteria & *Proteus mirabilis*& *P. aeruginosa*, as well as Enterobacter spp. were shown to be the most common causes for wound infection following head and neck surgery.[4] NSAIDs are often used to treat inflammatory conditions. However, long-term use frequently results in toxic or adverse consequences on the liver, as well as the gastrointestinal system as well, cardiovascular system, as well as kidneys.

Tissue conditioners (TCs) have been manufactured from amorphous polymers. They are formed in situ using a mixture of polymer powder and liquid plasticiser. They are frequently utilised for enhancing the fit and functioning of a poorly fitting

denture before replacement. They can temporarily cure harmed mucosal tissues below ill-fitting acrylic dentures. When powder and liquid are mixed, the polymer chains entangle and create a cohesive gel with viscoelastic properties suitable for clinical applications.

The soft resilient nature of TCs on the acrylic denture surface facilitates a whole range of diagnostic and treatment modalities. They are used to restore the condition of inflamed denture bearing oral mucosa, and in taking functional impressions. They may also be used as provisional liners to improve the fit of the acrylic dentures, to prevent mechanical irritation from the denture plate, and for trial evaluation of border extensions. TCs may also be used to modify dentures during implant surgery and rehabilitate cancer patients requiring obturation. Their physical properties of TCs, such as viscoelastic properties and dimensional stability, vary depending on the materials used.

Tissue conditioners are utilised in a range of applications in prosthodontics. They are used as a cushion separating the denture-bearing mucosa along with immediate surgical obturators, for improving the fit of loose dentures, and to protect the mucosa from severe damage.[6] Tissue conditioner is used during the wound healing process and has been shown to predispose the area to accumulation of bacteria as a result of deterioration. As previously indicated, *Staphylococcus aureus* is closely related to tissue inflammation, which impairs wound healing. The use of herbal compounds to tissue conditioners has the aim to counterbalance this disadvantage or reduce the incidence of wound infection. Medicinal herbs are helpful because of their bactericidal actions, antiulcerogenic, healing of wounds, antibacterial, anti-inflammatory, and antioxidant properties, as well as their safety and cost.

Punica granatum L. is native to the Mediterranean region and has long been used in traditional medicine in the Indian subcontinent and other countries. The exterior layers (pericarp, rind, and hull) make up approximately sixty percent of the complete weight of pomegranate fruit. It contains tannins, flavonoids, alkaloids, & organic acids that offer various health advantages. Pomegranate peel contains higher levels of phenolic compounds and antioxidants. The peel contains anti-inflammatory effects due to the presence of gallagyldilacton, gallic acid, & granatin B, plus anti-oxidative capabilities due to the availability of punicalin, punicalagin & pedunculagin, gallic acid, & casuarinin. It also possesses anti-bacterial activities due to the inclusion of flavonoids, luteolin, and luteolin derivative 7-O-glucoside, and Naringin. [8]

Given these advantages and features, this study was done to assess the antibacterial, anti-inflammatory, & anti-oxidative effectiveness of tissue conditioners containing Punica granatum at different concentrations.

NEED FOR THE STUDY

In prosthodontics, tissue conditioners are used on the intaglio surface of denture to repair abused tissues, record functional impressions, and reline ill-fitting and immediate dentures. These materials contain amorphous polymers and a liquid plasticiser. Tissue conditioners believed to have desirable qualities such as low water absorption, enhanced colour stability, stain and tear resistance, good bond strength with denture base, dimensional stability, resistance to bacterial and fungal growth, ease of wound healing, ease of processing, good shelf life, biocompatibility, and low elastic modulus.

Maxillofacial obturators are used in the treatment of maxillofacial deformities arise due to congenital, developmental anomalies, or acquired defects. Obturators fall into three categories, definitive (permanent), intermediate, and surgical (immediate or temporary). They have been used at various stages of the healing process. After the surgical packing is removed, the surgical obturator is usually replaced with a transitional obturator 1-2 weeks later. The surgical obturator is put right after surgery to promote wound healing. This stage potentially lowers the risk of infection and helps minimise contamination of the surgical site. Postoperative wound infections contain anaerobic and aerobic microbes. Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, & Enterobacter species are the aerobic pathogens most frequently identified as the main causative pathogens. Despite the fact that corticosteroids as well as non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to reduce inflammation, prolonged use of these medications is linked to serious systemic toxicity, which may have an impact on the gastrointestinal tract, liver, kidneys, and heart. Also, these medications can cause drug resistance against the pathogens.

Pomegranate (Punica granatum L.), is used extensively in traditional medicine. The fruit's peel, which makes up over 60% of its weight, is full of polyphenols like organic acids, flavonoids, alkaloids, and tannins. These substances provide the peel strong antibacterial, anti-inflammatory, and antioxidant properties. Punicalagin, punicalin, & casuarinin promote its antioxidative activity, while gallagyldilacton, gallic acid, & granatin B are mainly responsible for its anti-inflammatory properties. Furthermore, flavonoids with powerful antibacterial properties as luteolin, quercetin, & naringin are present. [8]

In consideration of these qualities, the purpose of this in vitro investigation is to evaluate the antimicrobial, anti-inflammatory, and antioxidant efficacy of a tissue conditioner integrated with varying concentrations of Punica granatum peel extract.

HYPOTHESIS

NULL HYPOTHESIS:

- Addition of pomegranate extract in tissue conditioner does not show antimicrobial activity against staphylococcus aureus and does not have effect on anti-inflammatory and anti-oxidative efficacy.

RESEARCH HYPOTHESIS:

- Addition of pomegranate extract in tissue conditioner shows antimicrobial activity against staphylococcus aureus and have effect on anti-inflammatory and anti-oxidative efficacy.

AIMS AND OBJECTIVES

AIM OF THE STUDY

- To evaluate and compare the antimicrobial, anti-inflammatory and anti-oxidative efficacy of tissue conditioner incorporated with *Punica granatum* in different concentrations.

OBJECTIVES

- To assess antimicrobial efficacy of tissue conditioner after incorporating *Punica granatum* extract against staphylococcus aureus.
- To assess anti-inflammatory and anti-oxidative efficacy of tissue conditioner incorporated with *Punica granatum* extract.
- To compare antimicrobial, anti-inflammatory and anti-oxidative efficacy of tissue conditioner incorporated with different concentrations of *Punica granatum* extract.

REVIEW OF LITERATURE

1) **Dhamodharan Bakkiyaraj et al (2013)** evaluated the anti-biofilm activity of a pomegranate methanolic extract against bacterial and fungal pathogens. This study concluded that Pomegranate methanolic extract demonstrated to suppress biofilm formation by *Staphylococcus aureus, methicillin-resistant S. aureus, Escherichia coli, & Candida albicans*. In addition to decreasing biofilm formation, pomegranate extract destroyed pre-formed biofilms and decreased germ tube growth, a virulence feature in *C. albicans*.[10]

2) **Malviya (2014)** conducted an in-vitro study to evaluate antioxidant and antibacterial potential of pomegranate peel extracts. Pomegranate peels were subjected to extraction using different solvents viz. water, methanol and ethanol either alone or in combination with water. The extraction yield, antioxidant activity (DPPH and ABTS inhibition) and total phenolic contents were evaluated. Higher antioxidant activity and phenolic content were found in 100 % water and 70 % ethanol: 30 % water. The antibacterial activity was also found significant against various pathogenic strains. The maximum antibacterial activity was found against the *S. aureus* and minimum activity was against *K. pneumoniae.* [11]

3) **Elwej A et al (2016)** in their study evaluated the biochemical composition and protective effects of Pomegranate peel with hematotoxicity and genotoxicity caused by barium chloride (BaCl2) in adult rats. The study showed that barium therapy caused chromosomal abnormalities and the production of micronuclei in adult rats. Pomegranate peel reduces toxicity by scavenging free radicals & protecting DNA. Its antioxidant as well as antimutagenic properties suggested that it could be used to prevent disease.[12]

4) **Selvan k (2018)** conducted a study involving a comparative clinical and biochemical evaluation of punicalagin loaded gel along with Scaling and Root Planning (SRP) and SRP alone in patients with Chronic Periodontitis. The results showed that punicalagin has the potential to serve as a therapeutic agent to treat chronic periodontitis patients. Compared to Scaling and Root Planning alone, adjunctive use of punicalagin showed greater probing Pocket Depth reduction, Clinical Attachment Level gain and favorable changes in anti-inflammatory and anti-oxidant markers. [13]

5) **Chincholikar et al (2019)** concluded in their study that Fluconazole demonstrated a considerably better absorption profile than neem. Fluconazole had a considerably greater mean zone of inhibition compared to neem, indicating superior antifungal activity against *Candida albicans* in vitro. The permanent silicone tissue conditioner released the most antifungal agents, followed by auto polymerising acrylic resin and heat polymerising acrylic resin in decreasing order. The permanent silicone tissue conditioner was found to be the most effective polymeric system for sustained antifungal agent release, with higher mean zones of inhibition and higher elution (up to 21 days).[14]

6) **Vildan Celiksoy (2020)** conducted as in-vitro study to evaluate oral wound healing effects of pomegranate (punica granatum) rind extract and punicalagin, in combination with Zn (ii). This study evaluated the antioxidant and in vitro gingival wound healing effects of pomegranate rind extract (PRE) and punicalagin, alone and in combination with Zn (II). Punicalagin demonstrated superior antioxidant capacities to PRE, although Zn (II) exerted no additional influences. PRE, punicalagin and Zn (II) reduced gingival fibroblast viability and migration at high concentrations, but retained viability at lower concentrations without Zn (II). Fibroblast speed and distance travelled during

migration were also enhanced by punicalagin with Zn (II) at low concentrations. Therefore, punicalagin in combination with Zn (II) may promote certain anti-inflammatory and fibroblast responses to aid oral healing.[15]

7) **Margita Belusic-Gobic (2020)** conducted an in vivo study to investigate the most common infection pathogen found in the postoperative wounds, following surgical treatment of oral and oropharyngeal cancer, in order to identify the most suitable antibiotic treatment. The patients were divided into two groups: 1) Patients with no postoperative wound infection (n = 80); 2) Patients with postoperative wound infection (n = 115). In those 115 patients they have identified 11 different bacterial species in 3 different types of wounds. The most common bacteria, at all three sites, was *Enterobacteriaceae* (G-), found in the wound on the neck in (32/115 patients), in the cultures taken from the cannula (61/115 patients) and in the wound in the oral cavity (32/115 patients). Other frequently isolated pathogenic bacteria were *Staphylococcus spp. (G+), Pseudomonas aeruginosa (G-), Corynebacterium spp. (G+) and Acinetobacter baumanii (G-).* [16]

8) **Abutayeh RF et (2020)** conducted a study utilising Jordanian pomegranate to investigate the antimicrobial efficacy of various pomegranate peel extracts individually as well as in combination of antibacterial treatments against four bacterial strains. In conclusion author stated that pomegranate peels, an entirely natural and harmless byproduct, show intriguing antibacterial properties. Furthermore, mixing PPEs with conventional antibiotics indicates promise in combating antibiotic resistance, indicating their potential use in infectious illness treatment.[17]

9) **Reham M Abdallah (2021)** conducted study to assessed the antifungal efficacy of a tissue conditioner/curcumin formulation based on surface roughness and tensile binding strength to the denture base. Surface roughness decreased significantly when the tissue conditioner was treated with 20 v/v% curcumin compared to the unmodified liner.

   Curcumin at 10 and 20 v/v% dramatically improved denture liner strength and antifungal activity. Curcumin has the properties as a natural powerful antifungal agent against oral candidiasis when used with denture tissue conditioners. Furthermore, it has been shown to reduce surface roughness and improve Tissue conditioner adhesion to denture bases.[18]

10) **Monisha Singhal(2021)** evaluated effectiveness of silver nanoparticles biosynthesised with Punica granatum leaves against bacteria and biofilms, The study concluded that silver nanoparticles (AgNPs) were biosynthesized using Punica granatum leaf extract under optimized conditions (31.4 °C, 1.5 mM $AgNO_3$, 55.55 µL extract, 15 min reaction). Characterization (SEM, DLS) confirmed spherical nanoparticles (~37.5 nm) with a zeta potential of −34 mV. AgNPs exhibited antibacterial efficacy against Gram-positive and Gram-negative bacteria, with inhibition zones of 13–14 mm at 200 µg/mL. Antibiofilm assays showed increased inhibition with concentrations from 12.5 to 100 µg/mL. FT-IR and SEM analyses revealed bacterial membrane disruption, highlighting PGL-AgNPs as potential antibacterial and antibiofilm agents.[19]

11) **Nichakorn Songsang (2022)** evaluated antibacterial qualities, cell cytotoxicity, and surface hardness of soft lining materials (GC tissue conditioner, Viscogel, and Coe comfort) were investigated with different concentrations of Litsea cubeba essential oil (LCEO). Based on this in vitro investigation, LCEO could be employed as a novel antibacterial additive to soft lining materials to combat oral pathogens, with an appropriate concentration of 10% v/v for C. albicans & 30% v/v for S. mutans. When in comparison with materials without additives, soft lining materials containing LCEO showed no cytotoxicity to the HGF cell line.[20]

12) **Cindy Ruiz Garcia et al (2022)** in their study on the effect of surface modification for denture liners with Equisetum giganteum and Punica granatum on the Candida albicans biofilm inhibition was explored, with the goal of using it as a sustained-release therapeutical delivery method for Candida-associated denture stomatitis. The study concluded that after 14 days, the efficacy of Equisetum giganteum and Punica granatum was comparable to that of nystatin (nearly 100% suppression). The proposed strategy is a promising alternative to allopathic medications in Candida-associated denture stomatitis treatment.[21]

13) **A Faisal Madhloom et al (2022)** Conducted a study was to assess the antibacterial activity of red pomegranate extracts & M. oleifera L. against *Porphyromonas gingivalis*. Superior antibacterial and anti-biofilm properties against *P. gingivalis* were demonstrated by the combination of red pomegranate albedo & M. oleifera L. seeds, followed by red pomegranate albedo & M. oleifera L. seeds. This might point to a viable substitute for conventional chemicals that can be applied as a supplement to treat periodontal disorders.[22]

14) **Eman M AL Hamdan (2022)** investigated how photodynamic therapy (PDT), chemical disinfectants, and herbal disinfectants affected the adherence of

bacteria that cause infections, including E. coli, Candida albicans, S. aureus, & S. mutans, to soft denture liners. On a denture Tissue conditioner, the study found that 0.12% CHX and TTO had improved antibacterial action in reducing adherent bacterial colonies including E. coli, C. albicans, S. aureus, and S. mutans (CFU)/mL.[23]

15) **Sri et al (2023)** conducted study to examine determine the anti-inflammatory and antimicrobial activity of *Glycyrrhiza glabra* incorporated in a soft-liner that can be used as denture relining material. A statistically significant antibacterial effect was observed ($P < 0.05$). The highest observed concentration of *G. glabra* exhibited an anti-inflammatory activity of 81%, with no significant alteration to its physical or mechanical properties ($P > 0.05$). The incorporation of *Glycyrrhiza glabra* into soft-liner materials presented a promising alternative to conventional tissue conditioners.[24]

16) **Gülten Ökmen et al (2023**) investigated in vitro antioxidant and antibacterial activities of pomegranate flower extracts on different *Staphylococcus* species linked with bovine mastitis. The study concluded that the methanol extract had the largest inhibitory zones against coagulase-negative *Staphylococcus-37 (CNS-37) & S. aureus-18.* The lowest MIC value was 6500 μg/mL. The strongest antioxidant activity was found in methanol extracts. As a result, pomegranate flower extracts showed strong antioxidant and antibacterial activity against the mastitis pathogens tested.[25]

17) **Amine Trabelsi(2023)** conducted a study and the aim was to evaluate the phytochemical composition, antibacterial properties, and antibiotic-modifying potential of Punica granatum leaf extracts within hexane, ethyl acetate, ethanol, as well as aqueous solvents. The best synergistic interaction against penicillin-resistant S. aureus and E. coli was achieved when TOF extract and amoxicillin

were combined. The tannins, flavonoids, & phenolic acids found in the leaves of P. granatum are responsible for these results. Extracts from pomegranate leaves or active compounds made from them can be used to stop resistant bacterial strains from emerging and spreading. [26]

18) **Acharya A et al (2024)** conducted study which compared maxillofacial obturators lined using a soft-liner with respect to of masticatory efficiency, speech characteristics, as well as quality of life of patients having maxillofacial abnormalities to routinely used obturators. The study concluded whether soft-liner relined obturators outperform traditional obturators in terms of masticatory performance & speech results in patients with craniofacial abnormalities. These findings indicated that using soft-liner relined obturators might be a more successful choice for rehabilitating individuals with craniofacial abnormalities, increasing both functional & aesthetic outcomes. Participants with soft-liner relined obturators had greater quality of life scores, but their difference was not statistically significant. Further research with sample volumes and extended follow-up times are required to corroborate these findings. [27]

19) **El Demerdash FM et al (2024)** investigated hepatoprotective effect of Ethanolic Pomegranate Peel Extract against Levofloxacin by suppressing oxidative stress, inflammation, as well as apoptosis in male rats. In conclusion author stated that PGPE has substantial anti-inflammatory, antiapoptotic, as well as antioxidant capabilities that protect rat livers from the harmful effects of LEV and provide a new perspective on the use of fruit waste products[28].

20) **Alessia Silla et. Al (2025)** investigated the biological properties of tomato skin (HP) & pomegranate peel extracts onto oral mucosa to determine their potential usage in mouthwashes. This study concluded that PPE & HP emerge as potential alternatives as antioxidant-rich dental products, in accordance with the

circular economy structure. These extracts' double benefits presented by promoting good oral hygiene with promoting sustainable practices and present an intriguing opportunity for research and development. By tackling the current difficulties, PPE and HP have the potential to enable an entirely fresh wave of beneficial, eco-friendly dental solutions that benefit the oral health and the environment. [29]

21) **Ayala-Flores F et al (2025)** evaluated the antioxidant & antibacterial activities with pomegranate peel extracts taken from Wonderful & Valenciana types. The study concluded that the utilisation of plant sources for extracting of compounds that enhance human health has become scientifically relevant over the past few years; thus, in this study, the researchers utilised pomegranate peels along with two pomegranate different types to extract polyphenolic compounds that demonstrated antioxidant & antibacterial activity. The Valenciana variety appeared to be the most suitable for this application. The inhibition of different microbial strains resulted in a substantially bigger diameter inhibitory halo, and total phenolic compounds as well as flavonoids showed a high association.[30]

MATERIALS & METHODOLOGY

SOURCE ABOUT DATA:

Using a procured RAW 264.7 cell line, this in vitro study was carried out at the KAHER KLE VKIDS Department of Prosthodontics and Crown & Bridge as well, KAHER's Shri BM Kankanawadi Ayurveda Mahavidyalaya, Post Graduate Studies and Research Centre Belagavi, KAHER's Dr Prabhakar Kore's Basic Science Research Centre and National Centre for Cell Science Pune.

This research was aimed to evaluate and compare the antimicrobial, anti-inflammatory and anti-oxidative efficacy of tissue conditioner incorporated with Punica granatum in different concentrations.

SAMPLE SIZE ESTIMATION:

At 95% confidence and 80% power, the sample size was estimated to be

- Sample size for each subgroup = 7
- 6 sub-groups for each parameter. 3 parameters are taken. (Antibacterial activity, Antioxidant and Anti-inflammatory activity)
- Total sub-groups=18
- Total Sample Size = 18*7=**126**

INCLUSION CRITERIA:

- Pomegranate peel extract authenticated by KLE college of Ayurveda
- Discs made (6 mm diameter and 1 mm thickness) of tissue conditioner and ethanolic Punica granatum peel extract in 3 different concentrations

EXCLUSION CRITERIA:

- Discs with inaccurate size, shape and dimension (more than 6 mm diameter and 1 mm thickness)

MATERIALS AND ARMAMENTARIUM:

Materials:

- *Punica granatum* peel extract
- GC tissue conditioner
- S. aureus (ATCC 23235)
- BHI Agar
- RAW 2 6 4.7(Murine macrophages)
- Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Thermofisher Scientific)- (Cat No-11965-092)
- Antimycotic 100X solution (Thermofisher Scientific)-Cat No-15240062
- The Dulbecco's Modified Eagle's Medium

  Fetal bovine serum FBS (Cat No. -10270106, Gibco, Invitrogen)
- 4.5 mg/mL l-glutamine,
- 100 units/mL penicillin,
- 100 µg/mL streptomycin
- 4.5 mg/mL glucose solution
- 2',7'-dichlorofluorescein diacetate (DCFDA)
- Griess reagent
- mouse fibroblast cells (L929)
- Chloramphenicol
- Nimesulide
- Vit C Tablet

Armamentarium:

- Incubator
- Weighing machine
- Laminar air flow
- Aerobic Jar
- Microtiter Plate Reader
- Fluorescence microplate reader
- Spectrophotometer

METHODOLOGY FLOW CHART

Preparation of ethanolic Punica granatum peel extract

↓

Preparation of mould for sample fabrication

↓

Determination of MIC, MBC, Cytotoxicity of ethanolic Punica granatum peel extract

↓

Determination of 3 concentrations of ethanolic Punica granatum peel extract referring MIC, MBC, Cytotoxicity values

↓

Fabrication of disc sample in already prepared mould for test and control group

↓

FOR CONTROL SAMPLES

Mixing tissue conditioner with

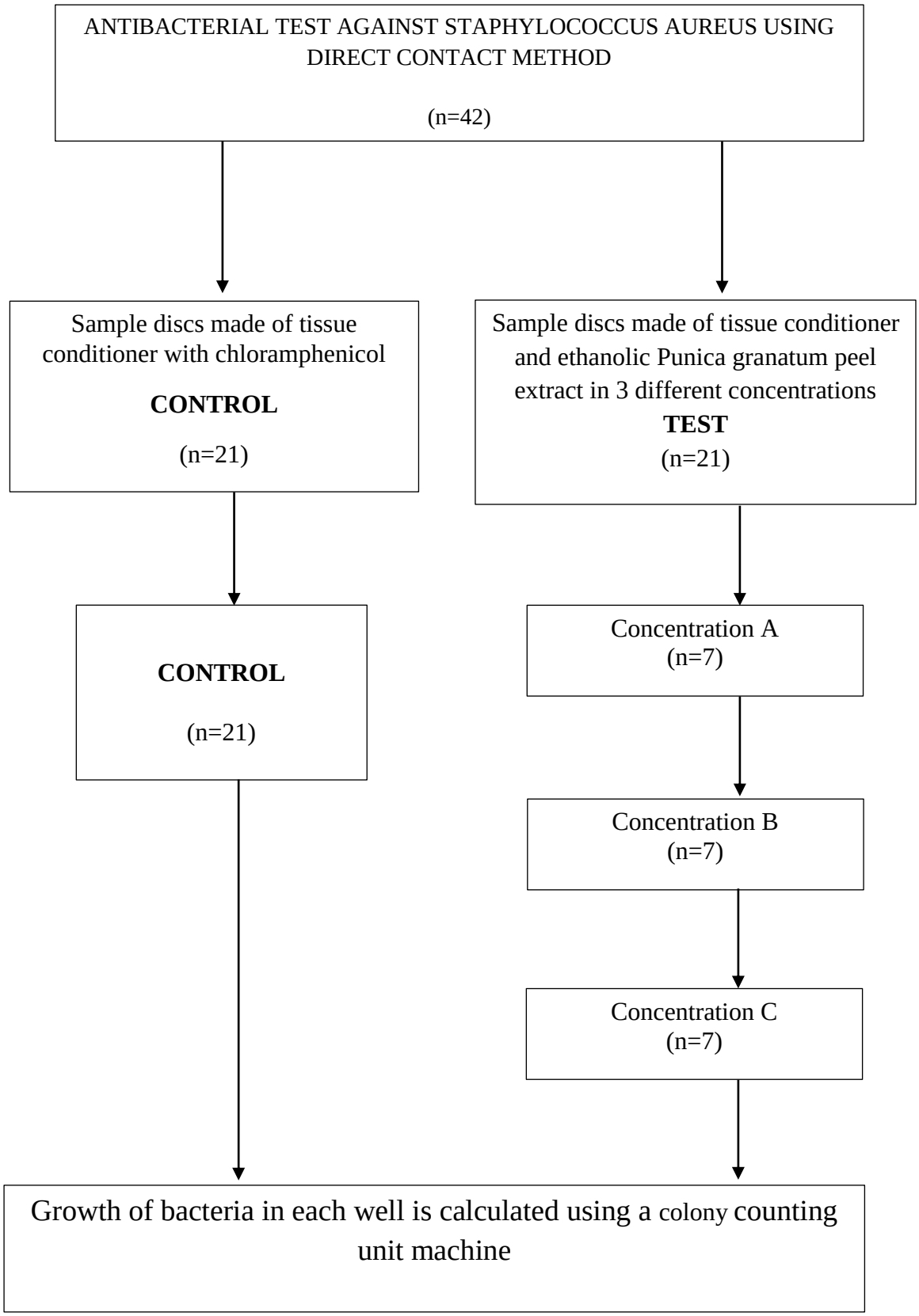
1)Chloramphenicol

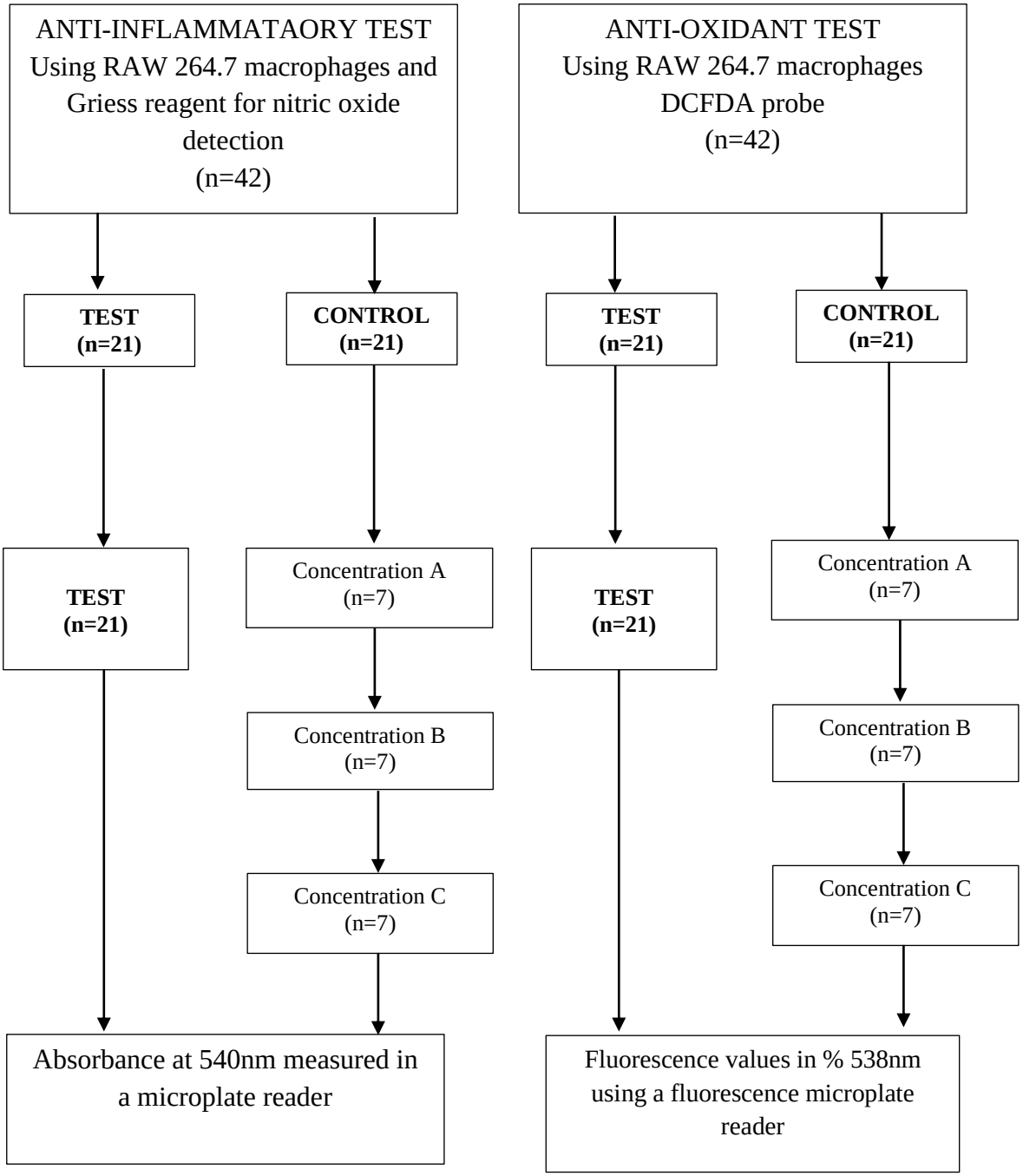
2) NSAID

3)Vit. C

FOR TEST SAMPLES

Mixing tissue conditioner and ethanolic Punica granatum peel extract in 3 different concentrations


ANTIBACTERIAL TEST AGAINST STAPHYLOCOCCUS AUREUS USING DIRECT CONTACT METHOD
(n=42)
Sample discs made of tissue conditioner with chloramphenicol
CONTROL
(n=21)
Sample discs made of tissue conditioner and ethanolic Punica granatum peel extract in 3 different concentrations
TEST
(n=21)
CONTROL
(n=21)
Concentration A
(n=7)
Concentration B
(n=7)
Concentration C
(n=7)
Growth of bacteria in each well is calculated using a colony counting unit machine


ANTI-INFLAMMATAORY TEST
Using RAW 264.7 macrophages and Griess reagent for nitric oxide detection
(n=42)
ANTI-OXIDANT TEST
Using RAW 264.7 macrophages
DCFDA probe
(n=42)
TEST
(n=21)
CONTROL
(n=21)
TEST
(n=21)
CONTROL
(n=21)
TEST
(n=21)
Concentration A
(n=7)
TEST
(n=21)
Concentration A
(n=7)
Concentration B
(n=7)
Concentration B
(n=7)
Concentration C
(n=7)
Concentration C
(n=7)
Absorbance at 540nm measured in a microplate reader
Fluorescence values in % 538nm using a fluorescence microplate reader

METHODOLOGY

Preparation of ethanolic extract pomegranate peel-

Fresh pomegranate fruits verified through KLE College of Ayurveda were bought at a nearby market. They were ground into a powder using a grinder after being allowed to air dry at room temperature. 200 g of each powder was left to soak in a solution of 70% ethanol (1:10 ratio) within a sealed container and agitated for 24 hours in a dark room to create the extract. A rotary machine was used to concentrate the extracts under vacuum at 40°C after they had been filtered using the Whatman. No. 41 filter paper.

Determination of Minimum Inhibitory Concentrations

A minimum inhibitory concentration analysis was carried out according to standard protocol. After being resurrected by plating on Brain Heart Infusion Agar, Staphylococcus aureus was cultured in an aerobic room. After being moved into sterilised Brain Heart Infusion broth, isolated colonies were cultured aerobically for 24 hours at 37 ^{0}C.

The growth concentration was altered using 0.5 McFarland's turbidity standards to 105 organisms/ml. One gram of pomegranate extract was dissolved in 100 millilitres of solvent (1 percent DMSO and 99 percent distilled water) to create a stock solution. In six tubes of Minimum Inhibitory Concentration, 1 millilitre of the Brain Heart Infusion broth was introduced. One millilitre of extract (stock solution) was introduced to the initial Minimum Inhibitory Concentration the tube that held one millilitre of broth.

One millilitre was added to a second lowest inhibitory concentration tube after thoroughly mixing. This went on until the sixth tube. The level of concentration in the extract of pomegranates was obtained by serial dilution. 50 µl of previously produced S. Aureus strains were then added to each test tube. After that, the tubes will be aerobically incubated over 24 hours at 37°C. After that, the turbidity and the control will be contrasted. Growth of S. Aureus is shown by turbidity inside the Minimum Inhibitory Concentration tube, suggesting that the bacteria proved resistive to pomegranate extract.

Determination of Minimum Bactericidal Concentrations-

The MIC dilution tubes having no obvious development (no turbidity) & the control tube was sub cultured onto BHI agar, incubated with 24 hours at 37.0C in an aerobic chamber, and the colonies that resulted were counted in order to calculate the bactericidal concentration. The test group's colony numbers were compared to those of the control group.

The following was the interpretation of the test: A smaller number of colonies suggests a partial or moderate bactericidal activity, whereas an equivalent number of colonies shows merely bacteriostatic activity. A full bactericidal effect is indicated if no growth is seen.

Determination of Cytotoxicity -

The Cytotoxicity Assay was conducted using mouse fibroblast stain L929. The MTT solution was made using 5 mg of MTT reagent within 1 ml with Phosphate Buffer Saline (PBS, pH 7.4). By measuring the conversion of MTT into "Formazan blue" by living cells, the ELISA reader evaluated the in vitro growth suppression

impact of pomegranate extract. In a 96-well micro titre plate, 50 µl of a 4,000 cells/ml suspension with cells was put in in each well. DMEM (Dulbecco's Modified Eagle Medium) fluid was added to bring the final volume up to 150 µl. At a dosage of 10% DMEM media, pomegranate extract was diluted. After adding 100µl of each pomegranate extract to each well, the wells were incubated during 24 hours with 5% CO_2, in incubator.

Twenty microliters of 5 mg/ml MTT reagent were added to each well after a 24-hour period. The plate was then incubated at room temperature for four hours in a dark environment. Because the MTT reagent is photosensitive, aluminum foil was placed over the plate.

After carefully removing the supernatant without disturbing the Formazan crystals that had precipitated, 100 µl of DMSO was then added for dissolving the crystals.

At a wavelength of 570 nm, the optical density, or OD, was evaluated. Three duplicates of the study were conducted. The average of three readings is represented by the result.

Formula**:**

$$\text{Surviving cells (percentage)} = \frac{\text{Test compound's Mean OD} \times 100}{\text{Control's (untreated cells) Mean OD}}$$

Fabrication of disc samples using mould-

Ethanolic extract of pomegranate peel in 3 different concentrations that was 30 µg/mL,60 µg/Ml and 90 µg/mL (referring MIC, MBC, Cytotoxicity values, 7 µg/ml,15µg/ml and 100 µg/ml respectively) with GC tissue conditioner monomer was mixed with monomer of tissue conditioner according to manufacturer's instructions. Control agents (chloramphenicol, AG Plus) was added to tissue conditioner to make a homogeneous mixture. Mixture was poured into mould to prepare both test and control disc samples.

Microbial analysis against Staphylococcus aureus

Direct contact test-

Both control and test samples discs were added in 24 well plate. 10 µg of 0.5 Mc Farland standard bacteria, *Staphylococcus aureus* (ATCC 23235) was added on top of each disc for 20 mins. Afterward, each well received 1 millilitre of sterile BHI broth. Loop full of broth was taken from each well and streaked on BHI Agar Plates. After 24 hours results were checked using colony counting unit machine.

Anti-inflammatory test in response to lipopolysaccharide (LPS) stimulated-RAW 264.7 cells-

The 26 4.7 RAW cells into a 96-well flat-bottom microplate, 4.7 cells were seeded at an average density of roughly 5×103 cells/well. They were then kept at 37 ^{0}C for the entire night with 95% humidity and 5% CO_2. Different ethanolic pomegranate peel extract concentrations (90, 60, and 30 µg/mL) were treated, along with a positive control concentration of 5 µg/mL. A further hour was spent incubating the cells. LPS (0.5 mg/mL) was then used to stimulate the cells

for 24 hours while maintaining the same conditions. The same volume of media was placed in the microplate well as a negative control. After measuring the nitrite concentration in the cell culture supernatant, 100 ml of it was combined with a similar volume of the Griess reagent and allowed to sit at room temperature for 10 minutes. A microplate reader was used to detect absorbance at 540 nm.

Formula:

NO inhibition (%) = Test compound means OD /negative control mean OD × 100

Graph Pad Prism Version 5.1 is used to determine a compound's IC 50.

Anti-oxidant activity test-

The measurement of reactive oxygen species (ROS) using DCFDA relies on a simple yet effective principle. DCFDA, or 2',7'-dichlorofluorescein diacetate, is a non-fluorescent molecule that readily enters cells. Once inside, cellular esterases remove the acetate groups, transforming it into DCFH, or 2',7'-dichlorodihydrofluorescein. This DCFH then acts as a substrate for ROS. When ROS, primarily hydrogen peroxide (H_2O_2) and other reactive species, are present, they oxidize DCFH, converting it into DCF, or 2',7'-dichlorofluorescein. This oxidized form is highly fluorescent, and the intensity of this fluorescence directly corresponds to the amount of ROS within the cell. Essentially, the brighter the fluorescence, the higher the concentration of ROS.

A 96-well flat-bottom microplate was used to seed 26 4.7 RAW cells at a density of roughly 5×103 cells/well, and the cells were kept at 37 ^{0}C with 95% humidity and 5% CO_2 for the entire night. After treating the cells with varying concentrations of ethanol-based pomegranate ethanolic extract (90, 60, and 30

µg/mL) and a positive control (5 µg/mL), the diluted DCFDA working solution was added to the cells, permitting the cell-permeable dye to get inside. The incubation period and DCFDA concentration were then adjusted according to the particular cell type and experimental conditions, and a critical washing step was carried out to remove any remained extracellular DCFDA, minimising background fluorescence, respectively the cells incubated for an additional hour, and the negative control used the same amount of medium in the microplate well.

To test nitrite concentration in cell culture supernatant, 100ml was combined using the same amount of Griess reagent for 10 minutes at room temperature. The absorption value (540 nm) was measured utilising a microplate reader.

FIGURES

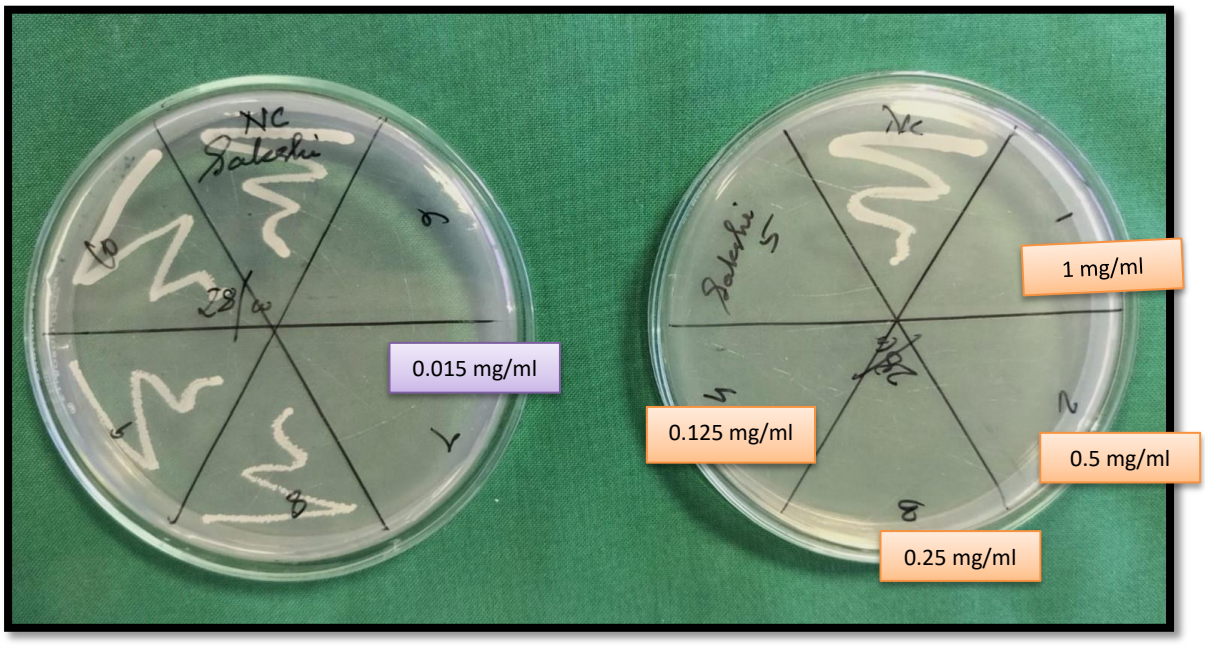


Figure 1: MBC Results (Bacterial growth is absent at a conc. of 15µg/ml hence it is selected as MBC value for deciding 3 concentrations for disc fabrication)

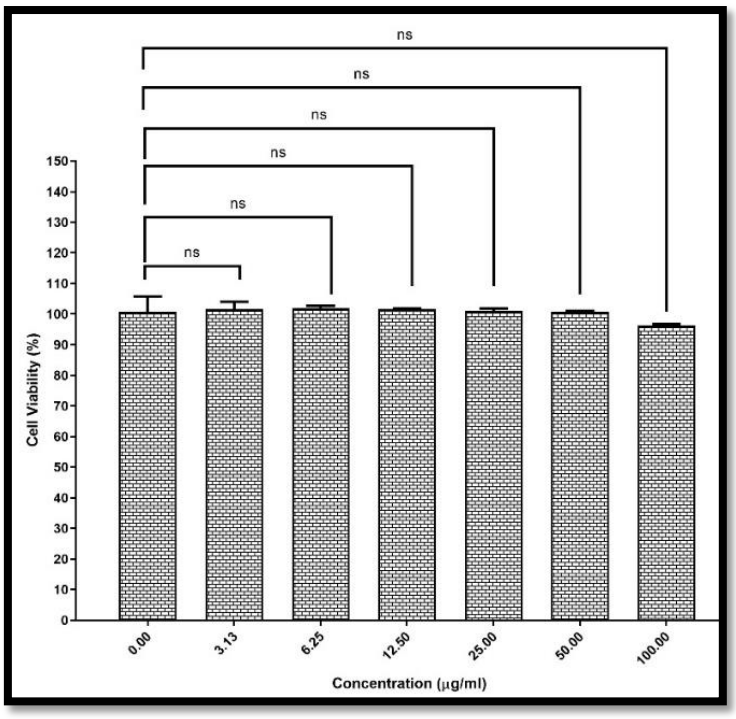


Figure 2: Cytotoxicity Graph

Figure 3 :GC Tissue Conditioner

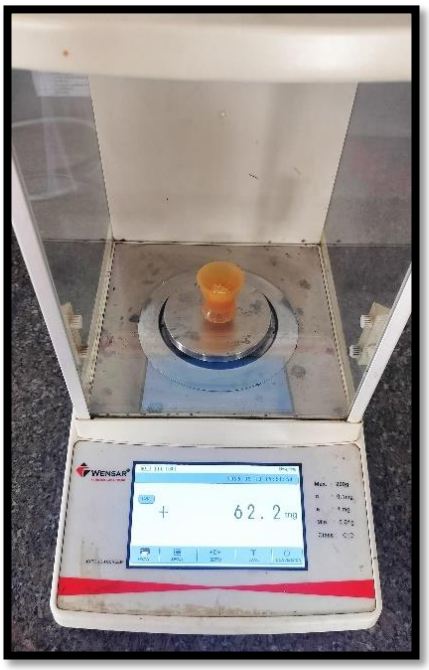


Figure 4: Weighing Machine

Figure 5: Incubator

Figure 6: Control Discs

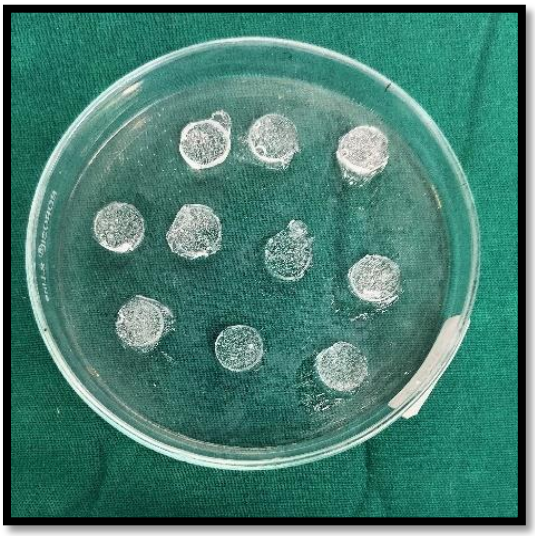


Figure 7: Plain Discs

Figure 8 : Discs In 3 Different Concentrations Reffering Mic, Mbc, Cytotoxicity Values (30 µg/ millilitres, 60 µg/ millilitres, 90 µg/ millilitres)

Figures 9: Anti-Inflammatory Control (NSAIDS)

Figures 10: anti-oxidative control

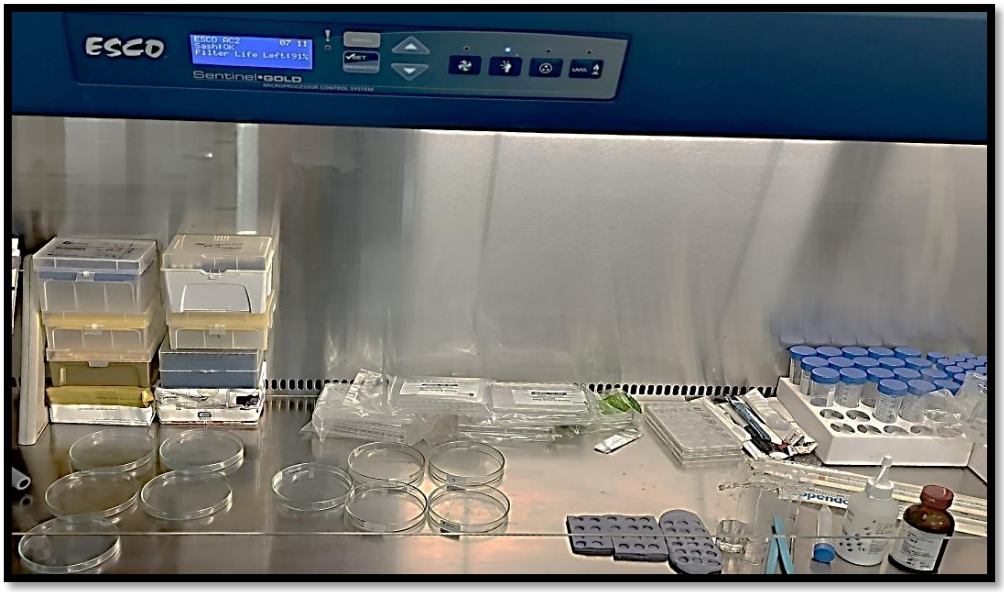


Figure 11: Fabrication of Discs Samples Under Sterile Environment

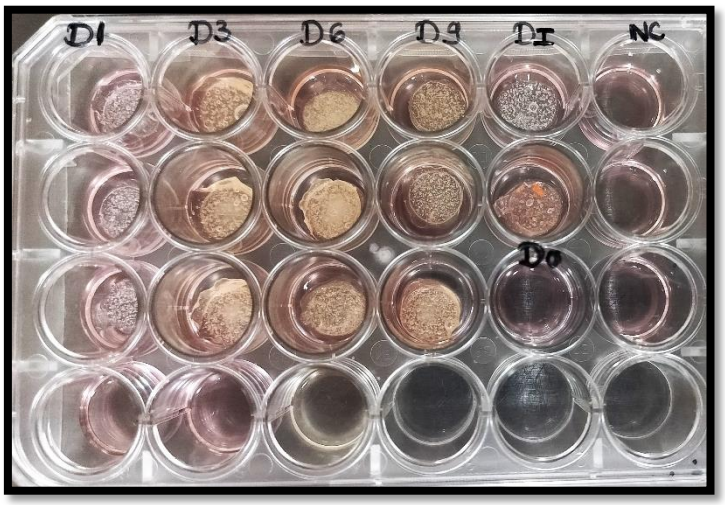


Figure 13: Anti-Inflammatory Test

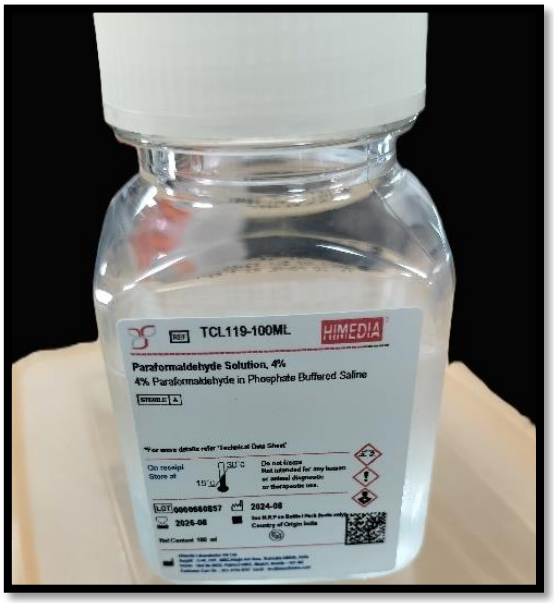
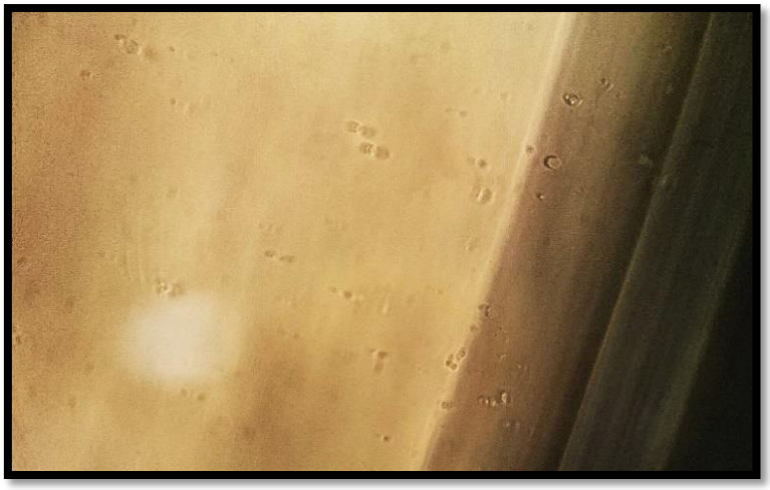
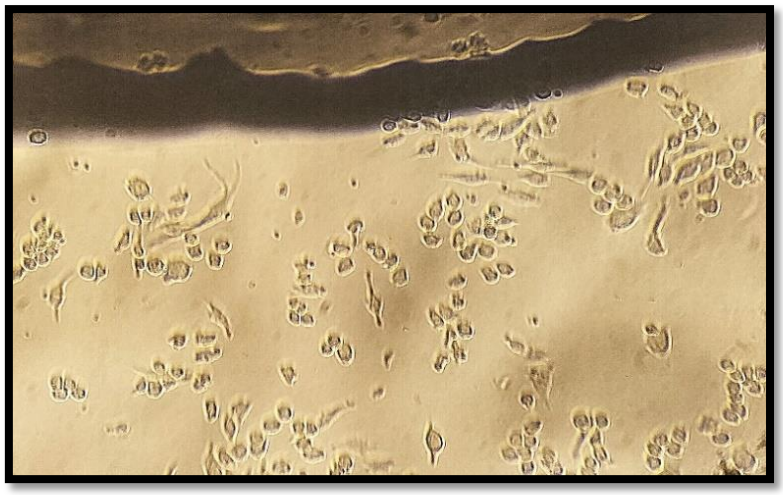


Figure 14: Paraformaldehyde

Figure 14: DMEM Media



NEGATIVE CONTROL



POSITIVE CONTROL

D3

Figure 15: Anti-Inflammatory Results

D6

D9

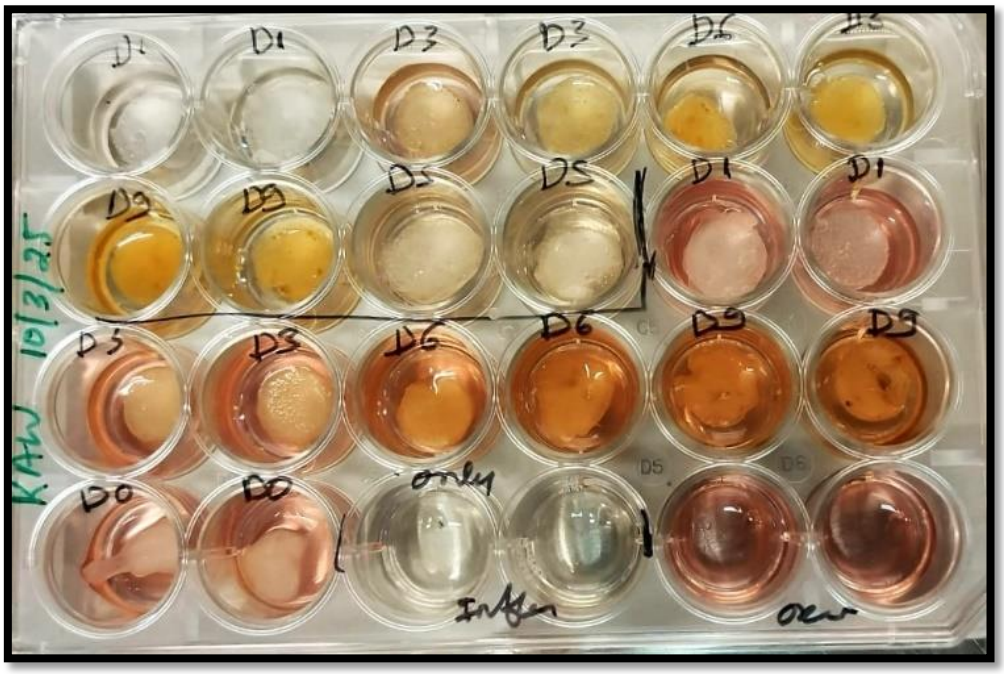
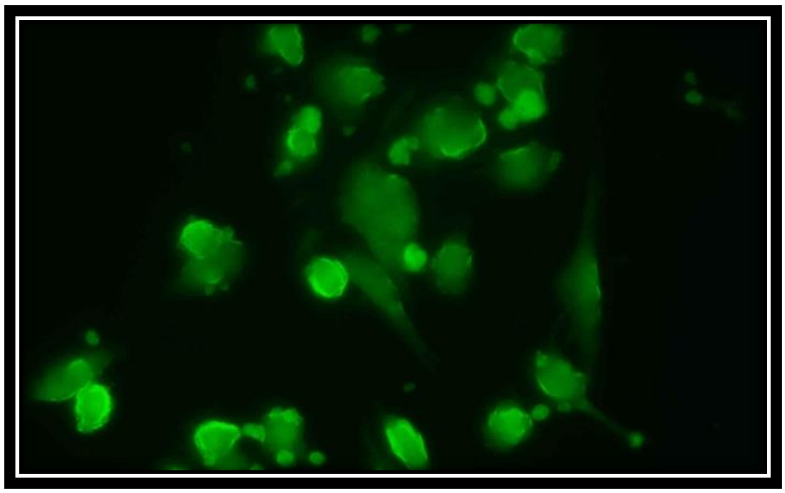
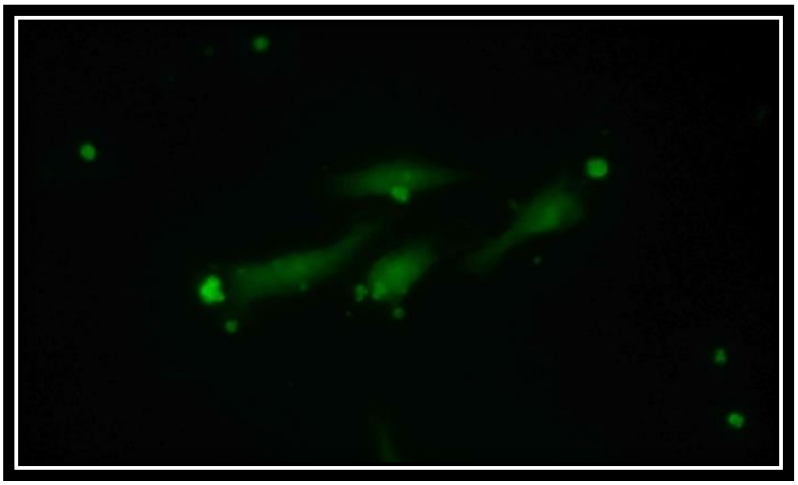


Figure 16: Anti-Oxidative Test

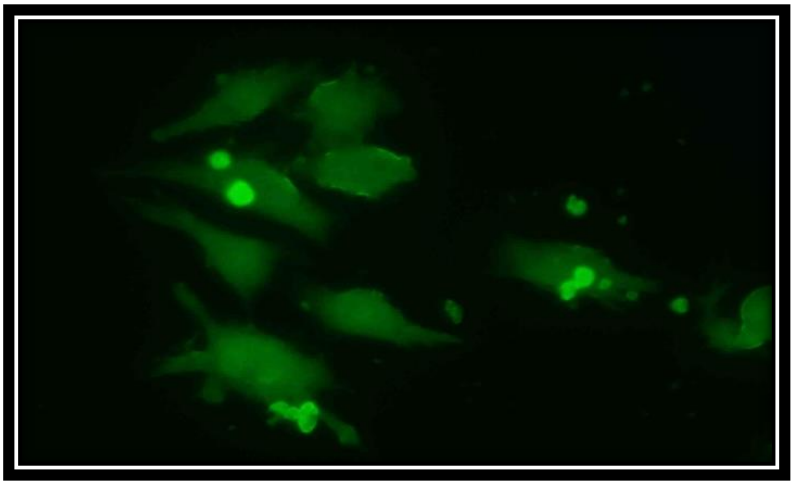
Figure 17: DCF Dye



POSITIVE CONTROL

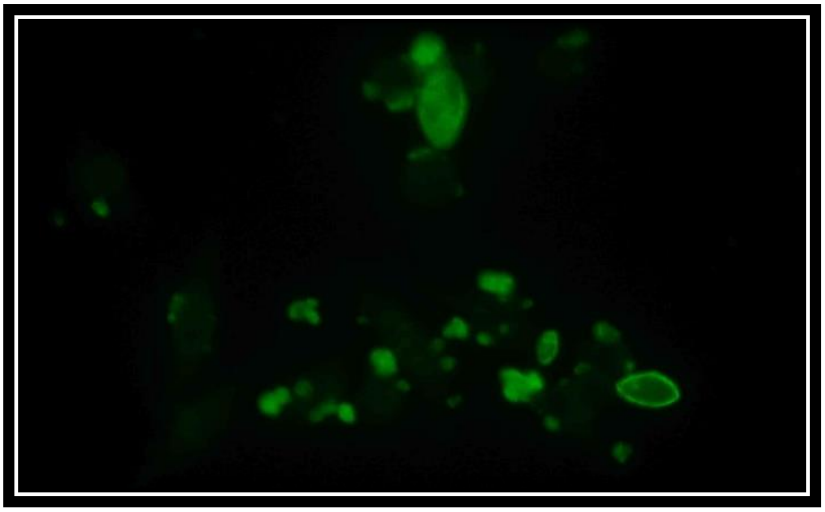


NEGATIVE CONTROL

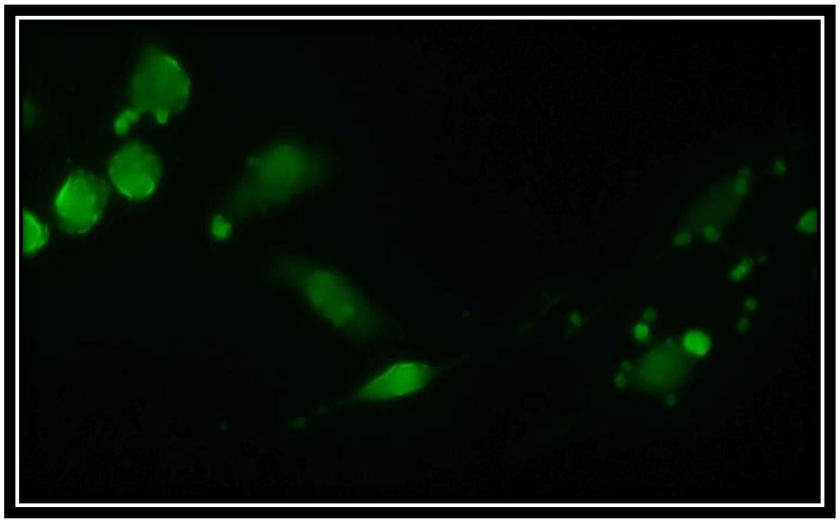


D9

Figure 18: Anti-Oxidative Test Results



D6



D3

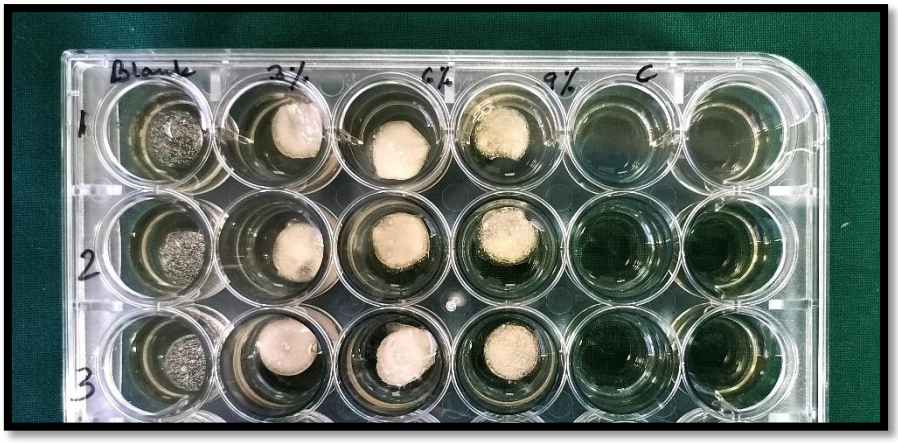


Figure 19: Anti-Bacterial Test

Figure 20: BHI Media

Figure 21: Staphylococcus aureus bacteria

Figure 22: Anti-Bacterial Test Results

RESULTS

- **The value of the minimum inhibitory concentration -** 7 µg/ml
- **The value of the minimum bactericidal concentration - -** 15µg/ml
- **Cytotoxicity Value** -100 µg/ml
- **3 Concentration Values Based On MIC, MBC And Cytotoxicity Values-**

  30 µg/milliliters, 60 µg/milliliters, and 90 µg/milliliters

Inhibiting NO_2 %, Inhibiting ROS production % and colony forming units (CFU) respectively are calculated to measure anti-inflammatory, anti-oxidative and antimicrobial efficacy of tissue conditioner incorporated with *Punica granatum* in 3 various concentrations,30 microgram/millilitres, 60 microgram /millilitres, and 90 microgram /millilitres. To get conclusion based on experimental data, statistical analysis was applied to the recorded values.

- **ANTI-INFLAMMATORY TEST**

Descriptive statistical measures like Mean, Standard deviation of Inhibiting NO_2 % scores in six compounds were calculated. By one way ANOVA, 2 group comparison that is with PPE extract and without PPE extract was done. Pair wise comparisons of six compounds with Inhibiting NO_2 % scores by Tukeys multiple post hoc procedure were performed.

- **ANTI-OXIDATIVE TEST**

Descriptive statistical measures like Mean, Standard deviation of Inhibiting ROS production % scores in six compounds were calculated. By one way ANOVA in

between 2 group comparison with PPE extract and without PPE extract was done. Pair wise comparisons of six compounds with Inhibiting ROS production % scores by Tukeys multiple post hoc procedure were done.

- **ANTI-BACTERIAL TEST**

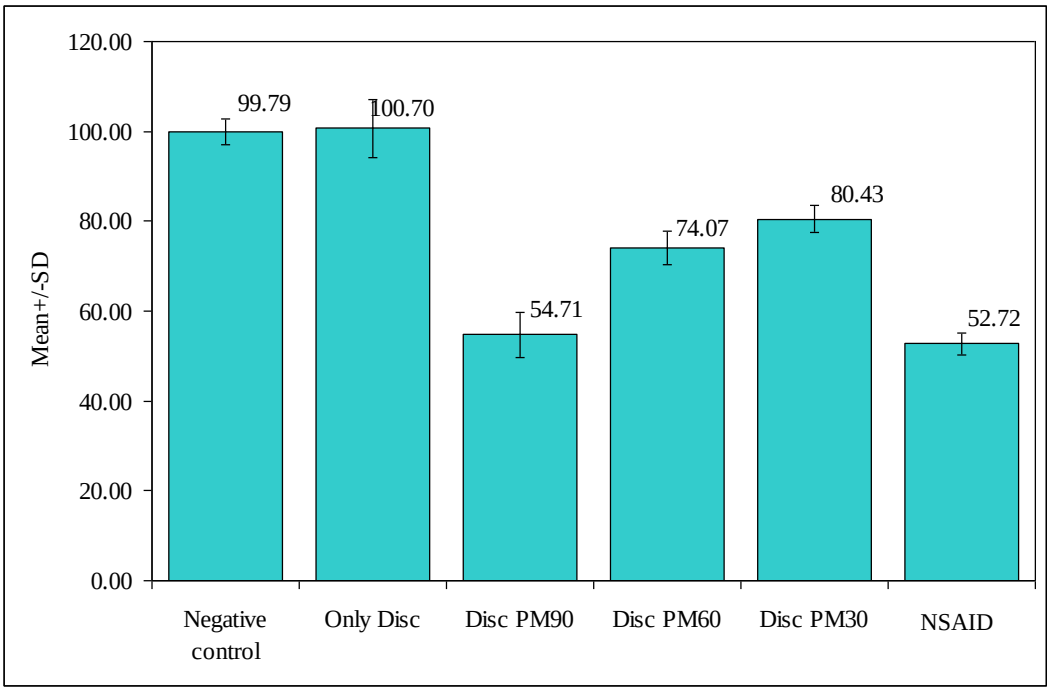
Descriptive statistical measures like Mean, Standard deviation of log (CFU) counts in six compounds were calculated. By performing one way ANOVA test, in between 2 group comparison that is with PPE extract and without PPE extract was done. Pair wise comparisons of six compounds with log (CFU) counts by Tukeys multiple post hoc procedure was performed.

ANTI-INFLAMMATORY TEST

Table 1: Summary of Inhibiting NO2 % scores in six compounds

Compounds	Means	SD	SE	95% CI for mean	
				Lower	Upper
Negative control	99.79	2.85	1.08	97.16	102.43
Only Disc	100.70	6.44	2.43	94.74	106.66
Disc PM90	54.71	5.12	1.93	49.98	59.44
Disc PM60	74.07	3.76	1.42	70.59	77.55
Disc PM30	80.43	3.04	1.15	77.61	83.24
NSAID	52.72	2.50	0.95	50.40	55.03

Graph 1: Comparison of six compounds with Inhibiting NO2 % scores



This table and figure (Table 1, Figure 1) indicates mean, standard deviation of Inhibiting NO2 % scores in six compounds namely, negative control, only disc, positive control (NSAID-5µg/mL) and discs in 3 various concentrations (30 µg/millilitres, 60 µg/millilitres, and 90 µg/millilitres)

Mean values and standard deviation values were highest for negative control and only disc, 99.79 &2.85 and 100.70&6.44 respectively. The mean values and standard deviation values were lowest for positive control (NSAID-5µg/mL) and disc concentration 90 µg/mL, 52.72&2.50 and 54.71&5.12 respectively.

Table 2: Pair wise comparisons of six compounds with Inhibiting NO2 % scores by Tukeys multiple post hoc procedures

Compounds	Negative control	Only Disc	Disc PM90	Disc PM60	Disc PM30	NSAID
Mean	99.79	100.70	54.71	74.07	80.43	52.72
SD	2.85	6.44	5.12	3.76	3.04	2.50
Negative control	-					
Only Disc	P =0.9985	-				
Disc PM90	P = 0.0001*	P =0.0001*	-			
Disc PM60	P = 0.0001*	P = 0.0001*	P= 0.0001*	-		
Disc PM30	P = 0.0001*	P = 0.0001*	P = 0.0001*	P= 0.0741	-	
NSAID	P = 0.0001*	P =0.0001*	P= 0.9468	P =0.0001*	P = 0.0001*	-

*p<0.05

When two groups were compared pairwise—negative control and only disc—no statistically significant variation was found (p is 0.9985); however, there was a statistically significant variation in negative control as well as positive control (NSAID-5µg/mL), as well as between negative control and discs at three different concentrations, (where p is 0.0001) 30 microgram/millilitres, 60 microgram/ millilitres, and 90 microgram /millilitres.

When two groups were compared pairwise, a statistically significant difference was observed in only disc and positive group (NSAID-5 µg/mL) and only disc & discs at three different concentrations (30 µg/millilitres, 60 µg/millilitres, and 90 µg/millilitres) with p -0.0001.

When two groups, Disc PM90 & Disc PM60, Disc PM90 and Disc PM30, were compared pairwise, there was a statistically significant difference among them (p is 0.0001), but there was no statistically significant difference among Disc PM90 & the positive control (NSAID-5µg/mL) (p is 0.9468).

Statistically significant difference was seen among Disc PM60 & Disc PM90 (p is 0.0001), but not between Disc PM60& Disc PM30. (p is 0.0741)

A pairwise analysis of Disc PM30 and NSAID groups showed a statistically significant difference (p is 0.0001). (Table 2).

Table3: Comparison of six compounds with Inhibiting NO2 % scores with one way ANOVA

Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Between compounds	5	15318.4233	3063.6847	174.3587	0.0001*
Within compounds	36	632.5619	17.5712		
Total	41	15950.9852			

*p<0.05

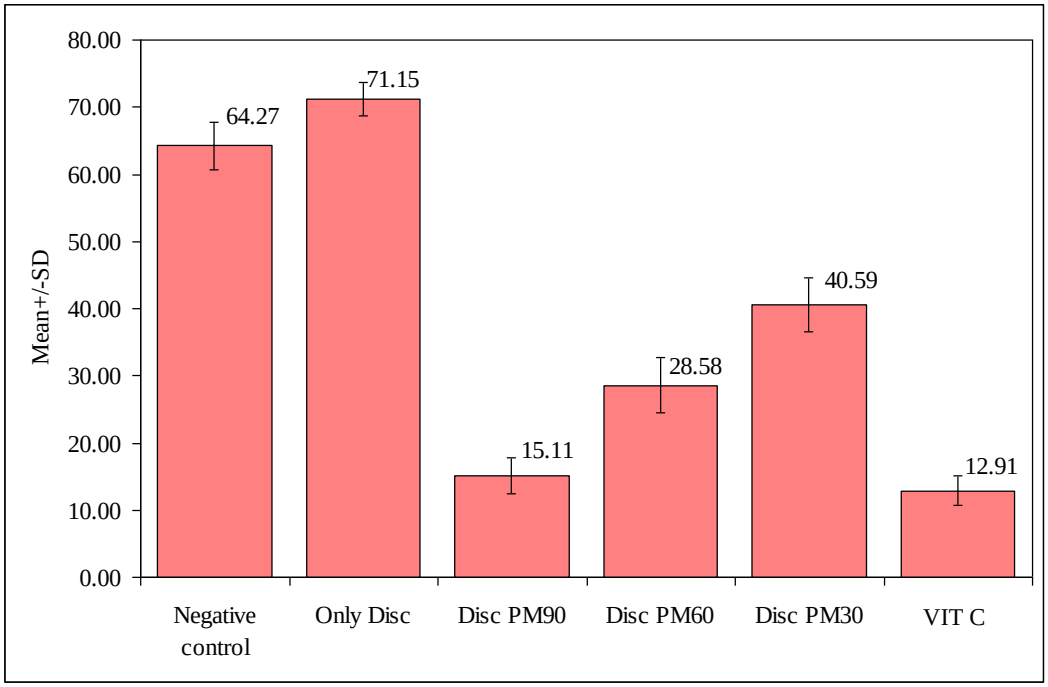
Here in Table 3, comparison of six compounds in 2 independent groups, with compound and without compound with Inhibiting NO2 % scores with one way ANOVA resulted statistically significant difference among between them. (p is 0.0001)

ANTI-OXIDATIVE TEST

Table 4: Summary of Inhibiting ROS production % scores in six compounds

Compounds	Means	SD	SE	95% CI for mean	
				Lower	Upper
Negative control	64.27	3.54	1.34	61.00	67.54
Only Disc	71.15	2.44	0.92	68.89	73.41
Disc PM90	15.11	2.69	1.02	12.62	17.60
Disc PM60	28.58	4.12	1.56	24.77	32.39
Disc PM30	40.59	4.08	1.54	36.81	44.37
VIT C	12.91	2.14	0.81	10.93	14.88

Graph 2: Comparison of six compounds with Inhibiting ROS production % scores



This table and figure (Table 4, Figure 2) indicates mean, standard deviation of Inhibiting ROS production % scores in six compounds namely, negative control, only disc, positive control (NSAID-5µg/mL) and discs in 3 different concentrations such as (30 microgram/millilitres, 60 microgram millilitres, and 90 microgram /millilitres)

Mean values and standard deviation values were highest for negative control and only disc ,71.15&3.54 and 64.27&2.44 respectively. The mean values and standard deviation values were lowest for disc concentration 90 µg/mL and positive control (VIT C-5µg/mL), 15.11&2.69 and 12.91&2.14 respectively.

Table 5: Pair wise comparisons of six compounds with Inhibiting ROS production % scores by Tukeys multiple post hoc procedures

Compounds	Negative control	Only Disc	Disc PM90	Disc PM60	Disc PM30	NSID
Mean	64.27	71.15	15.11	28.58	40.59	12.91
SD	3.54	2.44	2.69	4.12	4.08	2.14
Negative control	-					
Only Disc	P= 0.0046*	-				
Disc PM90	P = 0.0001*	P= 0.0001*	-			
Disc PM60	P = 0.0001*	P =0.0001*	P= 0.0001*	-		
Disc PM30	P =0.0001*	P = 0.0001*	P = 0.0001*	P = 0.0001*	-	
VIT C	P = 0.0001*	P = 0.0001*	P = 0.8036	P = 0.0001*	P = 0.0001*	-

*$p<0.05$

On pairwise comparison of two groups negative control and only disc, a statistically significant difference was observed (p is equal to 0.0001) in between them.

In between negative control and positive control (VIT C -5µg/mL), negative control and discs in concentrations (30 µg/millilitres, 60 µg/millilitres, and 90 µg/millilitres) varied statistically significant. (p = 0.0001)

When two groups were compared pairwise, only disc & positive control VIT C-5µg/mL, and only disc and discs in three different concentrations (30 µg/millilitres, 60 µg/millilitres, and 90 µg/millilitres). A statistically significant difference was observed at p is equal to 0.0001 in between them.

When two groups, Disc PM90 & Disc PM60, Disc PM90 and Disc PM30, were compared pairwise, there was a statistically significant difference between them (pis0.0001), however there was no statistically significant difference was observed in between them. Disc PM90 and the positive control (VITC-5µg/mL) (p=0.8036).

When two groups were compared pairwise, a statistically significant difference was observed at p is equal to 0.0001 in between them. Disc PM60 & Disc PM90 & Disc PM60 and Disc PM30, with pis0.0001.

When two groups were compared pairwise, Disc PM30 and VIT C exhibited, a statistically significant difference was observed at p is equal to 0.0001 in between them

Table 6: Comparison in six compounds with Inhibiting ROS production % scores with one way ANOVA

Sources in variation	Degrees in freedom	Total of squares	Mean total of squares	F value	P value
Among compounds	five	21242.6354	4248.5271	398.5881	0.0001*
Within compounds	36	383.7219	10.6589		
Total	41	21626.3573			

*p<0.05

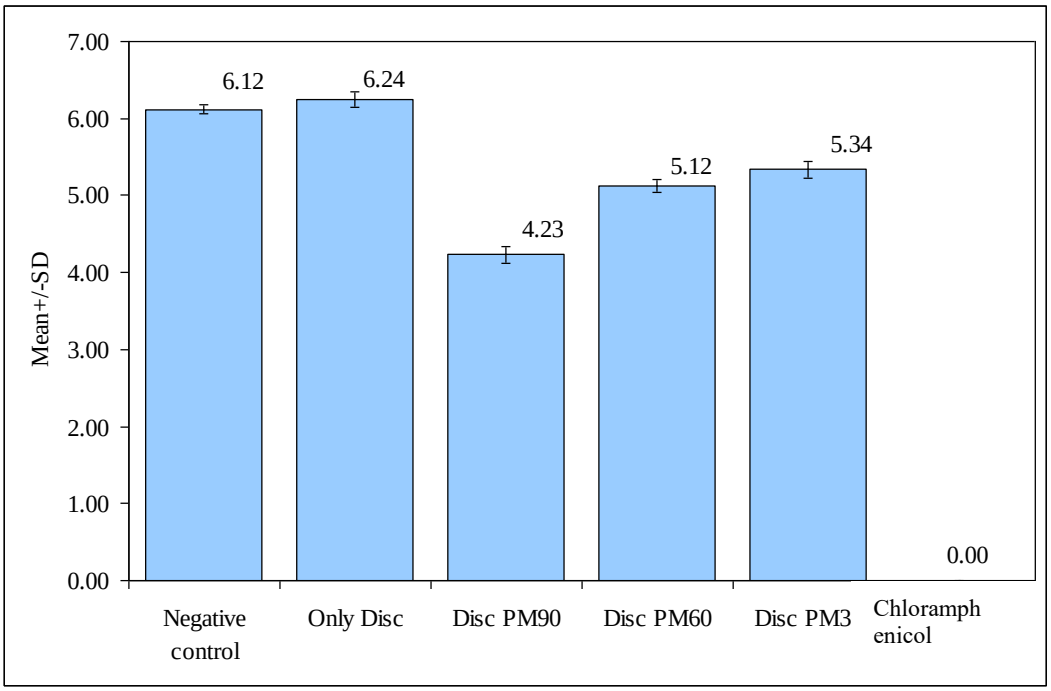
Here in Table 6, comparison of six compounds in 2 independent groups, with compound and without compound with Inhibiting ROS production % scores, statistically significant difference between them was revealed using a one-way ANOVA test. (p is 0.0001)

ANTI-BACTERIAL TEST

Table 7: Summary of log (CFU) counts in six compounds

Compounds	Means	SD	SE	95% CI for mean	
				Lower	Upper
Negative control	6.12	0.06	0.02	6.06	6.18
Only Disc	6.24	0.10	0.04	6.15	6.33
Disc PM90	4.23	0.11	0.04	4.14	4.33
Disc PM60	5.12	0.08	0.03	5.05	5.20
Disc PM30	5.34	0.11	0.04	5.24	5.45
Chloramphenicol	0.00	0.00	-	-	-

Graph 3: Comparison of six compounds with log (CFU) counts



This table and figure (Table 7, Figure 3) indicates mean, standard deviation of log (CFU) counts in six compounds namely, negative control, only disc, positive control (Chloramphenicol-5µg/mL) and discs in different quantities (30 µg/millilitres, 60 µg/millilitres, and 90 µg/millilitres).

Mean values and standard deviation values were highest for negative control and only disc ,6.12&0.06 and 6.24&0.10 respectively. The mean values and standard deviation values were lowest for disc concentration 90 µg/mL and positive control (Chloramphenicol -5µg/mL), that was 4.23&0.11and 0 respectively.

Table 8: Pair wise comparisons of six compounds with log (CFU) counts by Tukeys multiple post hoc procedures

Compounds	Negative control	Only Disc	Disc PM90	Disc PM60	Disc PM30	NSAID
Mean	6.12	6.24	4.23	5.12	5.34	0.00
SD	0.06	0.10	0.11	0.08	0.11	0.00
Negative control	-					
Only Disc	p = 0.1210	-				
Disc PM90	P = 0.0001*	P = 0.0001*	-			
Disc PM60	P = 0.0001*	P = 0.0001*	P = 0.0001*	-		
Disc PM30	P = 0.0001*	P = 0.0001*	P = 0.0001*	P = 0.0005*	-	
Chloramphenicol	P = 0.0001*	P = 0.0001*	P = 0.0001*	P = 0.0001*	P = 0.0001*	-

*p less than 0.05

When two groups were compared pairwise, no statistically significant difference found among between the negative control & only disc group (p - 0.1210); however, when, negative control and discs in three different concentrations (30 µg/millilitres, 60 µg/millilitres, and 90 µg/millilitres) & positive control, Chloramphenicol - 5µg/mL, a statistically significant difference was observed at p is equal to 0.0001 in between them

On pairwise comparison of two groups, Disc PM90 and Disc PM60 & Disc PM90 and Disc PM30, Disc PM90 and positive control (Chloramphenicol -5µg/mL) where a statistically significant difference was observed at p is equal to 0.0001 in between them

On pairwise comparison of two groups, Disc PM60 with positive control (Chloramphenicol 5µg/mL) and Disc PM60 with Disc PM30 where a statistically significant difference was observed at p is equal to 0.0001 in between them.

On pairwise comparison of two groups, Disc PM30 and Chloramphenicol A statistically significant difference was observed at p is equal to 0.0001 in between them. (Table 8)

Table 9: Comparison of six compounds with log (CFU) counts with the help of one-way ANOVA

Sources in variation	Degrees in freedom	total of squares	Mean total of squares	F =	P =
Among compounds	five	189.4398	37.8880	5168.0214	0.0001*
Within compounds	36	0.2639	0.0073		
Total	41	189.7037			

*p<0.05

Here in Table 9, comparison in six compounds in 2 independent groups, with compound and without compound with log (CFU) counts, difference of statistical significance was found using a one-way ANOVA. (p =0.0001)

DISCUSSION

Herbal extracts have acquired popularity in dentistry due to their antibacterial characteristics. Medicinal plants contain natural chemicals such polyphenols, flavonoids, and alkaloids that have potent antibacterial activity against oral infections like *Streptococcus mutans* and *Candida albicans*, *Staphylococcus aureus* [31]. Herbal extracts such as Cinnamomum verum (cinnamon), Curcuma longa (turmeric), and Azadirachta indica (neem) have been demonstrated in studies to dramatically lower bacterial adherence and biofilm formation on tissue conditioners and improving oral hygiene. These plants have active ingredients which interfere with quorum sensing systems necessary for microbial colonisation, break down bacterial cell membranes, and suppress enzyme activity. Herbal-based additions in tissue conditioners offer a sustainable, biocompatible substitute for synthetic antimicrobials with adverse effects [32].

Herbal extracts have been shown antibacterial and anti-inflammatory qualities which plays important role in wound healing. Phytochemicals like curcumin, tannins, and terpenoids may help to reduce mucosal inflammation. For example, curcumin suppresses the nuclear factor-kappa B (NF-κB) signalling pathway, which lowers the synthesis of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α). Acemannan, a compound found in aloe vera, another powerful anti-inflammatory, speeds up wound healing and lowers inflammatory reactions in oral tissues [33]. By reducing tissue irritation and promoting mucosal adaptability, the addition of these extracts to tissue conditioners can improve patient comfort. However, the effectiveness of herbal additions in tissue conditioners depends on variables such as extract content, polymer compatibility, and disintegration kinetics of the extract. To identify the best formulations for sustaining anti-

inflammatory benefits during extended usage, further carefully monitored research is required. [34]

To maximise ability of these plant-derived extracts, long-term antibacterial efficacy of the extract in clinical settings, regulated release of the extract is important. Also to standardise their compositions and assess their long-term effectiveness in dental biomaterials, additional investigations are required [35].

Antioxidants are vital for improving wound healing since oxidative stress is a major factor in bacteria colonisation and oral mucosal inflammation. By scavenging free radicals and preventing lipid peroxidation in soft tissues, herbal extracts such quercetin, resveratrol, and green tea polyphenols demonstrate potent antioxidative qualities [36]. Oxidative stress in the oral cavity can worsen inflammatory problems linked to denture stomatitis and cause tissue degradation. Research has indicated that adding herbal extracts high in antioxidants to Tissue conditioners can support tissue regeneration and preserve cellular homeostasis [37]. For instance, green tea catechins promote collagen production and fibroblast proliferation, which aids in mucosal healing. Furthermore, these substances work with antibacterial and anti-inflammatory substances to improve the general biocompatibility of dental materials. The regulated release and stability of these herbal extracts may be further enhanced by upcoming developments in nanotechnology and bioactive delivery systems, guaranteeing their continued effectiveness in prosthetic dentistry.[37]

In prosthodontics, tissue conditioners are vital because they offer a resilient, temporary lining that helps to evenly distribute masticatory stresses throughout the mucosa that supports the denture. These materials are used mainly to help patients with traumatised or inflamed oral tissues because they promote mucosal healing and

reduce functional stress. Viscoelastic properties of tissue conditioners enable them to adjust to the dynamic changes in the oral cavity, enhancing patient comfort and assuring improved denture base adaption[38] .In order to reduce post-operative difficulties, they are also commonly used in immediate surgical obturators to create a barrier of protection between the prosthesis and healing surgical site[38] .However, as the material hardens and plasticisers leak out, their effectiveness is reduced and they must be replaced on a regular basis .The goal of recent developments in tissue conditioner compositions is to increase their lifetime and therapeutic advantages in prosthodontic applications by using bioactive and antibacterial ingredients . Denture stomatitis and other oral infections might result from tissue conditioners' susceptibility to microbial colonization, especially by *Candida albicans, Staphylococcus aureus* regardless of their benefits. Because of the porous structure, promotion of microbial adhesion and biofilm formation occurs[39]. These are the reasons for frequent replacement and stringent oral hygiene practices while using tissue conditioner. To reduce microbial contamination and increase efficacy of tissue conditioners, researches have been done earlier adding antifungal and antibacterial ingredients including silver nanoparticles and herbal extracts. Additionally, tissue conditioners have been employed as drug delivery vehicles, specifically for the management of oral mucosal inflammation and fungal infections [40]. They are a promising adjuvant in prosthodontic rehabilitation because of their capacity to absorb and release therapeutic substances gradually, especially for elderly and immunocompromised patients. To guarantee the long-term safety and efficacy of modified tissue conditioners, additional research is necessary to address concerns about their mechanical stability and biocompatibility[41]. To maximise their use in prosthodontic treatment, future research should concentrate on creating tissue

conditioners with better mechanical qualities, regulated drug-release mechanisms, and increased resistance to microbial colonisation.

Pomegranate extract, or Punica granatum, has shown strong antibacterial activity, especially against oral microorganisms that cause periodontal diseases and dental cavities.[42] By breaking down bacterial cell walls and preventing the formation of biofilms, the bioactive substances found in pomegranates, including flavonoids, punicalagins, and ellagic acid, demonstrate potent antibacterial qualities[43]. The growth of *Streptococcus mutans, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Staphylococcus aureus*—all of which are major causes of oral diseases—is successfully inhibited by Punica granatum extract, as demonstrated by studies [44]. Its capacity to disrupt bacterial adhesion, suppress glucosyltransferase activity, and modify bacterial quorum sensing processes is thought to be responsible for the antibacterial action [45]. Pomegranate extract exhibits similar efficiency with fewer side effects and reduced resistance development when compared to traditional antibacterial medicines, according to research. Additionally, it has been demonstrated to dramatically reduce the microbial burden and enhance dental hygiene when added to mouthwashes and gels [46]. These results provide credence to Punica granatum's potential as an alternative medicine for bacterial infections in place of synthetic antimicrobials. In addition to its antibacterial qualities, Punica granatum extract has strong anti-inflammatory effects, which makes it a useful treatment for inflammatory tissue healing in the mouth. Polyphenolic compounds in Pomegranate, especially punicalagins, decrease the expression of pro-inflammatory cytokines including interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) and prevent nuclear factor-kappa B (NF-κB) from activating during wound healing [47]. Pomegranate extract has been shown in studies to modulate inflammatory pathways,

hence reducing gingival inflammation and the severity of periodontitis [48]. Its capacity to inhibit the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which are essential components of the inflammatory cascade, is another example of its anti-inflammatory properties[49]. Pomegranate-based mouthwashes and gels have demonstrated notable improvements in gingival index scores and a reduction in inflammatory biomarkers in saliva in clinical trials [50] Furthermore, by increasing fibroblast proliferation and extracellular matrix formation, Punica granatum extract facilitates tissue repair. This implies that formulations made from pomegranates may be used as an adjuvant therapy to treat surgical wounds, hence lowering the need for artificial anti-inflammatory medications.

Antioxidative properties of Punica granatum are essential for protecting oral tissues against harm due to oxidative stress. Many oral disorders, such as periodontitis and lesions of the oral mucosa, delayed wound healing are linked to oxidative stress, which is defined by an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defence systems[51]. Because of the increase in the levels of endogenous antioxidants like glutathione peroxidase (GPx) and superoxide dismutase (SOD), pomegranate polyphenols, including ellagitannins and anthocyanins, have strong free radical scavenging activity. Pomegranate extract has been shown in studies to dramatically lower lipid peroxidation, which protects gingival fibroblasts and oral epithelial cells from cellular damage. By reducing oxidative damage and promoting collagen formation in soft tissues, the antioxidant qualities also aid in better wound healing[52]. Furthermore, pomegranate extract has demonstrated protective properties against inflammatory reactions and UV-induced oxidative stress in oral keratinocytes. These results demonstrate its potential as a useful component in dental products, like

tissue conditioner sand mouth rinses, to improve oral tissue resistance to oxidative damage [53].

A significant disadvantage of traditional tissue conditioners is that they are susceptible to microbial colonisation, namely by Candida albicans and Staphylococcus aureus, which can result in mouth diseases like denture stomatitis [54]. One possible tactic to improve the biological qualities of tissue conditioners is the use of herbal extracts with antibacterial, anti-inflammatory, and antioxidative qualities. Strong antibacterial action against a range of pathogens, including Staphylococcus aureus, has been shown by extract from Punica granatum, which is well-known for its high polyphenolic concentration[43]. Research has also demonstrated that Punica granatum extract can decrease the production of pro-inflammatory cytokines, which in turn can modify inflammatory pathways and promote mucosal healing [55]. Furthermore, its antioxidative qualities are essential for scavenging reactive oxygen species (ROS), which are known to aggravate surgical wound inflammation and cause tissue damage.

In a study conducted by Valentina Parisi et al (2022), results indicated that pomegranate peel possess potential bioactive Phyto complexes, attributed to their notable antioxidant and anti-inflammatory properties. Specifically, bioinformatical analyses revealed a strong correlation between the bioactivity of the extracts and various compounds, including galloyl, pedunculagin, and ellagic acid derivatives.[56] Also, in study conducted by Fabio Mastrogiovanni (2019) stated that the 5 µg/mL concentration of Pomegranate Peel Extract consistently demonstrates significant anti-inflammatory action. These findings highlight the potential of bioactive chemicals derived from pomegranate waste peel regarding their anti-inflammatory effects in gastrointestinal cells and tissues.[57] According to the results of the present study, the

cytotoxicity of tissue conditioner containing Punica granatum extract, the extract does not significantly harm mammalian cells at different concentrations (90, 60, and 30 µg/mL), which makes it a promising option for clinical implications. Punica granatum extract has substantial anti-inflammatory potential, as evidenced by the nitric oxide (NO) inhibition assay, which showed that the maximum dose of the extract (90 µg/mL) significantly suppressed NO production. Lipopolysaccharide (LPS)-induced inflammatory pathways are suppressed, lowering oxidative stress and cytokine release in cells, which accounts for the anti-inflammatory effectiveness.

In a study conducted by Shalini Malviya et al in the year 2013 evaluated Pomegranate Peel extracts against four bacterial strains: Staphylococcus aureus, Enterobacter aerogenes, Salmonella typhi, and Klebsiella pneumoniae, and the extracts revealed outstanding antibacterial activity against all of them. The extract was made using six different solvents, including ethanol, methanol, water, 30% ethanol: 70% water, 50% ethanol: 50% water, and 70% ethanol: 30% water. The 70% ethanol: 30% water and 100% water extracts demonstrated increased antioxidant activity and phenolic content, indicating potential for nutritional use. Nebojša Mandić-Kovačević et al (2023) concluded in the study that Pomegranate peel extract exhibits notable antioxidant activity both in vitro and in vivo, according to the study. Pomegranate peel extracts high content of total phenols, flavonoids, flavanols, and monomeric anthocyanins with high radical scavenging potential is responsible for the high antioxidant capacity =s measured in vitro, the notable decrease of TBARS and NO2 radicals, and the increase in reduced glutathione levels were shown. (Thiobabrituric acid reactive substances - TBARS as an index of lipid peroxidation, Nitrites - NO2). [58] In the present study, measurement of reactive oxygen species (ROS) using DCFDA was done to assess the anti-oxidant activity of tissue conditioner containing Punica

granatum. The highest concentration (90 µg/mL) showed similar anti-oxidant activity to the positive control.

In a study conducted by Mohammad Anees (2023) has shown that PPE has antibacterial properties against S. aureus, MRSA, P. aeruginosa, K. pneumonia, P. vulgaris, and Candida albicans. Extract activity varies based on the technique of preparation (aqueous, ethanolic, or methanolic solvents) and the tested microorganisms. The methanolic extract demonstrated the strongest effectiveness against Candida albicans.[59] In the present study, direct contact test against Staphylococcus aureus was used to assess the antibacterial efficiency of tissue conditioner containing Punica granatum in addition to its anti-inflammatory and antioxidative qualities in this study. The highest concentration (90 µg/mL) showed similar antimicrobial effectiveness to the positive control, and the results showed a considerable reduction in bacterial growth. Because it can break down the integrity of bacterial cell walls and prevent the formation of biofilms, Punica granatum extract has antimicrobial properties that stop harmful microbes from adhering to and growing on the surface of tissue conditioners. These results demonstrate how tissue conditioners containing Punica granatum may lower microbial contamination and enhance the general clinical performance of prosthetic materials [60].

Long-term clinical trials and additional in vivo research are necessary to confirm the effectiveness and biocompatibility of tissue conditioners based on Punica granatum in a variety of patient populations. An important trend in prosthodontic research is the incorporation of bioactive natural substances into dental biomaterials, which opens the door to the creation of new therapeutic tissue conditioners with improved biological qualities.

SCOPE OF THE STUDY

- The study aimed at comparative evaluation of antibacterial, anti-inflammatory, anti-oxidative efficacy of tissue conditioner incorporated with *Punica granatum* extract in 3 concentrations 90, 60, and 30 µg/ml.
- Since this is an in-vitro study, in-vivo studies can be carried out since intraoral parameters such as pH, presence of saliva, different loading parameters can affect the properties of tissue conditioner.
- Further research on the color stability and mechanical properties of Pomegranate Peel extract incorporated into tissue conditioner can be conducted.
- Further research can be conducted on the various other biological properties such as anti-fungal property, anti-cancer property.
- Furthermore, long-term studies can be conducted for validation of the results.

LIMITATIONS OF THE STUDY

- The primary limitation is that the results obtained through in vitro investigation might not accurately reflect on the outcomes under clinical conditions.
- Several other biological properties, such as anti-fungal properties, antibacterial effects against other micro-organism than Staphylococcus aureus, were not evaluated.
- Also, other mechanical properties like flexural strength, wear resistance, and color stability, were not evaluated.
- The comparison was conducted using only one type of Tissue conditioner, and different tissue conditioners may yield different outcomes.
- The assessment of pomegranate peel extract was limited to only three concentrations. Evaluating additional concentrations could lead to varying results.
- Since this is an in-vitro study, in-vivo studies may yield different outcomes, since intraoral parameters such as pH, presence of saliva can affect the properties of Tissue conditioner.

CLINICAL IMPLICATIONS

Addition of pomegranate extract in tissue conditioner shows antimicrobial activity against staphylococcus aureus and has positive effect on anti-inflammatory and anti-oxidative efficacy. On integrating such herbal agent during the manufacturing process these Tissue conditioners could show increased efficiency, to fight with issues like secondary infections and delayed wound healing thereby enhancing the effectiveness of tissue conditioner especially in wound healing of surgical area when used with immediate surgical obturators.

CONCLUSION

Considering the limitations of the study, it can be inferred that,

1. The anti-microbial efficacy against *staphylococcus aureus* of tissue conditioner after incorporating *Punica granatum* extract in concentration 90 µg/mL and positive control (Chloramphenicol -5µg/mL) showed lowest number of colonies forming units. At increasing PPE concentrations, the numbers of bacterial colony forming units (CFU) were considerably decreased.

2. The anti-inflammatory efficacy of tissue conditioner after incorporating *Punica granatum* extract in concentration 90 µg/mL and positive control (NSAID-5µg/mL) showed lowest NO_2 % scores. At increasing PPE concentrations, the NO_2 % scores were considerably decreased.

3. The anti-oxidative efficacy of tissue conditioner after incorporating *Punica granatum* extract in concentration 90 µg/mL and positive control (VIT C -5µg/mL) showed lowest ROS production % scores. At increasing PPE concentrations, the numbers of ROS production % scores were considerably decreased.

4. *Punica granatum* extract in concentration 90 µg/mL had a considerable antibacterial, anti-inflammatory, anti-oxidative activity comparable with the positive control -chloramphenicol, NSAID, VIT C respectively.

5. At concentrations below the level of cytotoxicity threshold, there were statistically significant improvements with respect to the anti-inflammatory, antioxidative, and antibacterial qualities of tissue conditioners containing Punica granatum extract.

SUMMARY

The goal of this study was to compare & assess the antibacterial, anti-inflammatory, and antioxidative efficacy of a tissue conditioner incorporating extract from Punica granatum at 30 µg/mL, 60 µg/mL, and 90 µg/mL concentrations.

Nitric oxide (NO_2) & reactive oxygen species (ROS) inhibition assays were used to determine the anti-inflammatory and antioxidative characteristics, respectively, while Staphylococcus aureus was used to test the antibacterial activity. The selection of the test concentrations was determined by the analysis of the Minimum Bactericidal Concentration (MBC) and the Minimum Inhibitory Concentration, or MIC, & cytotoxicity threshold, which were 15 µg/milliliters, 7 µg/milliliters, and 100µg/milliliters, in that order.

Using SPSS software, statistical analysis was carried out to evaluate intra- and inter-group changes using Tukey's post-hoc test with one way ANOVA. 90 µg/mL concentration (Disc PM90) demonstrated lowest CFU counts of 4.23 units, which was comparable to the positive control (Chloramphenicol), indicating a dose-dependent trend in the anti-microbial action.

90 µg/mL concentration (Disc PM90) demonstrated a nitric oxide inhibition of 54.71%, which was comparable to the positive control (NSAID, 52.72%), indicating a dose-dependent trend in the anti-inflammatory action. Similar to this, ROS inhibition showed an antioxidant effect that was concentration-dependent, with Disc PM90 (15.11%) having activity that was equivalent to that of the vitamin C (12.91%).

The anti-inflammatory, antioxidative, and antibacterial qualities of Tissue conditioners were consequently improved by adding Punica granatum extract at various concentrations, showing significant possibilities for therapeutic uses in prosthodontics.

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ANNEXURES

ANNEXURE I- ETHICAL CLEARANCE

Research and Ethics Committee
KLE VK INSTITUTE OF DENTAL SCIENCES
A Constituent Unit of KLE Academy of Higher Education & Research
Accredited 'A' Grade by NAAC | Placed in Category 'A' by MHRD (GoI)
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Sl. No. : 1659

CERTIFICATE

This is to Certify that the synopsis titled

Comparative evaluation of antibacterial, anti inflammatory, anti oxidative efficacy of tissue conditioner incorporated with Punica granatum extract in different concentration - An vitro study. *Submitted by*

Dr. **REG. NO- IM0222001** _____ *P. G. Student / Staff, Guided by* _ *_from Department of* Prosthodontics Crown and Bridge *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committe
KLE VK Institute of Dental Scienc
Belgaum

ANNEXURE II

AUTHENTICATION CERTIFICATE

SHRI B.M.K. AYURVEDA MAHAVIDYALAYA
A constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University
Central Research Facility
DRUG AUTHENTICATION REPORT

Outward: - BMK/CRF/M19/2024-25

Submitted By:
Submitted Date : 09/09/2024

Date of Issue: 10/09/2024

SLNo	Sample Name	Scientific Name	Family	Part submitted	CRF Code	Authenticated as			
						Common Name	Scientific Name	Family	Part Authenticated
1.	Dadima	*Punica granatum* Linn.	Punicaceae	Phala Twak	CRF/Auth/524/2024	Dadima	*Punica granatum* Linn.	Punicaceae	Phala Twak

Signature:
Authentication Exper
Date: 10/09/2024

Signature of Coordinator
ASU Drug Testing Labotaratory