"EVALUATION OF ANTIMICROBIAL EFFICACY AND OSTEOGENIC POTENTIAL OF TITANIUM COATED WITH HYDROGEL OF CALENDULA OFFICINALIS: AN INVITRO STUDY."

By

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LIST OF ABBREVIATIONS USED IN THE STUDY

ABBREVATIONS	FULL FORMS
STUDY GROUP	Titanium discs treated with calendula officinalis hydrogel
$CONTROL_{MA}$ GROUP	Titanium discs treated with 1 % chlorhexidine gel
$CONTROL_{OP}$ GROUP	Machined Titanium discs
ANOVA	Analysis of variance
BIC	Bone Implant Contact
C.V.	Coefficient of Variation
CO_2	Carbon dioxide
°C	Degree Celsius
CHX	Chlorhexidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
gms	Grams
hrs	Hours
IL	Interleukin
L	Litre
M	Milli
MAPK	Mitogen associated protein kinases
MBC	Minimum Bactericidal Concentration

MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
PBS	Phosphate Buffer Saline
RANKL	Receptor activator of nuclear factor kappa B ligand
S.D.	Standard Deviation
S.E.	Standard of error
Ti6Al4V	Titanium-6 aluminum-4 vanadium
TNF	Tumor Necrosis Factor

ABSTRACT

STATEMENT OF PROBLEM

Success of a dental implant relies on its osseointegration process. Ever since osseointegration concept aroused, there have been number of techniques and materials that have been developed to increase the bone implant contact and decrease the time required for osseointegration. One of the reasons for Implant failures are related to periimplantitis and others related to inadequate bone to implant contact eventually causing mobility and implant loss. Hence, the probability of successful tissue integration would be greatly enhanced if the tissue integration occurs before bacterial adhesion could take place. Therefore, it was imperative that an implant/ coating should have both antibacterial and osseointegrative properties. Surface modification in the form of hydrogel scaffolds which promote bone growth as well as impart an antibacterial effect could be an advantage by reducing the time for osseointegration and helping in immediate loading protocols. The limited spectrum and toxicity of available antimicrobial agents have concerned for alternative therapies. The use of herbal agents has emerged as an alternative for treating periimplant bacteria compared to commercially available Chlorhexidine.

AIM: To assess the antimicrobial efficacy of *Calendula officinalis* hydrogel against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus* and the osteogenic potential of Calendula officinalis hydrogel coated on titanium

MATERIALS AND METHODS

A total of 270 titanium disc shaped specimen measuring 10 mm x 2 mm (ASTM B348) which were free of voids and porosities were used in the study. The discs were sandblasted and their surface roughness was evaluated quantitatively using Profilometer and qualitatively using Scanning Electron Microscopy. MIC, MBC was evaluated for *Calendula officinalis* extract against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Staphylococcus aureus*. At the desired concentration, the gel was formulated using Carbopol 940, glycerine and Triethanolamine. The cytotoxicity of the gel to L929 cells was evaluated. The gel was coated on the titanium discs by dipping method and the antimicrobial efficacy of the gel coated on the discs was assessed using disc diffusion assay against the three organisms in comparison to the control which consisted of titanium discs coated with 1 % Chlorhexidine gel. Osteogenic potential was evaluated by assessing the cell attachment using hemocytometer and Cell proliferation by MTT Assay using MG-63 cells on Calendula officinalis treated titanium discs in comparison to machined titanium discs at time intervals of 24, 48, 72 hours. The resultant data was tabulated and subjected to statistical analysis.

RESULTS

The results showed that the hydrogel showed antimicrobial efficacy against the three organisms tested. *Aggregatibacter actinomycetemcomitans* showed the highest zone of inhibition as compared to the other two organisms for the hydrogel treated group. *Porphyromonas gingivalis* and *Staphylococcus aureus* showed similar zone of inhibition. However, the study group showed lesser zone of inhibition as compared to specimens on the $control_{MA}$ group. The cell attachment was higher for the study group at all three time intervals as compared to the $control_{OP}$ group. There was a statistically significant difference in the cell attachment between the time intervals for the study group. The cell proliferation was higher in the study group with statistically significant results at all three time intervals studied.

CONCLUSION

Within the limitations of this invitro study, titanium surfaces treated with Calendula officinalis hydrogel was found to be effective against periimplantitis and had increased cell attachment and cell proliferation of MG-63 cells at all three time intervals with a greater effect size as compared to machined titanium surfaces. This opens the door to further investigations for an implant coating having osseoconductive as well as antibacterial properties.

KEYWORDS: calendula officinalis, chlorhexidine, coated materials, osseointegration, quercetin, titanium.

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INTRODUCTION

Natural teeth are commonly lost due to caries, periodontal diseases and trauma that affect the patient's appearance, speech, and the masticatory system. Dental implants have become a popular solution to restore missing teeth, masticatory function, and esthetics thereby improving patient's quality of life.[1] Dental implants are fixtures that are surgically embedded in the jaw bone to substitute the root part of the tooth. [2] The dental implants are biocompatible and made of titanium and its alloys. Ti-6 aluminum-4 vanadium (Ti6Al4V) is the most commonly used titanium alloy and is preferred due to its superior mechanical properties and excellent biocompatibility.[3] They are left undisturbed where bone healing takes place in which bone grows around the implant which is termed as osseointegration and is critical for long term prognosis. Osseointegration may be affected by a number of factors including the material properties, surface topography, and geometrical features of dental implants [4]

Osseointegration may also be affected in cases of low bone density and quantity. This can be caused by systemic disorders like osteoporosis or be encountered as a side effect of radiotherapy. Such circumstances imply a challenging bone healing situation. Under these conditions, the survival rate of dental implants may decrease to 55% which would require additional surface modifications that would stimulate new bone formation and promote early osseointegration. [5]

Considering that the biological tissues establish an interface with the materials' surface, the surface modifications enhance the materials' biological response and osseointegration without affecting the bulk properties [4].

Therefore, enhancement of the osteoconductive properties of dental implants is always desired to achieve clinical success.

There are various techniques pertaining to surface modifications that have been researched and applied to improve biological surface properties, that support the mechanism of osseointegration, these include subtractive methods such as sandblasting, acid etching or a combination of both. The additive methods include plasma spraying, hydroxyapatite coating and also coating with biomimetic agents. [6]

Most of these implant surface treatments aim at enhancing the activity of bone forming cells and their mediators to increase the new bone formation hence promoting early osseointegration and higher secondary implant stability. [6]

One of the surface chemical modifications to further stimulate osseointegration is the coating of implant surfaces with biological components. Many different types of surface coatings have been proposed in recent years. These include coatings with peptides, growth factors, calcium phosphates and lipids. Different organic coatings, biopolymers and biomimetic agents have been explored on biomaterials used for dental implants which can potentially influence cellular activity during peri-implant healing, and promote an intimate bone-implant contact, promoting osseointegration by the proliferation of osteoblasts.[7] Biomaterials such as polymeric matrices, hydrogels and nanoporous matrices have also been evaluated as a means of surface modification. [8]

Hydrogels used as one of the implant surface modifications are polymeric networks onto which matrices for tissue engineering or drugs can be incorporated which have a sustained release directly at the site of interest which can be used as an antimicrobial, to accelerate bone healing or both. [9]

The two main areas of concern in dental implant therapy are biomaterial centered infection and successful tissue integration. Infection after implant placement still remains one of the major complications in dental implants even though optimal aseptic surgical practices are followed and modern antibiotic regimes are used. [10]

In the competition for colonization of the implant surface, the probability of successful tissue integration would be greatly enhanced if tissue integration occurs before bacterial adhesion. [11]

Significant advancements have been made in implant surface modification as a result of requirement to mitigate biofilm formation to reduce the occurrence of peri-implantitis, accelerate osseointegration for rapid loading, or in cases of deficient bone at the recipient site.[12] Therefore an ideal implant/ implant coating should have both antibacterial and osseointegrative properties. [11]

With growing resistance to antibiotics and side effects associated with certain antimicrobials which impair osseointegration and cause cell toxicity there is a search for natural products with less or no side-effects and traditional medicines are now being considered as alternatives.[13] Plants are known to synthesize aromatic substances, usually phenols or their derivatives which are intensely antimicrobial and antioxidant in nature [14]

Calendula officinalis commonly called marigold, belongs to the Asteraceae family. *Calendula officinalis* contains various phytochemicals, the flavonoid quercetin is considered of great importance due to its anti-inflammatory, antitumor, antioxidant and cicatrizing effects.[14] These phenolic structures namely quercetin complex with extracellular soluble proteins and bacterial cell walls and are capable of disrupting microbial membranes based on their lipophilic nature[14]

Quercetin is also known to enhance human osteoblast like MG 63 proliferation and differentiation through MAPK signaling and increase the expression of some of the prominent markers of osteoblast differentiation process such as osteopontin, osterix, RunX2 , osteoprotegrin and osteocalcin.[15]

The anti-inflammatory effect of *Calendula officinalis* extract might be due to the inhibition of anti-inflammatory cytokines and cyclocoxygenase-2 and subsequent prostaglandin synthesis. Quercetin has shown to significantly increase osteoblast differentiation and induced mRNA expression of sialoprotein and osteocalcin in the osteoblast culture. It increased serum osteocalcin levels and the activity of alkaline phosphatase, contributing to bone tissue preservation [16]

Considering these advantages and properties of *Calendula officinalis* this study was undertaken with an intend to assess and evaluate the antimicrobial efficacy and osteogenic potential of titanium when surface treated with a hydrogel of *Calendula officinalis*.

NEED FOR STUDY

Implant dentistry is unique because it has the ability to accomplish the goals of normal function, contour, aesthetics, speech and health regardless of atrophy, disease, or injury to stomatognathic system. [17]

Ti_6Al_4V, are the most popular titanium alloys used for implants due to their excellent mechanical properties and superior biocompatibility in comparison to conventional materials such as stainless steel 316L and cobalt–chromium alloys. However, titanium in its use as implant materials have some limitations. Titanium as an alloy is bioinert lacking the ability to form any chemical bond with surrounding tissues, it only forms physical bond with the bone tissue and the stability of this physical bond is less than chemical osseous bonding leading to a higher risk of failure or implant loosening. Titanium lacks natural antimicrobial properties, easily allowing bacterial colonisation during the early stages of implantation. Following colonisation, the bacteria can grow to form a biofilm that interferes with the function of antibiotics leading to subsequent infection. [18]

The titanium implant requires 4-6 months for Osseointegration with the adjacent bone using the current materials and techniques, however in cases of impaired quality of bone, systemic diseases longer waiting periods are required. Bone response refers to the rate, the quantity and the quality and is attributed to the implant surface behaviour. Therefore, majority of the implant surface treatments aim at increasing the activity of osteoprogenitor cells and their mediators to increase the new bone formation and promote early osseointegration and higher secondary implant stability [19]

The purpose of surface modification is to simultaneously maintain the excellent mechanical properties and biocompatibility of the implant, while also optimising its antibacterial properties, resistance to corrosion, and bioactivity. [18]

Various commercially available antimicrobials are used to treat early periimplantitis, however these antimicrobials released at suboptimal concentrations are likely to promote bacterial resistance, whereas high doses of antimicrobials may alter the oral microflora, generate cell toxicity and impair osseointegration. [20] The ethnopharmacological approach by using natural phytochemicals isolated from medicinal plants are considered to be a safe, effective, and good alternative to synthetic drugs. [20]

Calendula officinalis is a well-known therapeutic herb that was used for decade. There are numerous biologically active compounds that are found in different components of the plant such as in its leaves and flowers. These compounds include carotenoids,flavonoids, saponins, sterols, phenolic acids, lipids which are therapeutic and are used both in-vitro and in-vivo. It is reported to have medicinal properties and is widely used as an anti-inflammatory, analgesic, antiseptic and in healing wounds. In dentistry, it is effective in the treatment of oral mucositis, gingivitis, periodontitis. Calendula extract promotes wound healing by boosting neovascularisation and the rate of hyaluronic acid deposition. In bone wounds, hyaluronic acid can accelerate new bone creation by promoting mesenchymal cell differentiation. [21]

It is a very promising plant that needs indepth research that can be used to extract active ingredients, to synthesize different drugs, to prevent various diseases, and also used to manage different pathologies.[21]

The research related to the antimicrobial and osteogenic potential of *Calendula officinalis* hydrogel on titanium surfaces are at sparse. Hence it is required to evaluate the effect of *Calendula officinalis* on periimplant pathogens and its osteogenic potential for early osseointegration of dental implants. Therefore, this invitro study was undertaken with an intent to evaluate the antimicrobial efficacy and osteogenic potential of *Calendula officinalis* hydrogel coated on titanium disc surface

HYPOTHESIS

NULL HYPOTHESIS: There is no significant difference in the antimicrobial efficacy and osteogenic potential of titanium coated with hydrogel of calendula officinalis.

ALTERNATIVE HYPOTHESIS: There is a significant difference in the antimicrobial efficacy and osteogenic potential of titanium coated with hydrogel of calendula officinalis.

AIM AND OBJECTIVES

AIM OF THE STUDY:

- To assess the antimicrobial efficacy of Calendula officinalis hydrogel against Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus and the osteogenic potential of Calendula officinalis hydrogel coated on titanium.

OBJECTIVES:

- To assess the antimicrobial efficacy of Calendula officinalis hydrogel against Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus.
- To compare the antimicrobial efficacy of Calendula officinalis hydrogel with chlorhexidine gel (CHX) against Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus.
- To assess and compare the osteogenic potential and proliferative nature of osteoblast like cell on titanium with and without subjecting it to hydrogel of Calendula officinalis.

REVIEW OF LITERATURE

1. **K. J. Bundy et al in 1980** investigated the bacteriostatic properties of pure metals. The effect of 16 pure metals on the in vitro growth of *Streptococcus mutans* was studied under both aerobic and anaerobic conditions. With less consistency nickel, titanium, iron, and vanadium exhibits the ability to inhibit growth of the organism. Threshold concentrations above which growth did not occur were identified. As measured by threshold concentrations, wide variability between metals exists in the ability to inhibit the growth, with cobalt being particularly effective at small concentrations. This implies that, sensitivity on the part of the organism is different for different metals. The threshold concentration varied between aerobic and anaerobic conditions. *Streptococcus mutans* appeared more resistant to the effects of the metals under anaerobic conditions even though little difference in the amount of corrosion was detected. [22]

2. **Gregory R. Parr et al in 1985** highlighted the important dental materials aspects of titanium and its alloys. A successful long-term implant requires biocompatibility, toughness, strength, corrosion resistance, wear resistance, and fracture resistance. Titanium and its alloys are important in dental and surgical implants because of their high degree of biocompatibility, their strength, and their corrosion resistance. The alloys most commonly used for dental implants are of the alpha-beta variety. Of these, the most common contains 6% aluminum and 4% vanadium (Ti-6Al-4V). After heat treatment these alloys possess many favorable physical and mechanical properties that make them excellent implant materials. [23]

3. **Mombelli A et al in 1987** compared the microbiota associated with successful and failing osseointegrated titanium implants. The microbiota associated with oral endosteal titanium hollow cylinder implants (ITI) were studied using microscopic, immunochemical and cultural methods. Microbiological samples from five edentulous patients with successfully incorporated implants serving as abutments for overdentures for more than one year were compared with samples from 7 patients with clinically failing implants. These peri-implant sites harbored a complex microbiota with a large proportion of gram- negative anaerobic rods. Control sites in the same patients harbored small amounts of bacteria. The microbiota in control sites of unsuccessful patients and in site of successful patients were very similar. On the basis of these results, it was suggested that "peri-implantitis" be regarded as a site-specific infection, which yields many features in common with chronic adult periodontitis. [24]

4. **Sennerby L et al 1991** conducted a study to examine key implant factors that determine the bone-metal interface reactions that occur around a Titanium screw. At the ultrastructural level, interfacial reactions to experimental Titanium implants are studied. Tissue reactions to CP Titanium versus Titanium-6aluminum-4Vanadium were examined, and relevant surface characteristics and surface structure for achieving reliable osseointegration, as well as probable bonding processes over the bone-to-Titanium interface, are outlined. This article indicates that elements linked to the implant alone do not dictate the bone-metallic interfacial responses, but that other factors such as surgical technique and loading circumstances are equally significant for establishing a reliable osseointegration. [25]

5. **Ong et al in 1992** selected 19 patients for evaluation of periimplant space with age ranged between 22 to 77 years with a minimum requirement of two osseointegrated branemark titanium implants. All clinical variables were assessed for *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia* and total of 37 sites were examined. Patients were prescribed 0.2% chlorhexidine gluconate mouthwash daily as oral homecare hygiene regimen. The results showed 22 of 37 sites had more proportion of anaerobes than aerobes. [26]

6. **Gerald Mcdonnell et al in 1999** studied antibacterial activity of Chlorhexidine. It is bactericidal and fungicidal, however it does not kill or limit the growth of bacterial spores or mycobacteria. It has a low order of effectiveness against viruses, however it is effective in destroying cysts of *Acanthamoeba* species at high doses. [27]

7. **Lauk et al in 2003** studied the antibacterial efficacy of medicinal plant extracts against periodontopathic bacteria. The alcoholic extract of *Calendula officinalis* was found to be antibacterial against *Porphyromonas gingivalis* , *Prevotella intermedia, Fusobacterium nucleatum and Actinomyces* species. Among all the organisms, it inhibited *Fusobacterium and Actinomyces* at very high MIC. [28]

8. **Ethan Basch et al in 2006** putforth a systematic review which included statistical analysis of scientific literature, pharmacology, kinetics/dynamics, interactions, adverse effects, toxicology and dosing of Calendula. The constituents of calendula are triterpenoids and flavonoids with at least eight bioactive triterpendiol monoesters identified in the extracts of dried calendula

flowers. The active components of calendula's anti-inflammatory activity are thought to be the triterpenoids, particularly faradiol monoester. In case of wound healing there was a reduction of epithelization time, an increase in wound strength in wounds that were topically treated with calendula. [29]

9. **Lindhe J Meyle et al in 2008** conducted a study to determine the causes of peri-implant mucositis, peri-implantitis and their management. Poor oral hygiene, dental cements, a history of periodontitis, and cigarette smoking are all risk factors. Mechanical debridement is required in the treatment of peri-implant mucositis inorder to eliminate the plaque. In the treatment of peri-implant mucositis lesions, mechanical non-surgical treatment is beneficial. In the treatment of peri-implantitis, non-surgical mechanical cleaning of the implant surface with or without an adjuvant antibacterial treatment is ineffective. [30]

10. **Stefan Renvert et al in 2008** did a review of the literature to assess non-surgical therapy options for peri-implant mucositis and peri-implantitis. Mechanical nonsurgical therapy has been shown to be successful in the treatment of peri-implant mucositis lesions. Furthermore, using antibacterial mouth rinses in addition to mechanical therapy improved the outcome of such mucositis lesions. Nonsurgical treatment for peri-implantitis lesions was found to be ineffective. Clinical and microbiological characteristics were only little influenced by the use of chlorhexidine. Adjunctive local or systemic antibiotics, on the other hand, have been demonstrated to minimize bleeding during probing and probing depths. [31]

11. **Antonio Fernández-Barbero et al in 2009** studied the internal structure, refractive index, and mechanical properties of the polymer network in hydrogels. They are known as super absorbent materials since they can absorb hundreds of times their own weight in solvent. They react quickly to local environmental changes. Microgels have found its use as carriers of therapeutic medications and as diagnostic agents since size changes are followed with changes in interior dimensions. [32]

12. **Wennerberg A et al in 2010** in their study putforth that the roughness of an implant surface is a key factor in osseointegration and in the long-term success of the implant. The study concluded that the optimum roughness value is in the range of 1–2 μm, as this provides an optimum degree of roughness to promote osseointegration, compared to the smoother or rougher surfaces. The best clinical results have been obtained with moderately rough surfaces (Sa between 1.0–2.0 μm). [33]

13. **Krisztina Ungvari et al in 2010** evaluated three cleaning solutions in their study, 3% H2O2 (5 min), saturated citric acid (pH 14 1) (1 min), or chlorhexidine gel were used for commercially pure (grade 4) machined Titanium discs (5 min). The dimethylthiazolyl-diphenyltetrazolium bromide (MTT) and bicinchoninic acid(BCA) protein content assays were used to study human epithelial cell attachment (24-h observation) and proliferation (72-h observation). The Titanium surface is not harmed by these chemicals. In contrast to chlorhexidine gel, cleaning with H2O2 modestly promotes human epithelial cell development. [34]

14. **Raquel Lourdes Faria et al in 2010** conducted a study to compare the antimicrobial effect of mouthwashes containing *Calendula officinalis., Camellia sinensis* and 0.12% chlorhexidine digluconate on the adherence of microorganisms to suture materials after extraction of unerupted third molars. The microorganisms tested were *Staphylococcus, Streptocococcus mitis, Pseudomonas and Candida*. The three mouthwashes tested reduced the number of microorganisms adhered to the sutures compared to the control group. The study concluded that *calendula officinalis and camellia sinensis* presented antimicrobial activity against the adherence of microorganisms to sutures but was not as efficient as chlorhexidine digluconate. [35]

15. **Pragtipal Saini et al in 2012** conducted a study to evaluate the effects of *Calendula officinalis* on gingival fibroblasts mediated collagen degradation and MMP activity. Lactate dehydrogenate assays were performed to determine the non-toxic concentrations of Calendula, doxycycline and quercetin. Cell-mediated collagen degradation assays were performed to examine the inhibitory effect on cell mediated collagen degradation. Gelatin zymography was performed to examine their effects on MMP-2 activity. The study concluded that Calendula inhibits HGF-mediated collagen degradation and MMP-2 activity more than the corresponding concentration of quercetin. This may be attributed to additional components in Calendula other than quercetin.[36]

16. **Leila Maria Leal Parente et al in 2012** studied the angiogenic activity of *Calendula officinalis* extract and fractions was evaluated through the chorioallantoic membrane and cutaneous wounds in rat models. The healing

activity of the extract was evaluated by the same cutaneous wounds model through macroscopic, morphometric, histopathologic, and immunohistochemical analysis. The antibacterial activity of the extract and fractions was also evaluated. This experimental study revealed that Calendula officinalis presented anti-inflammatory and antibacterial activities as well as angiogenic and fibroplastic properties acting in a positive way on the inflammatory and proliferative phases of the healing process. [37]

17. **Marcelo H. Napimoga et al in 2013** studied the effect on quercetin on bone loss and inflammation in a rat periodontitis model induced by *A. actinomycetemcomitans* infection. effects.Thus, Subcutaneous treatment with quercetin reduced *A. actinomycetemcomitans*-induced bone loss and IL-1β, TNF-α, IL-17,RANKL, and ICAM-1 production in the gingival tissue without affecting bacterial counts. These results demonstrated that quercetin exhibits protective effects in *A. actinomycetemcomitans*-induced periodontitis in mice by modulating cytokine and ICAM-1 production. [38]

18. **Jemat A et al in 2015** conducted a review which covered several basic methodologies of surface treatment and their effects on titanium implants. Significant surface roughness played an important role in providing effective surface for bone implant contact, cell proliferation, and removal torque, despite having good mechanical properties. An acid etched surface-modified and a coating application on commercial pure titanium implant was most preferable in producing the good surface roughness. Thus, a combination of a good surface roughness and mechanical properties of titanium could lead to successful dental implants. [39]

19. **Xureb M et al in 2015** conducted a systematic review of the current dental implant coating materials and novel coating techniques.Several materials have been identified to coat dental implants including calcium , hydroxyapatite, bone stimulating factors among others. The most common coating techniques include plasma spraying and hydrocoating in which the material was directly applied to the surface from the solution. [40]

20. **Amparo Mendoza-Arnau et al in 2016** conducted a study to characterize the surface topography of dental implants. A rough implant surface improves cellular adhesion however, too rough a surface hinders osseointegration and biological response. An ideal Ra roughness range (0.775µm± 0.058µm) and Rt range (5.258µm ± 0.554) have been proposed. All the implant systems analysed in this study had lower scores. This study also found that a medium R_a achieved the best cell adhesion, coming to the conclusion that high roughness is not necessary in order to achieve the best cell response. [41]

21. **Laura K. Uribe-Fentanes et al in 2016** conducted an in vivo study to evaluate the action of *Calendula officinalis* essence on bone preservation after extraction. *Calendula officinalis* 10% was used as an irrigant during surgical extraction of third molars and was continued as a mouthwash for a week while in the control group saline was used. Periapical radiographs were taken and alveolar ridges and depth of alveolar bone were measured and compared with control group. There was statistically significant evidence to state that *Calendula officinalis* favorably affects bone preservation after extraction. [42]

22. **Joanna Trycia M. Alexandre et al in 2017** conducted an animal study to evaluate the anti-inflammatory and antiresorptive effects of *Calendula*

officinalis on alveolar bone loss in rats. Rats received saline solution or Calendula (10, 30, or 90 mg/kg) 30 minutes before ligature and daily until the 11th day. Out of the concentrations of Calendula officinalis used, 90 mg/kg reduced bone loss, neutrophilia, the levels of pro-inflammatory mediators, and RANKL expression, while it increased OPG immunopositive cells and BALP serum levels which are important markers for bone regeneration. [15]

23. **Shrinidhi Maji Shankar et al in 2017** conducted a study to assess the efficacy of *Calendula officinalis* against five oral microbes as compared to gold standard Chlorhexidine gluconate. *Porphyromonas gingivalis* and *Prevotella intermedia* were sensitive to *Calendula officinalis*, while *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* exhibited reduced sensitivity. *Calendula officinalis* took longer than chlorhexidine digluconate 0.2% to exhibit lethality against all the organisms that it inhibited. *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* were inhibited by *Calendula officinalis* at 30 minutes, there was no inhibition *of Porphyromonas gingivalis* by *Calendula officinalis*. [14]

24. **Shiva Charan Yadav et al in 2017** conducted an invitro study to evaluate the antimicrobial activity of *Calendula officinalis* against various periopathogens *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Tanerella forsythia.* The Minimum Inhibitory Concentration was established by tube dilution method. *Porphyromonas gingivalis* showed sensitivity at the concentration of 0.8 μg/ml, *Aggregatibacter actinomycetemcomitans* showed sensitivity at the concentration of 25μg/ml, *Fusobacterium nucleatum* showed sensitivity at the concentration of 100µg/ml while *Tanerella forsythia* showed resistance through out. [43]

25. **Ramesh Chowdhary et al in 2018** conducted a study to evaluate the cell viability of MG-63 cells with the Hydrogel formulated at different concentrations from acemannan and curcuminoids coated on titanium discs. Discs were coated with hydrogel using dipping method and MTT Assay was used to assess the cell viability of osteoblast like MG-63 cells. The study concluded that acemannan and curcuminoid hydrogel coated on titanium discs had good cell viability at 24 hours however changing the concentration of the extracts had no effect on the proliferation of cells. [44]

26. **Darius Gleiznys et al in 2019** conducted a study with an intend to analyze the effect of polyphenols and flavonoids substrates from *Calendula officinalis, Salvia fruticosa, Achillea millefolium,* and propolis as immunomodulatory in the production of IL-1b and IL-10 in peripheral blood leukocytes of patients who were diagnosed with periimplant mucositis compared to patients with healthy implant tissue. The levels of IL-1b decreased more considerably in the mucositis group than in those of healthy group after the treatment with the plant extracts at only 10.0 mg/mL concentration. These results suggest that this herbal solution might offer a new potential for the development of a new therapeutic path to prevent and treat peri-implant mucositis. [45]

27. **Yanlong Yin et al in 2020** carried out a study to assess the specific and distinctive capabilities of hydrogels as carrier systems for the delivery of a variety of cargo molecules in comparison to other nanomaterials. These are soft materials having crosslinked networks that can store tiny molecular

medicines, biomacromolecules and inorganic nanoparticles, allowing them to be used for therapy and imaging of a variety of illness conditions. Nanogels' stimuli-responsive behaviour can be easily controlled by selecting constituent polymer and crosslinker components to achieve a desired response at the site of action, giving them the ability to actively participate in the carrier system's intended function rather than being passive cargo carriers. [46]

28. **Samira Camargo et al in 2020** evaluated the influence of titanium coatings Titanium Nitride (TiN), Quarternized Titanium Nitride (QTiN), Silicon Carbide (SiC), Quarternized Silicon Carbide (QSiC) on monomicrobial biofilm adhesion and the viability of human osteoblasts in contact with this coated titanium. All the coatings exhibited a lower biofilm coverage compared to the uncoated samples which was confirmed by Scanning Electron Microscope (SEM) images. Fluorescence images demonstrated that the osteoblast cells adhered and proliferated on the surfaces. These coatings may possess the ability to prevent the development of peri-implantitis and stimulate osteointegration, thereby an ideal implant coating should possess both antibacterial and osseointegrative properties. [47]

29. **Nader Tanideh et al in 2020** conducted a study to compare the effects of *Calendula officinalis* and *Hypericum perforatum* on antioxidant, anti-inflammatory and histopathologic indices of induced periodontitis in male rats. Each group was treated with a mix of two plants and saline. The plant group showed decreased IL-1β, increased the antioxidant parameter in comparison to the control group. There were significant histopathological differences between the treatment group and the control group. The study concluded that mixed hydroalcoholic extract of *Calendula officinalis* and *Hypericum perforatum* might be considered as an adjunctive treatment for periodontitis. [16]

30. **Raluca-Adriana Milutinovici et al in 2021** conducted a review which focused on the most used plants in the dental field, especially on active phytocompounds. The main phytocompounds found in *Calendula officinalis* are flavonoids, tannins, polysaccharides, phenolic acids, tannins and saponosides. Its therapeutic property is due to the increase in hyaluronic acid deposits due to the flavonoids contained in calendula. The main biological actions that give utility to calendula in dental treatment is due to anti-inflammatory action by reducing proinflammatory cytokines, antioxidant effects due to the content of flavonoids and carotenoids and immunomodulatory effects due to the content of polysaccharides. [48]

31. **Moustafa K. El-Sayed et al in 2021** conducted a study to assess the effects of Calendula based topical formula on palatal wound healing after free palatal graft surgery in comparison to oxidized regenerated cellulose. The surgical sites were divided into two groups based on topical formula used i.e Calendula based, oxidized regenerated cellulose. Palatal wound healing was assessed using photo-digital planimetry on the day of the surgery and; at seven and fourteen days, post-surgical and pain was assessed by visual analogue scale at one, four and seven days post-surgical.This study concluded that topical Calendula could be equivalent to oxidized regenerated cellulose to be used for faster and better healing. [49]

32. **Karthikeya Patil et al in 2022** gave an overview of the use of Calendula and its pharmacological effects. *Calendula officinalis* belonging to the Asteraceae

family is a fragrant herb that has been used in traditional medicine for centuries. Alkaloids, flavonoids, and saponins are found in ethanolic extract. Flavonoids and saponins are found in aqueous extract. Plastoquinone, phylloquinone, tocopherol, and ubiquinone are isolated from quinone in different sections of *Calendula officinalis*. Quercetin, isorhamnetin, isoquercetin, and other flavonoids were also extracted from *Calendula officinalis*. The methanol extract of *Calendula officinalis* showed better antibacterial activity than most of the bacteria tested and was better than the ethanol extract. *Calendula officinalis* preparations are mostly used as a wound healing medicine for inflammations of the skin, mucous membranes, tissue repair, scars, blisters. [21]

33. **Bharath Kumar Ayyanahalli Matta et al in 2022** conducted an in-vitro study to analyze the cytotoxicity and hemostatic properties of gelatin sponge and *Calendula officinalis*. The MTT assay showed 7% *Calendula officinalis* to be cytocompatible, and there was an increase in cell proliferation. This concentration when incorporated in the gelatin sponge took 4.60sec for clot formation. Hence, it could be concluded that when *Calendula officinalis* is incorporated into a gelatin sponge, it shows material compatibility and cytocompatibility, reduces the time for clot formation, and could be used as an alternative to other hemostatic agents. [50]

34. **Mayur Khairnar et al in 2022** conducted a study to evaluate the efficacy of *Calendula officinalis* in reducing dental plaque and gingival inflammation. The patients in the test group were advised to have 2 ml of tincture of calendula as a mouthwash for six months. Clinical parameters like the plaque index, gingival index, sulcus bleeding index and oral hygiene index-simplified were recorded at baseline, third month and sixth month. There was statistically significant reduction in the scores of all parameters, when the third month scores were compared with the sixth month scores in both groups. The study concluded that calendula mouthwash was effective in reducing dental plaque and gingivitis adjunctive to scaling. [51]

35. **Nian Liu et al in 2022** conducted a study to evaluate the antimicrobial and osseointegrative properties of quercetin coating on 3D-Printed Ti6Al4V Implant. The study concluded that quercetin-loading provided a feasible alternative for coating on 3d printed titanium implants with enhanced rapid osseointegration as shown by its rapid cell adhesion and proliferation and confirmed by Scanning Electron Microscopy images and anti-inflammatory properties, and the specific mechanisms of quercetin promoting osteogenesis and anti-inflammation through modulating polarization.[52]

MATERIALS AND METHODOLOGY

SOURCE OF DATA:

This in vitro study was conducted in KAHER KLE VKIDS Department of Prosthodontics and Crown & Bridge, Department of Pharmaceutics KAHER KLE College of Pharmacy, Gogte Institute of Technology, KAHER Dr. Prabhakar Kore's Basic Science Research Centre and Goa University.

This research was intended to evaluate the antimicrobial efficacy of *Calendula officinalis* hydrogel against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus* and the osteogenic potential of Calendula officinalis hydrogel coated on titanium surface.

METHOD OF COLLECTION OF DATA:

INCLUSION CRITERIA:

- Identical Titanium disc shaped specimens measuring 10 mm in diameter and 2 mm in thickness (ASTM B348).

EXCLUSION CRTERIA:

- Specimens with internal and external porosities.
- Specimens with surface irregularities. (Ra> 4μm)

Table No. 1: - List of Materials used for hydrogel formulation, microbial analysis and assessment of osteogenic potential

Material	Description	Manufacturer
Titanium Alloy	TYPE V (Ti-6Al-4V alloy)	Special Metals , Mumbai, India
Calendula Officinalis extract	Hydroalcoholic	Kuber Impex Ltd , Indore, India
Chlorhexidine Gel	Hexi gel 1%	ICPA Health products, India
Phosphate buffer solvent (6.8 pH)	Sodium chloride, sodium dihydrogen orthophosphate dihydrate, potassium chloride, distilled water	Himedia, Mumbai, India
Carbopol gel base	Carbopol 940	OEM manufacturers
Distilled water	Batch No.:-007M15	Rankem Chemicals, Avantor, India
Nutrient Agar	Culture media	Hi-media, Mumbai, India
Blood agar	Culture media	Hi-media, Mumbai, India
70% Ethanol	LOT No. : -20151011	Changshu Hongsheng Fine Chemicals Co., Ltd.
Phosphate Buffer Saline	LOT No.0000237353	Hi Media, Mumbai
Dulbecco’s Eagle Medium	LOT No.0000284902	Hi media, Mumbai
Trypsin EDTA	LOT No.0000297540	Hi media, Mumbai
MTT Reagent	LOT No.0000173715	Hi Media, Mumbai
Tryphan Blue	LOT No. : - 2024334	Hi Media, Mumbai
MG- 63 Cell Line		NCCS, Pune

Table No. 2: - Armamentarium used for evaluation of surface characteristics, microbial analysis & assessment of osteogenic potential of the test specimen

Material	Description	Manufacturer
Profilometer	Contact profilometer Model: - Surtronic S-128	Taylor Hobson,Brazil.
Scanning Electron Microscope	FE- SEM	Carl- Zeiss
Laminar Air Flow	Model: - Vertical	Quest International, Bangalore.
Micropipette	Model No. : - 299932	Riviera Glass Pvt, Ltd., Mumbai
Tissue Culture Plate	24 well plate	Tarsons, Korea
Electric Loop Sterilization	Model.:-i-therm A1-401	Hi-Media, Mumbai
Anaerobic Jar	LOT No.:- 14-1016	Hi-Media, Mumbai
Hemocytometer	-	Rohem, India
Microscope	TCM400	LABOMED,USA
CO2 Incubator	Galaxy 170R	Eppendorf, India
Micro Titre PlateReader	Epoch	BioTek, USA

METHODOLOGY:

1. **Preparation of specimen**

A total of 270 commercially available identical Titanium Grade V 10 mm x 2 mm (ASTM B348) disc shaped specimens were used in the study. (Fig 1)

For antimicrobial efficacy, 90 titanium discs were used and divided as study and $control_{MA}$ groups (n=45)

Study Group- Titanium Discs surface treated with calendula officinalis hydrogel

$Control_{MA}$ Group – Titanium discs treated with 1 % Chlorhexidine gel

The study and $control_{MA}$ groups were further subdivided into three subgroups (n=15) based on the microorganisms tested i.e *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus.*

For osteogenic potential, 180 titanium discs were used which were divided as Study and $Control_{OP}$ groups.

Study Group -Titanium Discs surface treated with calendula officinalis hydrogel

$Control_{OP}$ Group - Machined titanium discs

The study and $control_{OP}$ groups were further divided based on cell attachment and cell proliferation assays (n=45). These groups were subdivided (n=15) based on the time interval tested i.e. 24 hours, 48 hours, 72 hours.

These identical Titanium discs were sandblasted for 1 minute at a constant pressure of 4kg/cm^2 with 50 µm alumina. The discs were ultrasonically cleaned using acetone for 180 seconds to remove any residual contaminants.

2. Surface analysis

The specimens in each group were numbered from 1 to 45. The specimens were subjected to surface roughness evaluation quantitatively and qualitatively.

The quantitative analysis of Surface roughness of the specimens was carried out using Contact Stylus Profilometer (Surtronic S-128 – Taylor Hobson). An average roughness profile (R_a) was evaluated for each specimen to describe the overall roughness of the surface. Each disc was placed on a flat surface with surface to be tested facing upwards. The Profilometer determined the surface profile along 3 lines on the surface by means of a tracking device. The disc specimens were mounted and diamond point-stylus was made to run with a transverse length of 4 mm and cut off length of 0.8 mm and the arithmetical average value of all absolute distances of the roughness profile from the center line within the measuring length were calculated. (Fig 2) Each group had 45 test specimens with their R_a measured by the Profilometer. To evaluate the surface roughness qualitatively, specimens from each group were examined under field emission Scanning Electron Microscope (SEM , Carl Zeiss). The SEM photographs were made at 100X, 500X and 1000X magnification for better visualization. (Fig 3) Random specimen from each sub- group were selected and subjected to Scanning Electron Microscopy. The specimens were rinsed with distilled water, dried, and fixed onto an aluminum cylinder (13 mm in diameter and 10 mm in height). The topographic observations of the polished surface were compared with each other as a complement to the quantitative results obtained by surface roughness assessment.

3. **To assess the antimicrobial efficacy**

a. **Determination of Minimum Inhibitory Concentration (MIC) for *Calendula officinalis:***

A standard procedure for performing the Minimum Inhibitory Concentration test was followed. *Porphyromonas gingivalis* ATCC 33277 and *Aggregatibacter actinomycetemcomitans* ATCC 33384 were revived by plating on Blood Agar and was incubated in an anerobic chamber. *Staphylococcus aureus* ATCC 25923 were revived by plating on Brain Heart Infusion Agar and was incubated in an aerobic chamber. Isolated colonies were transferred to sterile Brain Heart Infusion broth and incubated at 37^oC for 48 hours anaerobically while for *Staphylococcus aureus* it was incubated at 37^oC for 24 hours aerobically. The growth concentration was adjusted to 10^5 organisms / ml by using 0.5 McFarland's turbidity standard. A stock solution was prepared by dissolving 1 gram *Calendula officinalis* extract in 100 ml solvent (1 % DMSO and 99% Distilled Water). A 1 ml of the Brain Heart Infusion broth was added in six Minimum Inhibitory Concentration tubes. In first Minimum Inhibitory Concentration tube containing 1 ml broth, 1 ml of *Calendula officinalis* extract (stock solution) was added. After mixing well, 1ml was transferred in second Minimum Inhibitory Concentration tube. This was continued till (6^{th}) tube. From the last well 1 ml was discarded, by following this serial dilution, the following concentration of the *Calendula officinalis* was achieved i.e., 10, 5, 2.5, 1.25, 0.625, 0.31mg/ml. Then to each such test tube 50 µl of earlier prepared strains of *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Staphylococcus aureus* were added. The tubes were then incubated. Turbidity in the Minimum Inhibitory Concentration tube indicates growth of the *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Staphylococcus aureus* implying that it was resistant to *Calendula officinalis*.

Based on the results MIC was established as 5mg/ml for *Porphyromonas gingivalis*, 2.5 mg/ml for *Aggregatibacter actinomycetemcomitans* and 5 mg/ml for *Staphylococcus aureus*

b. Determination of Minimum Bactericidal Concentrations (MBC) for *Calendula officinalis*

To determine bactericidal concentration, the MIC dilution tubes with no visible growth (no turbidity) were subcultured onto blood agar and incubated for 48 hours at 37 ^{o}C anaerobically. For *Staphylococcus aureus* it was subcultured on BHI agar and incubated for 24 hours at 37 ^{o}C in an aerobic chamber and the resultant colonies were counted. The resultant colony counts in the test group were compared with the control group. The test results were interpreted as follows: If there are similar number of colonies which indicated bacteriostatic activity only, while reduced number of colonies denotes a partial or slow bactericidal activity. If no growth is observed it indicated a complete bactericidal effect. Based on the results the MBC it was established as 5mg/ml for *Porphyromonas gingivalis* and *Staphylococcus aureus* and 2.5mg/ml for *Aggregatibacter actinomycetemcomitans.*

c. Cytotoxicity evaluation:

The MTT solution was prepared using 5 mg MTT reagent in 1 ml of Phosphate Buffer Saline (PBS – pH 7.4) and Cytotoxicity Assay was performed on mouse fibroblast strain L929. In vitro growth inhibition effect of *Calendula officinalis* was assessed by ELISA reader by determination of conversion of MTT into "Formazan blue" by living cells. The 50µl of 4000 cells/ml cell suspension were seeded into each well in a 96 well micro titer plate and final volume was made up to 150 µl by adding Dulbecco's Modified Eagle Medium (DMEM) media. Dilution of *Calendula officinalis* was prepared at a concentration of 10 % DMEM media.100µl of the *Calendula officinalis* was added to the wells and incubated for 24 hours, in presence of 5 % CO_2, at 37^0C into CO_2 incubator. After 24 hours, 20µl of 5 mg/ ml MTT reagent was added to the wells. The plate was incubated for 4 hours in dark place at room temperature. (The plate was covered with aluminium foil, since MTT reagent is photosensitive.). The supernatant was carefully removed without disturbing the precipitated Formazan crystals and 100 µl of DMSO was added to dissolve the crystals formed. The optical density (OD) was measured at wavelength of 570 nm. The study was performed in triplicates. The result represents the mean of three readings. Results demonstrated favourable cell viability with the tested concentration. The 10mg/ml Calendula concentration resulted in 108 % cell viability.

Formula**:**

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of test compound} \times 100}{\text{Mean OD at control (untreated cells)}}$$

4. **Formulation of hydrogel**

The weighed quantity of Carbopol 940 (3gms) was distributed in 100ml of distilled water with a help of magnetic stirrer for 2 hrs. The soaked polymer solution was kept for 24hrs for complete hydration. Weighed quantity of extract 10gms (2%) (Fig 4) was dispersed in the above polymer gel with constant stirring at high-speed propeller for half hour. To above mixture 5% Glycerin, 0.5% Sodium benzoate, and 0.01% Methyl paraben were added as preservatives. The pH was adjusted to 7.0 with Triethanolamine. (Fig 5) Final product was stored in air tight container at room temperature. (Fig 6).

Each titanium disc in the study group was then coated with *calendula officinalis* hydrogel by dipping method in which the disc was immersed in freshly prepared hydrogel for 45-60 seconds. (Fig 8)

5. **Microbial analysis-**

The disc-diffusion method was used to assess the effectiveness of 2% *Calendula officinialis* incorporated hydrogel and 1 % Chlorhexidine gel (Fig 7) which were the study and control$_{MA}$ group respectively against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* , *Staphylococcus aureus* standard bacterial strains.

The standard strain of *Porphyromonas gingivalis* (ATCC 33277) was inoculated on blood agar supplemented with haemin and vitamin K at 37°C for 72 - 96 hours. The standard strain of *Aggregatibacter actinomycetemcomitans* (ATCC 33384) was inoculated blood agar supplemented with haemin and vitamin K at 37°C for 72 - 96 hours. The standard microbial strain of *Staphylococcus aureus* (ATCC

25923) inoculated on Nutrient agar at 37°C for 18 - 24 hours. Fresh isolates were transferred to sterile saline and a microbial suspension was prepared and the turbidity adjusted to 0.5 McFarland standard to yield approximately 106 CFU/mL.The inoculum was evenly spread on Blood agar and Nutrient agar. The agar plate was streaked with standard bacterial strains. After the inoculum dries, a 12 mm diameter and 3mm depth well was punched with a sterile cork borer. The sterile discs were placed.

Each plate contained two discs:

1. Study group- The discs were surface treated with Calendula Officinalis hydrogel.
2. Control$_{MA}$ group- The discs were surface treated with 1% Chlorhexidine (CHX) gel.

The plates were incubated at 37°C for - aerobic – 18 to 24 hours for *Staphylococcus aureus* and anerobic – 72 to 96 hours for *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*

Zone Of Inhibition: There was absence of bacterial colonies around the antimicrobial agent forming a clear zone around this agent which was the zone of inhibition.(Fig 9) The zone of inhibition was measured in millimeters (mm), tabulated and subjected to statistical analysis.

6. **Assessment of Osteogenic Potential:**

A total of 180 titanium discs were subjected to surface characterization. The specimens were divided into study group (n=90) titanium discs coated with calendula officinalis hydrogel and control$_{OP}$ (n=90) titanium discs without any surface treatment. The resultant specimens were subdivided into two groups for cell attachment assay (n=45) and cell proliferation (n=45). The specimens were further subdivided into (n=15) each subgroup for cell attachment and cell proliferation for different time intervals studied i.e. 24 hours, 48 hours, 72 hours respectively. MG-63 cell lines (osteoblast like cells) were used to assess the osteogenic potential (Fig 10)

a. Cell attachment-

The tryphan blue exclusion test was used to assess the number of viable cells. The viable cells with an intact cell membrane mixed with dyes will take up the dye and will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. The MG 63 cells were cultured onto specimens in the control$_{OP}$ and study group (Fig 11A). The culture medium was removed after 24, 48 and 72 hour time interval and the wells were washed thrice using Phosphate-buffered solution (PBS) at 37ºC to eliminate unattached cells. Tryphan blue was added to the cell suspension and the adherent cells were then enzymatically counted using a hemacytometer.(Fig 11B) The unstained cells in the first set of 16 squares were counted followed by moving onto the next set. Cell attachment was expressed as the number of cells.

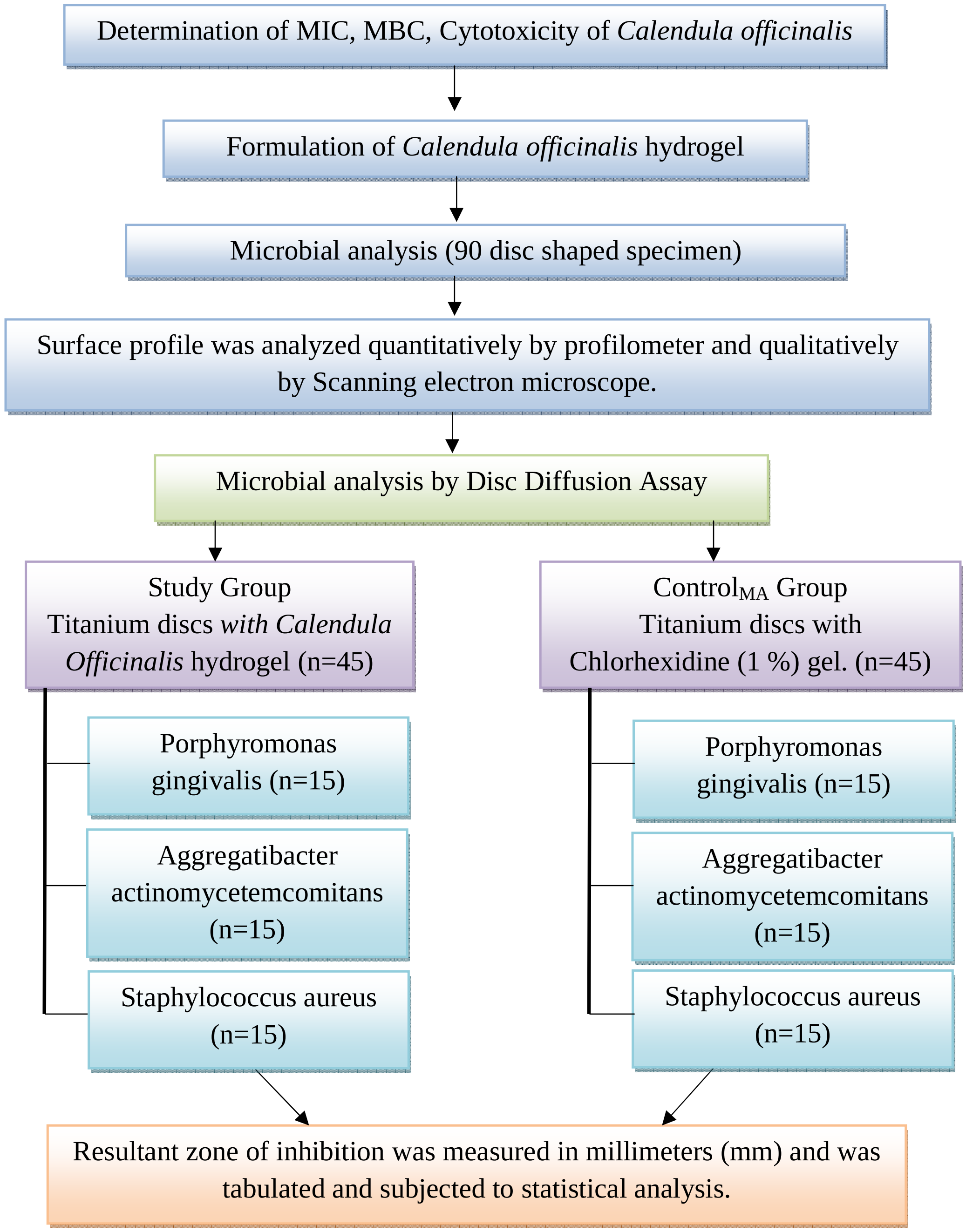
b. Cell Proliferation-

A 50µl of 1 × 10^5 cells/ml cell suspension was seeded into each well microtiter plate and final volume was made up to 150 µl by adding DMEM media.100µl of the above dilutions was added to the wells and incubated for 72 hours, in presence of 5 % CO2, at 37^0C into CO_2 incubator.

After 72 hours, 20µl of 5 mg/ ml MTT reagent was added to the wells. The plate was kept for 4 hours incubation in dark place at room temperature.(Fig 12) The supernatant was removed without disturbing the precipitated Formazan crystals and 200 µl of DMSO was added to dissolve the crystals formed. The optical density (OD) was measured using micro titre plate reader at a wavelength of 570 nm at 24,48 and 72 hours. (Fig 13)

$$\text{Formula: Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control (untreated cells)}} \times 100$$

The cell attachment and cell proliferation in study and control group was evaluated as percentage (%), tabulated and subjected to statistical analysis to draw the conclusion from resultant data.


Determination of MIC, MBC, Cytotoxicity of *Calendula officinalis*
Formulation of *Calendula officinalis* hydrogel
Microbial analysis (90 disc shaped specimen)
Surface profile was analyzed quantitatively by profilometer and qualitatively by Scanning electron microscope.
Microbial analysis by Disc Diffusion Assay
Study Group Titanium discs *with Calendula Officinalis* hydrogel (n=45)
$Control_{MA}$ Group Titanium discs with Chlorhexidine (1 %) gel. (n=45)
Porphyromonas gingivalis (n=15)
Aggregatibacter actinomycetemcomitans (n=15)
Staphylococcus aureus (n=15)
Porphyromonas gingivalis (n=15)
Aggregatibacter actinomycetemcomitans (n=15)
Staphylococcus aureus (n=15)
Resultant zone of inhibition was measured in millimeters (mm) and was tabulated and subjected to statistical analysis.

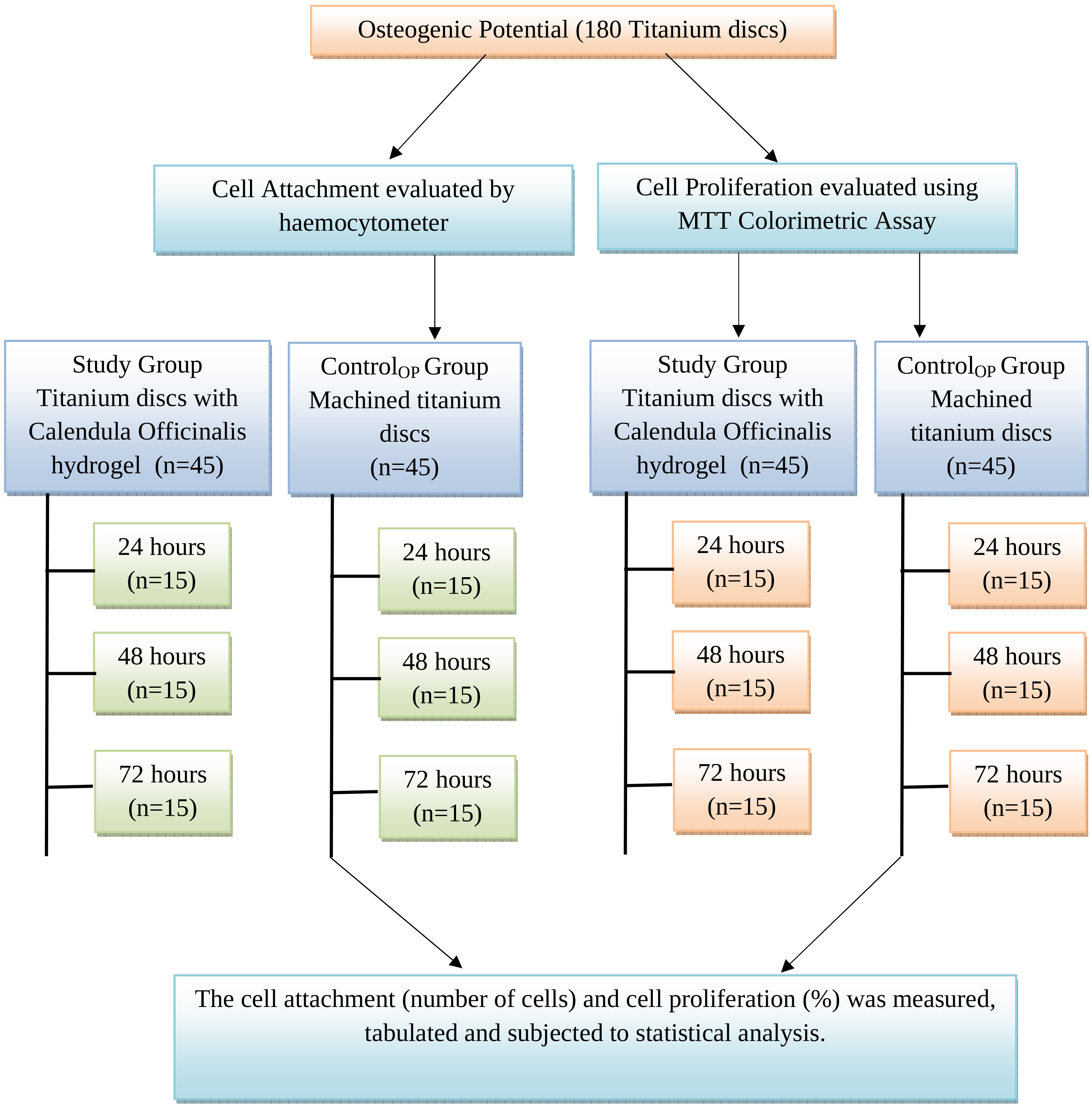

Osteogenic Potential (180 Titanium discs)
Cell Attachment evaluated by haemocytometer
Cell Proliferation evaluated using MTT Colorimetric Assay
Study Group Titanium discs with Calendula Officinalis hydrogel (n=45)
Control$_{OP}$ Group Machined titanium discs (n=45)
Study Group Titanium discs with Calendula Officinalis hydrogel (n=45)
Control$_{OP}$ Group Machined titanium discs (n=45)
24 hours (n=15)
48 hours (n=15)
72 hours (n=15)
24 hours (n=15)
48 hours (n=15)
72 hours (n=15)
24 hours (n=15)
48 hours (n=15)
72 hours (n=15)
24 hours (n=15)
48 hours (n=15)
72 hours (n=15)
The cell attachment (number of cells) and cell proliferation (%) was measured, tabulated and subjected to statistical analysis.

FIGURE NO 1 : DISC SHAPED TITANIUM SPECIMENS

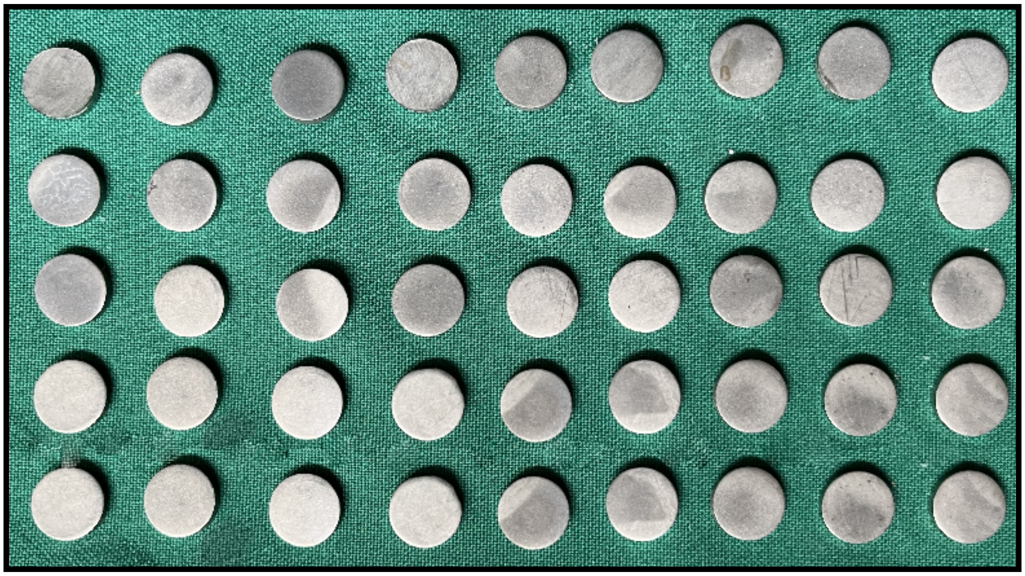


FIGURE NO 2 : QUANTITATIVE EVALUATION OF SURFACE ROUGHNESS OF SPECIMEN USING PROFILOMETER

FIGURE NO 3: QUALITATIVE EVALUATION OF SURFACE ROUGHNESS OF SPECIMEN USING SCANNING ELECTRON MICROSCOPY.

A: 100X B:500X C:1000X

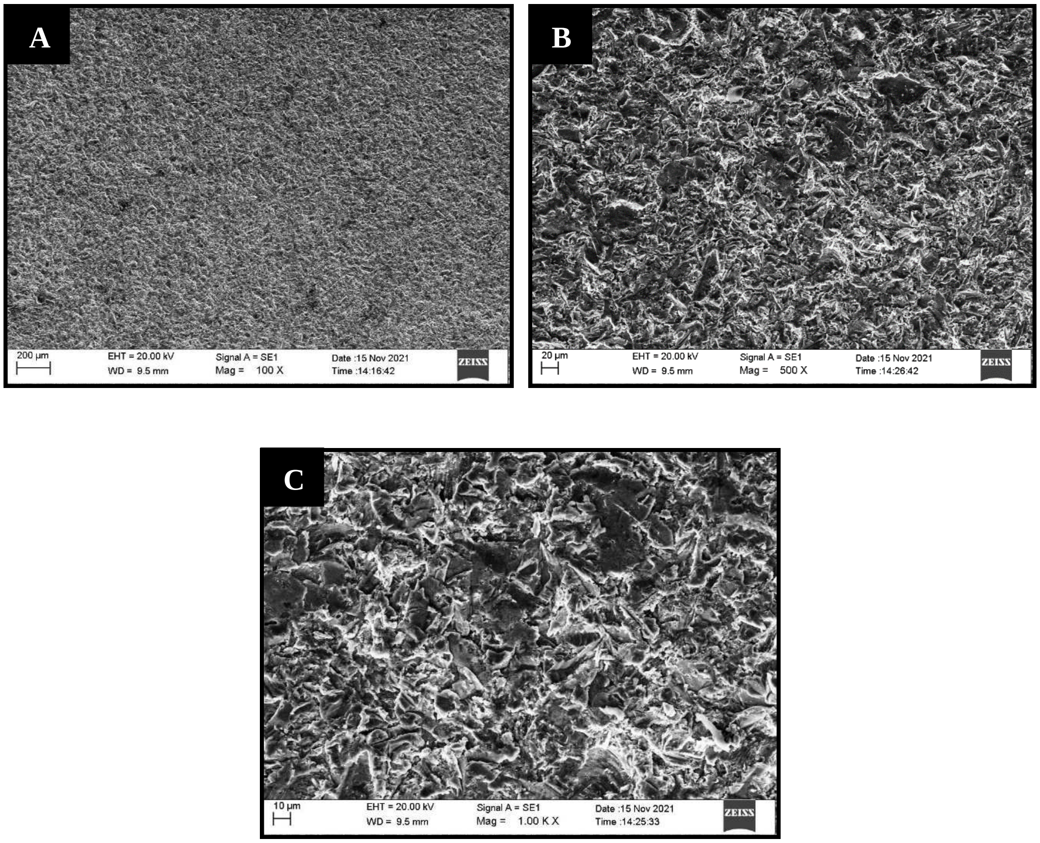


FIGURE NO 4: PREWEIGHED CALENDULA OFFICINALIS EXTRACT

FIGURE NO 5: ADJUSTED pH VERIFIED USING DIGITAL pH METER

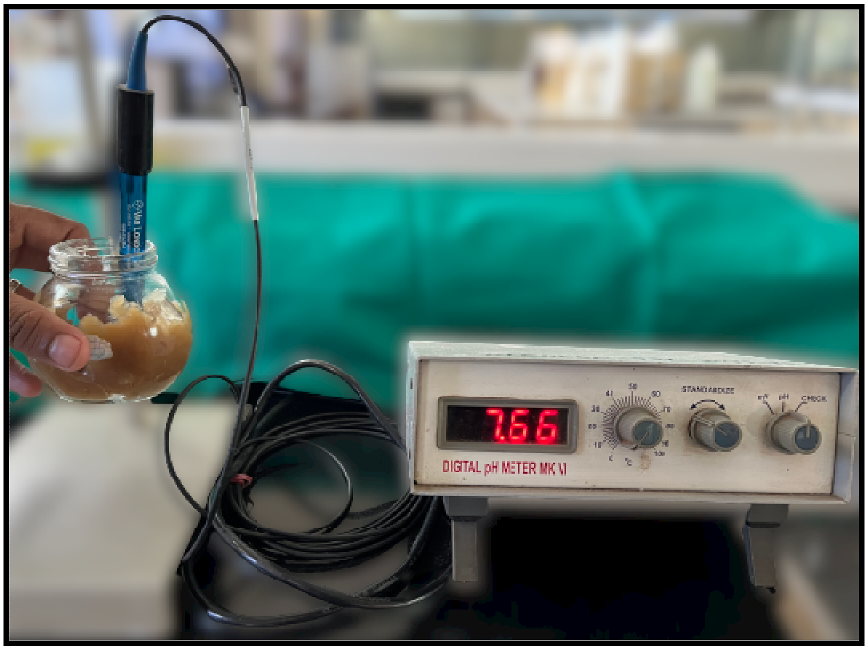


FIGURE NO 6: CALENDULA OFFICINALIS HYDROGEL

FIGURE NO 7: CHLORHEXIDINE GEL 1 %

FIGURE NO 8: COATING OF TITANIUM DISC WITH HYDROGEL BY DIPPING METHOD

FIGURE NO 9: ZONE OF INHIBITION AGAINST PERIIMPLANT PATHOGENS

A: STAPHYLOCOCCUS AUREUS B: PORPHYROMONAS GINGIVALIS

C: AGGREGATIBACTER ACTINOMYCETEMCOMITANS

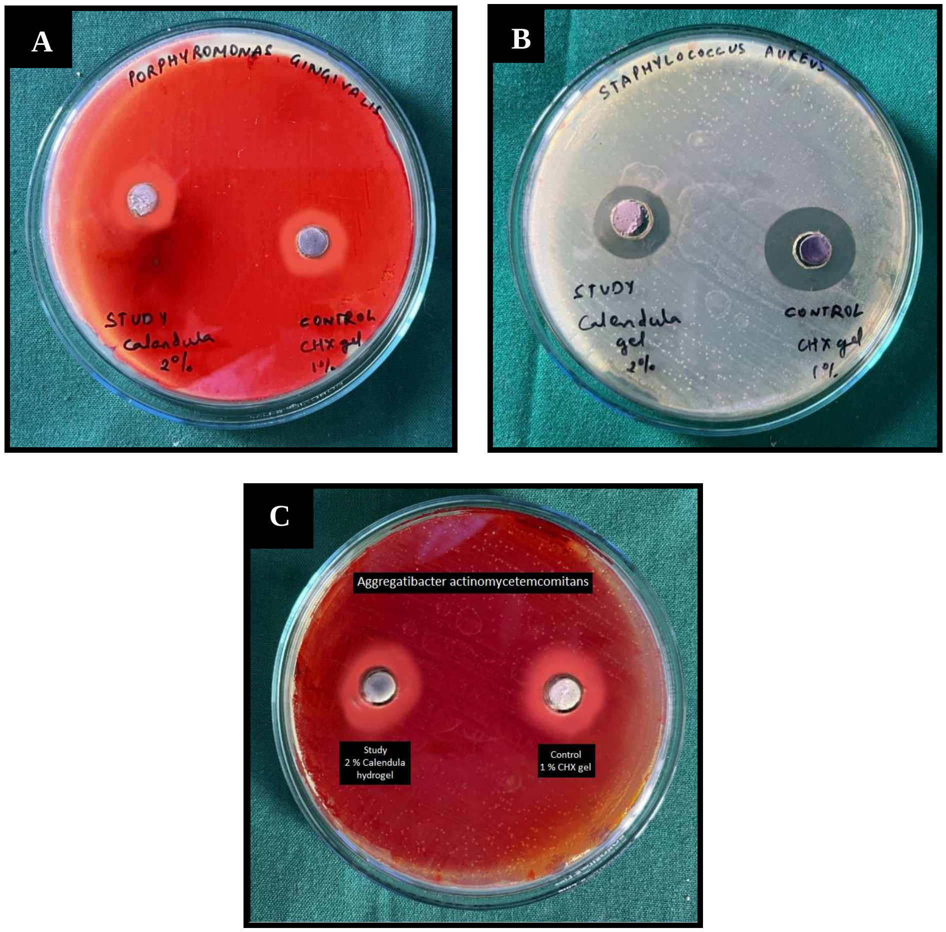


FIGURE NO 10: MG-63 CELL LINES USED TO ASSESS OSTEOGENIC POTENTIAL

FIGURE NO 11:

A: SPECIMENS SEEDED WITH MG-63 CELLS FOR ASSESSING CELL ATTACHMENT

B: EVALUATION OF ATTACHED CELLS ON HEMOCYTOMETER

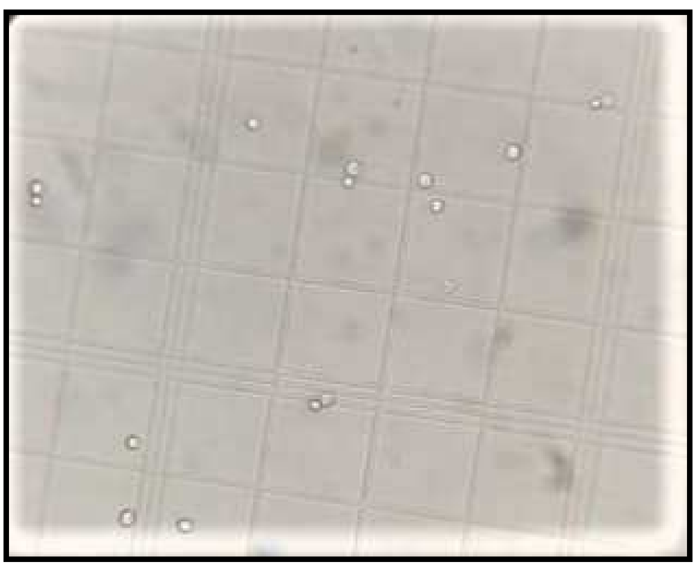
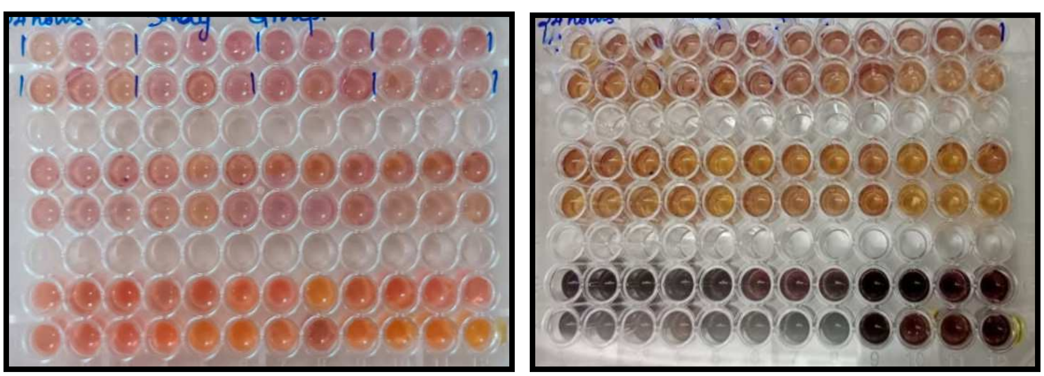


FIGURE NO 12: WELL PLATE WRAPPED IN ALUMINIUM FOIL FOR MTT ASSAY TO ASSESS CELL PROLIFERATION

FIGURE NO 13 : MTT ASSAY TO ASSESS CELL PROLIFERATION OF THE TEST AND CONTROL$_{OP}$ SPECIMENS AT 24,48 ,72 HOURS



RESULTS

This present study evaluated the antimicrobial efficacy of *calendula officinalis* hydrogel coated on Titanium discs against *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Staphylococcus aureus* and its osteogenic potential by assessing the cell attachment and cell proliferation of MG-63 cells on hydrogel coated surfaces at 24, 48, 72-hour time intervals. The antimicrobial efficacy was assessed by disc diffusion method and for osteogenic potential, the cell attachment was assessed using hemacytometer while cell proliferation was assessed by MTT Assay at 24, 48,72 hours.

Table 3: Summary of antimicrobial efficacy interms of zone of inhibition (in mm) in study and control$_{MA}$ groups and three organisms i.e *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Staphylococcus aureus*

Table 4: Comparison of two groups (Study and control$_{MA}$) and three organisms (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Staphylococcus aureus*) with mean zone of inhibition (in mm) by Two-way ANOVA

Table 5 and Graph 1: Pair wise comparison of study and control$_{MA}$ group with respect to zone of inhibition by Tukeys multiple post hoc test.

Table 6 and Graph 2: Comparison of study group and control$_{OP}$ group with respect to cell attachment at different time intervals 24,48,72 hours by independent t test

Table 7 and Graph 3: Comparison of different time intervals with respect to cell attachment in study group and control$_{OP}$ group by dependent t and repeated measures of ANOVA

Table 8 and Graph 4: Comparison of study group and control$_{OP}$ group with percentage of cell proliferation at different time intervals 24, 48, 72 hours by independent t test

Table 9 and Graph 5: Comparison of different time intervals with respect to percentage of cell proliferation in study group and control$_{OP}$ group by dependent t and repeated measures of ANOVA

The resultant values of antimicrobial efficacy in terms of zone of inhibition (mm) and osteogenic potential by means of cell attachment (number of cells) and cell proliferation (%) of specimens in Study Group, Control$_{MA}$, Control$_{OP}$ Group were subjected to statistical analysis to draw conclusion from the experimental data. Descriptive statistical measures such as mean, standard deviation were computed for all the groups. Pair wise comparison of study group for antimicrobial efficacy was done using two-way ANOVA. Pair wise comparison of study group for osteogenic potential was done using dependent t test and repeated measure ANOVA and comparison between different time intervals was done using independent t test. The p value of <0.05 was considered statistically significant.

The mean and standard deviation was calculated for the study group (titanium discs surface treated with *Calendula officinalis* hydrogel) and control$_{MA}$ group (titanium discs surface treated with 1 % Chlorhexidine gel).

Table 3 shows the summary of antimicrobial efficacy interms of zone of inhibition for the study group with discs treated with *Calendula officinalis* hydrogel and $control_{MA}$ group consisting of discs treated with 1 % Chlorhexidine gel against *Porphyromonas gingivalis*, *Aggregatibacter Actinomycetemcomitans, Staphylococcus aureus.*

The mean and standard deviation for the zone of inhibition (in mm) for the study and $control_{MA}$ group was calculated using Two way ANOVA. There was a statistically significant difference in the zone of inhibition between the two groups. (p=0.0001*) and between the three organisms (p=0.0005*). The pair wise comparison of the two groups for the mean zone of inhibition was calculated using Tukeys multiple posthoc. The mean value and standard deviation for the study group was 14.33±1.48 and 17.47±1.85 which was statistically significant (p=0.0001*) (Table 4,5 and Graph 1).

The mean and standard deviation for the cell attachment on study group and $control_{OP}$ group was calculated by independent t test. The mean values of the cell attachment of the number of cells in the study group (193200 ± 43515) at 24 hours, 48 hours (242488 ±38229) and 72 hours (280110 ± 33860) were statistically significant (p = 0.0001*) as compared to the $control_{OP}$ group showing an increased cell attachment in the study group at all three-time intervals. (Table 6 & Graph 2)

On comparison between the time intervals within the groups, there was a statistically significant difference in the cell attachment between 24-48 hours (49288.20 ±62595.74) (p= 0.0055*) and 24-72 hours (86910.00 ± 45513.34) (p= 0.0001*) between the study and $control_{OP}$ group. (Table 6)

The mean and standard deviation for the cell attachment for the study group and $control_{OP}$ group at different time intervals were calculated using dependent t test and repeated measure of ANOVA. There was a statistically significant difference in the cell attachment in the study group between 24-48 hours (0.0087*), 48-72 hours (0.0086*), 24-72 hours (0.0001*) with an effect size of 59 %. However, in the $control_{OP}$ group there was no significant change in the cell attachment and had an effect size of 19%. (Table 7 & Graph 3)

The mean and standard deviation for the cell proliferation for the study group and $control_{OP}$ group was calculated by independent t test. The mean values of the cell proliferation in the study group (88.27 ± 3.73) at 24 hours, 48 hours (85.07 ±5.28) and 72 hours (83.80 ± 2.54) were statistically significant (p = 0.0001*) as compared to the $control_{OP}$ group showing an increased cell proliferation in the study group. (Table 8 & Graph 4)

The mean and standard deviation for the cell proliferation for the study group and $control_{OP}$ group at different time intervals were calculated using dependent t test and repeated measure of ANOVA. There was a no statistically significant difference in the percentage of cells in the study group between 24-48 hours (p = 0.1117), 48-72 hours (p= 0.2766) however there was a statistically significant difference in the 24–72-hour time interval (p=0.0050*)(Table 9 & Graph 5).

Table 3: Summary of antimicrobial efficacy interms of zone of inhibition (in mm) in study and control$_{MA}$ groups and three organisms i.e *Porphyromonas gingivalis, Aggregatibacter Actinomycetemcomitans and Staphylococcus aureus*

Levels of Factor	N	Mean	SD	SE	95% CI for mean	
					Lower	Upper
Study group	45	14.33	1.48	0.22	13.89	14.78
Control$_{MA}$ group	45	17.47	1.85	0.28	16.91	18.02
Porphyromonas Gingivalis	30	15.73	3.02	0.55	14.61	16.86
Aggregatibacter Actinomycetemcomitans	30	16.77	1.81	0.33	16.09	17.44
Staphylococcus Aureus	30	15.20	1.56	0.29	14.62	15.78
Study group with Porphyromonas Gingivalis	15	13.60	1.06	0.27	13.02	14.18
Study group with Aggregatibacter Actinomycetemcomitans	15	15.53	1.60	0.41	14.65	16.42
Study group with Staphylococcus Aureus	15	13.87	0.92	0.24	13.36	14.37
Control$_{MA}$ group with Porphyromonas Gingivalis	15	17.87	2.83	0.73	16.30	19.43
Control$_{MA}$ group with Aggregatibacter Actinomycetemcomitans	15	18.00	1.00	0.26	17.45	18.55
Control$_{MA}$ group with Staphylococcus Aureus	15	16.53	0.64	0.17	16.18	16.89

Table 4: Comparison of study and control$_{MA}$ groups and three organisms (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Staphylococcus aureus)* with mean Zone of inhibition (in mm) by two way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value
Main effects					
Groups	220.90	1	220.9000	95.3852	0.0001*
Organisms	38.07	2	19.0333	8.2186	0.0005*
2-way interaction effects					
Groups x organisms	14.60	2	7.3000	3.1522	0.0479*
Error	194.53	84	2.3159		
Total	468.10	89			

*$p<0.05$

This table concluded that there was a significant difference in the antimicrobial efficacy in terms of zone of inhibition between the Study and Control$_{MA}$ group as well as between the three organisms

Table 5: Pair wise comparison of two groups (Study and control$_{MA}$) with mean Zone of inhibition (in mm) by Tukeys multiple posthoc procedures

Groups	Study group	Control$_{MA}$ group
Mean	14.33	17.47
SD	1.48	1.85
Study group	-	
Control$_{MA}$ group	P=0.0001*	-

*p<0.05

On pair wise comparison of the groups, it showed a statistically significant differences in zone of inhibition (in mm) among the Study and Control$_{MA}$ Group.

Graph 1 : Comparison of Study and Control$_{MA}$ groups with mean zone of inhibition (in mm)

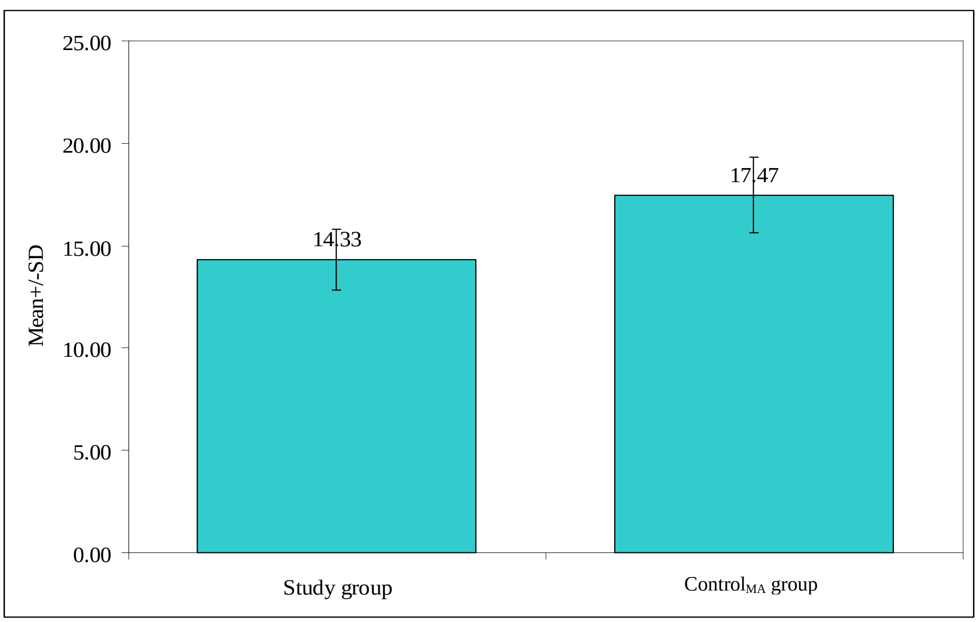


Table 6: Comparison of study group and control$_{OP}$ group with respect to cell attachment at different time intervals 24,48,72 hours by independent t test

Time points	Study group		Control$_{OP}$ group		t-value	p-value
	Mean	SD	Mean	SD		
24hrs	193200.00	43515.51	57121.33	8665.17	11.8781	0.0001*
48hrs	242488.20	38229.07	57097.33	6068.66	18.5497	0.0001*
72hrs	280110.00	33860.53	50725.00	7446.95	25.6248	0.0001*
24hrs-48hrs	49288.20	62595.74	-24.00	10959.79	3.0054	0.0055*
24hrs-72hrs	86910.00	45513.34	-6396.33	12775.81	7.6445	0.0001*

*p<0.05

This table demonstrates statistically significant difference in the cell attachment between the study and control groups at 24,48,72 hours. Also, there was a statistically significant difference in the cell attachment between 24-48 and 24-72 hour time intervals between the study and control$_{OP}$ group.

Graph 2: Comparison of study group and control$_{OP}$ group with respect to cell attachment at different time intervals of 24,48,72 hours.

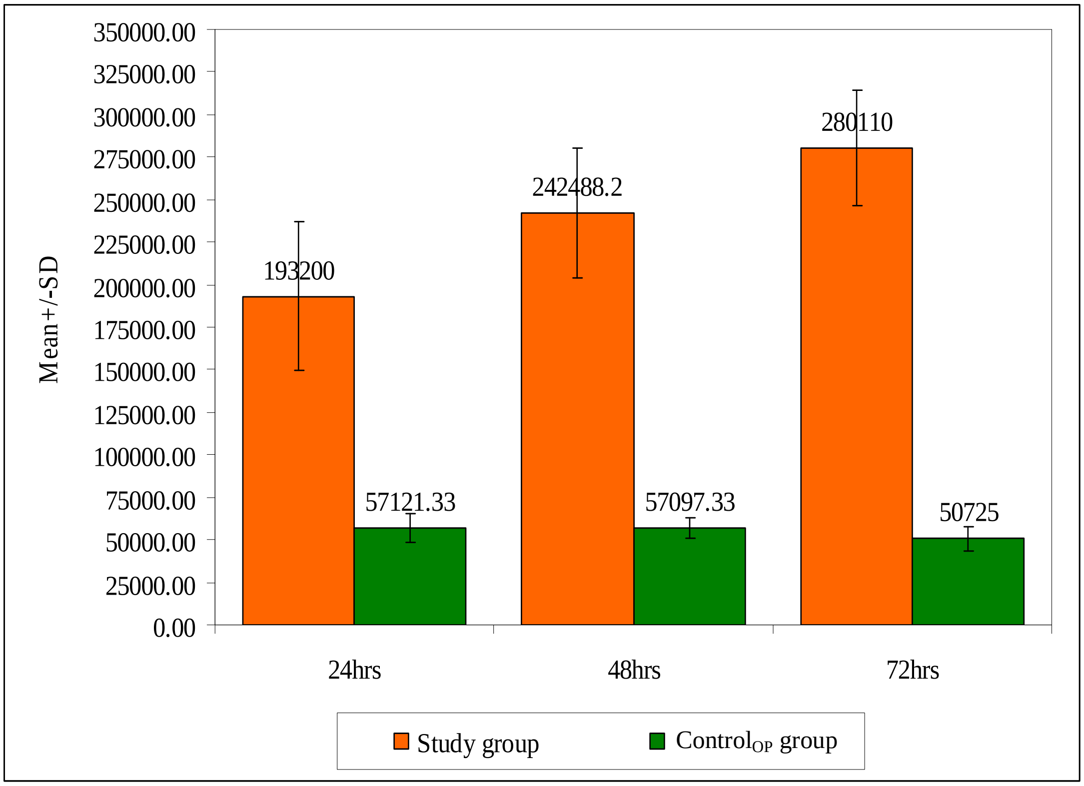


Table 7: Comparison of different time intervals with respect to cell attachment in study group and control$_{OP}$ group by dependent t and repeated measures of ANOVA

Group	Time point	Mean	SD	Mean Diff.	SD Diff.	% of change	t-value	p-value	F-value	Effect size
Study group	24hrs	193200.00	43515.51						20.671*	0.596
	48hrs	242488.20	38229.07	-49288.20	62595.74	-25.51	-3.0496	0.0087*		
	24hrs	193200.00	43515.51							
	72hrs	280110.00	33860.53	-86910.00	45513.34	-44.98	-7.3957	0.0001*		
	48hrs	242488.20	38229.07							
	72hrs	280110.00	33860.53	-37621.80	47765.39	-15.51	-3.0505	0.0086*		
Control$_{OP}$ group	24hrs	57121.33	8665.17						3.413*	0.196
	48hrs	57097.33	6068.66	24.00	10959.79	0.04	0.0085	0.9934		
	24hrs	57121.33	8665.17							
	72hrs	50725.00	7446.95	6396.33	12775.81	11.20	1.9390	0.0729		
	48hrs	57097.33	6068.66							
	72hrs	50725.00	7446.95	6372.33	8655.32	11.16	2.8514	0.0128*		

*p<0.05

This table demonstrates statistically significant difference in the cell attachment between 24-48 hours, 48-72 hours and 24-72 hours with respect to the study group with an effect size of 59 % as compared to 19% in the control$_{OP}$ group with statistically significant change seen only at 48-72 hour time interval.

Graph 3: Comparison of different time intervals with respect to cell attachment in study group and control$_{OP}$ group.

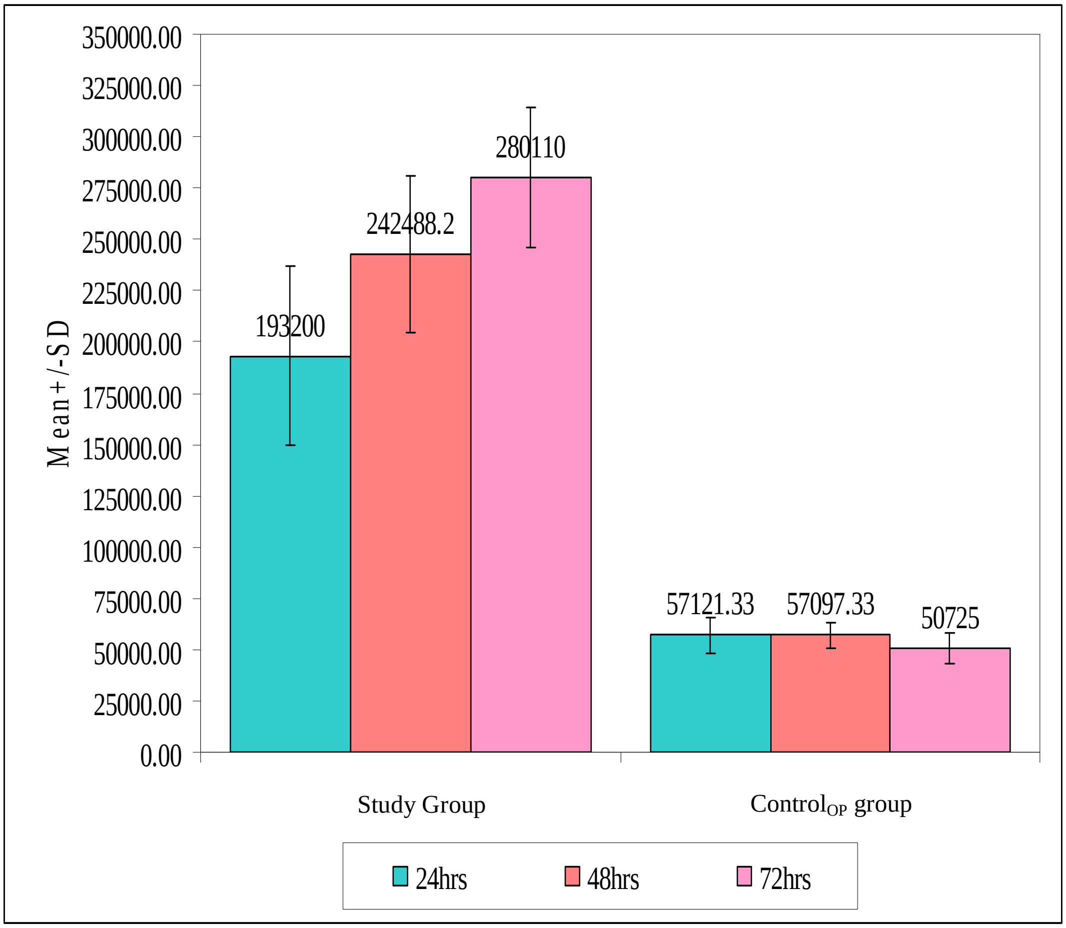


Table 8: Comparison of study group and control$_{OP}$ group with percentage of cell proliferation at different time intervals 24, 48, 72 hours by independent t test

Time points	Study group		Control$_{OP}$ group		t-value	p-value
	Mean	SD	Mean	SD		
24hrs	88.27	3.73	80.13	4.44	5.4328	0.0001*
48hrs	85.07	5.28	73.21	3.11	7.4902	0.0001*
72hrs	83.80	2.54	75.13	4.07	6.9975	0.0001*
24hrs-48hrs	-3.20	7.30	-6.93	5.68	1.5602	0.1299
24hrs-72hrs	-4.47	5.19	-5.00	4.94	0.2881	0.7754

*$p<0.05$

The table shows statistically significant difference in the cell proliferation at 24 , 48 , 72 hours between the study and control$_{OP}$ group. However there was no significant change was seen at 24-48 and 24-72 hours between the two groups.

Graph 4: Comparison of study group and control$_{OP}$ group with percentage of cell proliferation at different time intervals 24, 48 , 72 hours.

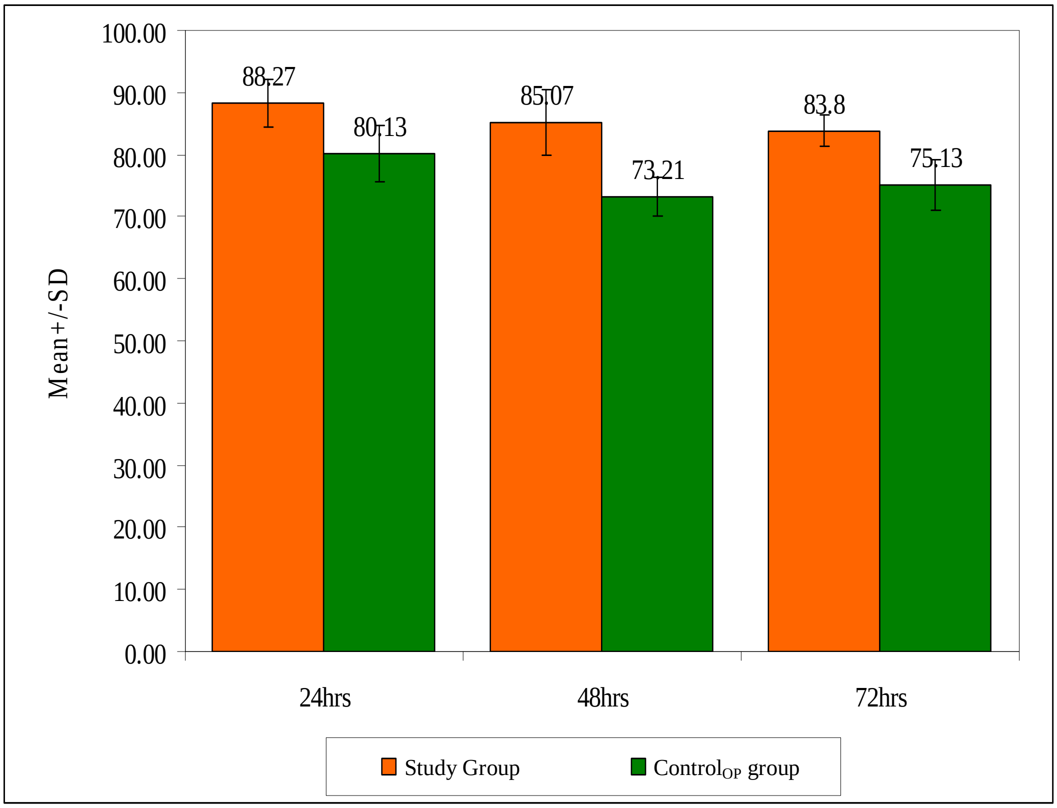
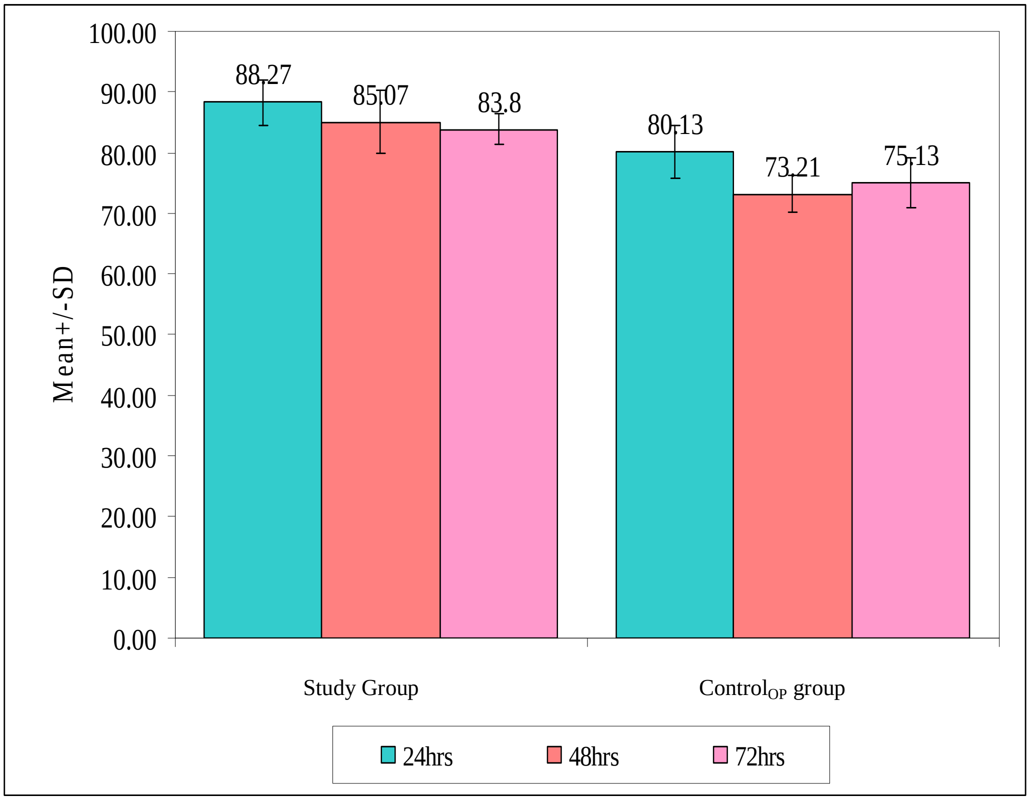


Table 9: Comparison of different time intervals with respect to percentage of cell proliferation in study group and control$_{OP}$ group by dependent t and repeated measures of ANOVA (*p<0.05)

Group	Time point	Mean	SD	Mean Diff.	SD Diff.	% of change	t-value	p-value
Study group	24hrs	88.27	3.73	3.20	7.30	3.63	1.6974	0.1117
	48hrs	85.07	5.28					
	24hrs	88.27	3.73	4.47	5.19	5.06	3.3304	0.0050*
	72hrs	83.80	2.54					
	48hrs	85.07	5.28	1.27	4.33	1.49	1.1320	0.2766
	72hrs	83.80	2.54					
Control$_{OP}$ group	24hrs	80.13	4.44	6.93	5.68	8.64	4.7227	0.0003*
	48hrs	73.21	3.11					
	24hrs	80.13	4.44	5.00	4.94	6.24	3.9180	0.0015*
	72hrs	75.13	4.07					
	48hrs	73.21	3.11	-1.93	5.02	-2.63	-1.4874	0.1591
	72hrs	75.13	4.07					

This table demonstrated that there was no significant difference seen in the cell proliferation for the study group at 24-48 and 48-72 hours however statistically significant difference was seen at 48-72 hour time interval.

Graph 5: Comparison of different time intervals with respect to percentage of cell proliferation in study group and control$_{OP}$ group.



DISCUSSION

Currently, dental implants have become an integral part of dentistry. Enhancing of dental implant osseointegration has been an area of research for more than four decades now. Researchers have tried from macrogeometry to microgeometry, change in materials, drill sequences, etc., to enhance osseointegration. It is well acknowledged that the overall success and survival of implants depend on the quality and quantity of host bone, presence of sufficient primary stability at the time of implant placement, and formation of a direct BIC. [44]

Osseointegration consists of a series of bone modeling and remodeling processes. It has actually been defined as the direct structural and functional connection between living bone and the surface of a load-bearing artificial implant. The success of osseointegration depends on the quality, distribution, and amount of bone present at the site of the dental implant.[40]

However, osseointegration and BIC are mainly enhanced by implant surface characteristics, such as surface topography, chemistry, roughness, and energy. [40]

Titanium is the most widely used material for dental implants due to its minimal toxicity, resistance to corrosion, high mechanical resistance, and biocompatibility. Titanium implants have always demonstrated better biocompatibility results and a better long-term prognosis. [40]

Albrektsson and Wennerberg classified implant surfaces as Smooth surfaces with a surface roughness ($Sa < 0.5$ μm), Minimally rough surface ($Sa = 0.5–1$ μm) , Moderately rough surface ($Sa = 1–2$ μm), Rough surface ($Sa > 2$ μm) and the authors concluded that moderately roughened surfaces seem to had some clinical advantages over smoother or rougher surfaces. [33]

In the present study for the surface characterisation, quantitative evaluation of all the disc-shaped specimens was assessed using Contact stylus Profilometer (Surtronic S-128 – Taylor Hobson) respectively. The profilometer is a contact stylus instrument used to measure surface profiles and roughness. This parameter describes the overall roughness of a surface and is the average arithmetical value of all absolute distances of the roughness profile from the center line within the measuring length. Profilometer determined the profile along 3 lines on the surface by means of a tracking device. An average roughness profile (Ra) was evaluated for each specimen to describe the overall roughness of the surface. The results obtained by surface profilometry showed that all tested specimens had a mean average surface roughness (Ra) within the range of 1.34 to 1.54 μm. To compliment the surface roughness values resulted on quantitative evaluation, the specimens were subjected to Scanning Electron Microscopy (100X , 500X, 1000X) to evaluate the surface roughness qualitatively.

The optimum value of average roughness found in the literature is 1–2 μm, [53] which coincided with average roughness values which was obtained from profilometric readings of the current study.

According to Lukaszewska et al, surface topography influences osteoblastic morphology; on rough surfaces, cells have a smaller coverage area and are less dispersed than on smooth surfaces. However, they exhibit numerous cytoplasmic extensions, phylodapy, and interconnections, which indicate greater adhesion properties compared to smooth surfaces. An increase in cellular vitality was observed over time on rough surfaces with respect to smooth surfaces [54]

Lima et al suggested that implants with a rough surface induce osseointegration better than machined ones, it is important that they be manipulated carefully, as this surface treatment can have a negative influence or can modify the surface of titanium oxide. [55] Hence it is important to standardize the surface roughness of the titanium so that a constant surface roughness could be used to evaluate the characteristics of the hydrogel.

Although Titanium implants have high clinical success rates, coatings of various materials have been advocated. [40] An effective coating surface should be able to do the following: Improve cell attachment, cell differentiation, and bone apposition; allow bone fixation; limit the rate of dissolution in the body fluids; and function in a therapeutic way. [56,57]

The success of oral rehabilitation using dental implants depends on numerous factors. The implantation process requires good interactions between the titanium surface and surrounding bone tissue as well as resistance against bacterial colonization since implant-related infections are responsible for a large part of implant failure. [58]

Studies indicate that around 29.48% (implant-based) and 46.83% (subject-based) of dental implants suffer from peri-implant mucositis and around 9.25% (implant-based) and 19.83% (subject-based) develop peri-implantitis. Peri-implant mucositis is a biofilm-induced inflammation localized on the soft peri-implant mucosa, without any evidence of supporting bone loss. It develops from healthy peri-implant mucosa around osseointegrated dental implants after the accumulation of bacterial biofilms. The major clinical sign of peri-implant mucositis is bleeding on probing, although it can also present erythema, swelling, and suppuration. However, with good oral hygiene and biofilm control, it can be reversed. Nonetheless, if left untreated, the inflammatory process may progress and trigger the gradual destruction of the bone surrounding the implant, resulting in peri-implantitis. [58]

These types of infections, when untreated, result in implant loosening and require implant removal. The ideal dental implant should have both enhanced osseointegration properties and protection from the bacteria that cause peri-implant mucositis.[59,60] To achieve this, titanium and its alloys with surface modifications were chosen as the go-to material in commercialized implants due to its properties such as great resistance to corrosion, biocompatibility, and good tolerance by the biological environment, amongst others. [59,60,61]

Inorder to control and prevent the accumulation of bacteria around the implant since bacterial adhesion occurs immediately after implantation and results in biofilm formation. Biofilm is also resistant to many antimicrobial agents, making it difficult to treat once established. [61] In order to prevent bacterial infections, surface coatings with antimicrobial properties were hypothesized to be a reliable solution for this problem. The titanium surface may be treated by adding materials or agents in the form of coatings.[39]

Coating materials such as silver, copper, zinc, chlorhexidine, and some antibiotics presented to be a promising solution due to antimicrobial properties that would fight bacterial colonization. Nonetheless, the methods required to modify and incorporate coatings in the implant surface are complex and antibiotics can cause bacterial resistance and further generate cellular toxicity. Furthermore, while trying to achieve maximum antimicrobial properties, biocompatibility and osseointegration properties may be lost. Hence a balance will always be the key to determine the potential of a coating. [62]

Chlorhexidine is the most popular and the primary agent for chemical plaque control and has earned its eponym of gold standard. But it cannot be used on a long term basis due to its growing resistance and its side effects that can impair the oral microflora and generate cellular toxicity. Hence, the search for alternatives continues, and the focus has shifted toward biogenic agents. [50]

Calendula officinalis is well known by its anti-inflammatory activity, the flavonoid quercetin is considered of great importance due to its anti-inflammatory and antioxidant effects It has been reported that Calendula extract lowered lipopolysaccharide-induced IL-1, and -6, TNF-alpha, interferon and acute-phase proteins levels.[51] In periodontal diseases, as a gel or mouthwash, Calendula has shown positive effect on gingivitis treatment and has been used as specifically in periodontitis, and also has been used in the treatment of periimplant mucositis,[45] hence it was required to evaluate the antimicrobial efficacy and the osteogenic potential of *Calendula officinalis* as a hydrogel coated onto titanium surfaces.

The hydrogel-based treatment for bone tissue engineering seems rather promising. However, the hydrogel formulation biomaterials employed for bone regeneration are either synthetic biomaterials or are a combination of synthetic and natural biomaterials. When applied within the bone defect, these synthetic or natural non-modified hydrogels are generally not conducive to the cellular influx. However, creating hydrogels with positively charged domains have improved the hydrogel's ability to encourage cellular adhesion within in vitro models. [63]

Listgarten et al in their study concluded that bacterial flora around implant has found to be similar to that of microbial flora around natural teeth, include *Streptococci, Veillonella* species, *Capnocytophaga* species, *Fusobacterium nucleatum* etc. [64,65] While, *Actinomyces* species, *Prevotella intermedia*, and *Porphyromonas gingivalis* are the bacteria associated with peri-implantitis, similar to those associated with periodontitis.[65] High levels of periodontopathic bacteria including *Porphyromonas gingivalis, Prevotella intermedi*a and *Actinobacillus actinomycetemcomitans* confer an increased risk for periodontitis.[66] *Actinomyces* species are considered as early colonizers, while *Prevotella intermedia and Porphyromonas gingivalis* are considered as secondary colonizers of the surface of different prosthetic materials. [26,64,65]

In addition, others have reported on the presence of *Staphylococcus aureus* and enteric rods in cases with peri-implantitis. Failing dental implants have been associated with low antibody titer and avidity levels to *Staphylococcus aureus.*[26] Also, in vitro studies have confirmed its strong affinity to titanium surfaces. Thus, *Staphylococcus aureus* infection may be of importance in the development of peri-implantitis induced by bacterial infection.[26] In the absence of adequate oral hygiene, there is formation of the acquired pellicle followed by bacterial attachment with initial colonizers followed by cell-to-cell adhesion with secondary colonizers on the implant surface.

After surface roughness evaluation, all the test specimens from study group were coated with *calendula officinalis* hydrogel and specimens in the control$_{MA}$ group were treated with 1% Chlorhexidine gel. Three bacterial strains were used to assess the antimicrobial efficacy namely *Porphyromonas gingivalis* strain ATCC 33277, *Staphylococcus aureus* strain ATCC 25923, and *Aggregatibacter actinomycetemcomitans* strain ATCC 33384

The antimicrobial efficacy can be assessed by various methods such as adhesion methods, biofilm formation, however the most known and basic methods are the disk-diffusion and broth or agar dilution methods. These are simple and effective in which the diameter of the clear zone around an antibiotic-paper disk enables us to directly, and elegantly, visualize the inhibitory potency of an antimicrobial agent. The major advantages over other methods are its simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and the ease to interpret results provided. [67,68]

The antimicrobial efficacy of specimens in study and $control_{MA}$ group were comparable, the *Aggregatibacter actinomycetemcomitans* showed the greatest zone of inhibition compared to *Staphylococcus aureus and Porphyromonas gingivalis*. The zone of inhibition for the specimens in $control_{MA}$ group was higher compared to specimens in study group with respect to all organisms tested.

The findings were in accordance with the studies conducted Shankar et al[14], Lauk et al[43], Yadav et al[28] which concluded that *Calendula officinalis* showed antibacterial activity against periodontal pathogens, however its efficacy was not on par with chlorhexidine gluconate 0.2 %. *Calendula officinalis* contains flavonoids most important being quercetin. These are phenolic structures which contain one carbonyl group and complex with extracellular soluble proteins and bacterial cell walls. They are also capable of disrupting microbial membranes based on their lipophilic nature. [14] It was shown that the strong anti-inflammatory response of *Calendula officinalis* extract might be due to the inhibition of anti-inflammatory cytokines and cyclooxy-genase-2 and subsequent prostaglandin synthesis.[16] Like chlorhexidine, calendula also has the property of substantivity as demonstrated by Schmidgall et al in his ex-vivo laboratory model. [69]

In a study conducted by Khairnar et al [54] there was a significant reduction in the plaque and gingival index after using Calendula containing mouthwash which was in accordance to the study conducted by Yusoff et al., [70] which reported that a calendula-containing mouthwash was effective in reducing the plaque index and gingival index score during its study period, thereby these mouthwashes containing calendula were effective against the periodontal pathogens which are commonly associated with periodontal diseases.

Calendula exerts anti-inflammatory activity through reducing the level of proinflammatory cytokines like IL-1β, IL-.6, TNF-.α, in lipopolysaccharide-induced animals and also inhibits the expression of the Cox-2 gene. Flavonoids and carotenoids are potent antioxidant components of calendula, which have free radical-scavenging activity against the free radicals in a dose-dependent manner. Treatment with the extract of calendula enhanced the level of endogenous antioxidant catalase, superoxide dismutase, and glutathione in animals. The polysaccharide fraction of calendula has an immunomodulatory effect by stimulating the phagocytic activity of human granulocytes. [54]

Osteogenic potential for the specimens in the $control_{OP}$ and study group were evaluated by assessing their cell attachment and cell profileration using MG-63 cells (osteoblast like cells) on the hydrogel treated surfaces which acted as study group and machined titanium discs which were $control_{OP}$ group.

MG -63 cell lines were used because it can retain the differentiated phenotype over consecutive subcultures and shows a faster growth than the primary bone-forming lines making it a good in vitro model. These cell types are associated with initial cell material characterization that is known for its high proliferation potential due to its capacity to continuously divide and grow thereby finding its use in various invitro and biocompatibility studies [71,72]

In this study,cell adhesion was evaluated using the tryphan blue exclusion test. The viable cells with intact cell membranes mixed with dyes will take up the dye and will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm, this test is a simple and rapid technique measuring the cell viability and adhesion whereas the cell proliferation was assessed using MTT Assay, which measures the cellular metabolic activity and is an indicator of cell viability, proliferation and cytotoxicity. MTT Assay is a colorimetric reaction that can be easily measured from cell monolayers plated on multiwell plates, it is dependent on mitochondrial respiration and indirectly serves to assess the cellular energy capacity of a cell.[73]

The results of this study indicate that there was a higher cell adhesion in the hydrogel coated titanium surfaces at 24 , 48 and 72 hours. The essential finding of this investigation was that the hydrogel enhanced early adhesion of MG-63 to the titanium alloy surface. This can be attributed to the polyelectron complex which makes the surface hydrophilic and proteins interacting through functional groups which were consistent with findings of Studies done by Parfitt et al[74], Zhao et al. [75], Qu et al [76] who have shown that hydrophilic surfaces are more responsive to cell attachment using different kinds of models and materials.

The higher cell attachment may also be due to an increase in surface area and roughness of the surface. It is known that surface topography could also play a role in cell attachment and proliferation on implants as has been studied by Lukaszewska et al [55]

When compared between the time intervals within the groups there was a definite increase in the cell adhesion in the study group between 24-48 , 48-72 and 24-72 hours showing a greater effect size as compared to the $control_{OP}$ group.

Cell proliferation was evaluated by the MTT assay. Cells were seeded onto discs and evaluated at intervals of 24, 48 and 72 hours. There was a higher cell proliferation on the specimens in the study group at all the time intervals tested, however these changes were not significant at 24-48 and 24-72 hours between the study and $control_{OP}$ group.

When compared between time intervals there were no statistically significant results at 24-48 hours, however, significant difference was seen at 24-72 hour time interval indicating that there was a functional maturation as well as proliferation at 48 hour time interval. There was no statistical change seen between 48-72 hours which could be due to cell confluence in the study group.

These results were in agreement with the study conducted by Alexandre et al[15] in which there was a reduction of BALP enzyme (enzyme produced by osteoblast which is a specific indicator of global bone formation activity) that putforth an essential anabolic effect of *calendula officinalis* on the alveolar bone. Calendula has high content of flavonoid quercetin, which has been shown to enhance human osteoblast-like MG63 proliferation and differentiation, through MAPK signaling. It has also shown to increase the expression of markers associated with osteoblast differentiation process which include osteopontin, osterix, osteoprotegerin, and osteocalcin.[15] It was also reported that quercetin reversed the inhibition of osteoblast differentiation induced by lipopolysaccharide and increased alkaline phosphatase activity and mineralization.[77]

Saini et al reported that quercetin, the main flavonoid found in Calendula extract, has a major role in the pharmacological effects of this plant [36]. Zhou et al in his invitro study concluded that quercetin significantly increased osteoblast differentiation and induced mRNA expression of sialoprotein and osteocalcin in osteoblast culture. [78,79]

Napimoga et al in his in-vivo periodontitis model in rats showed that quercetin inhibited bone loss in periodontitis models, increased serum osteocalcin and the activity of alkaline phosphatase, contributing to bone tissue preservation. [38]

Alexandre et al in their ex-vivo study put forth that calendula officinalis extract has the potential to modulate the inflammatory responses and consequently prevent the osteoclast activation via the RANKL-OPG axis and also induce osteoblast activity.[15]

Collagen is the main constituent of periodontal ligament, which plays an important role in maintaining the architecture of the periodontium. De Almeida et al[80] showed that breakdown of collagen is one of the main markers in the progression of periodontal disease. Their study concluded that calendula extract reduced the collagen breakdown and increased the concentration of collagen. These results were in agreement with the study conducted by Tanideh et al [16] in which the lowest grade of collagen breakdown was seen in periodontitis induced rats treated with Calendula compared to control group.

Within the limitations the findings of this study suggest that Calendula officinalis extract showed antimicrobial activity with potential therapeutic uses in the prevention of early periimplantitis and to enhance the process of early osseointegration. The osteogenesis, induced by osteoblastic cells, is characterized by a sequence of events, involving cell attachment, cell proliferation and followed by the expression of osteoblast phenotype. [64]

The study investigated the response of osteoblast like MG-63 cells which were cultured on to the characterized titanium disc surfaces with novel hydrogel of *Calendula officinalis*. The results showed that all discs, coated with hydrogel independent of the surface roughness were effective against the predominant preimplant bacteria with enhanced cell attachment, cell proliferation compared to the specimens in machined titanium surface.

Even though Chlorhexidine is a gold standard antimicrobial agent, *Calendula officinalis* can be used as therapeutic alternative to the standard drugs without any adverse effects.

Based on the above findings the study showed that the tested *Calendula officinalis* product may represent an alternative method to reduce periimplantitis bacteria and also promote early osseointegration.

SCOPE OF THE STUDY

- The present study evaluated the microbial efficacy and osteogenic potential of titanium discs coated with a hydrogel of Calendula Officinalis.
- The results of the present study can be applied and investigated In Vivo conditions.
- Other implant materials such as zirconia and polyetheretherketone (PEEK), can be used to check the efficacy of this hydrogel on microbial efficacy and osteogenic potential.
- Since periimplantitis is a polymicrobial biofilm, the hydrogel can be evaluated for its efficacy against other periimplantitis bacteria.
- The hydrogel can be further tested for the duration of drug release, disintegration rate etc.
- Further research is suggested to assess the differentiation of cells with parameters like alkaline phosphatase activity (ALP), receptor activator of nuclear factor kappaB ligand (RANKL) , Alizarin Red staining and deposition of calcium deposits with Von Kossa Staining.

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LIMITATIONS OF STUDY

- This is an in vitro study; all the clinical conditions have not been studied and the sample size is relatively smaller.
- As peri-implantitis is caused by multiple complexes of microorganisms, the effect of the Calendula officinalis hydrogel on varying microbial strains was not carried out and only predominant microbial organisms in periimplantitis were included in the study.
- The study used MG-63 cells (osteoblasts like cells) is an osteosarcoma cell line which lack coherence in the capacity of cell differentiation
- Even though the dipping method is considered one of the commonly used hydrogel coating methods, there could be variation in the uniform thickness of the surface of the substrate. An ideal hydrogel coating method should have strong adhesion to the substrate and conformation to a substrate with an arbitrary shape.

CLINICAL IMPLICATIONS

In this study, the results showed that Calendula officinalis hydrogel and Chlorhexidine had a definite reduction in the periimplant bacteria. Commercially available Chlorhexidine had a more significant zone of inhibition as compared to Calendula.

Although various commercially available antimicrobials are used to treat early periimplantitis, these released at suboptimal concentrations are likely to promote bacterial resistance, whereas high doses of antimicrobials may alter the oral microflora, generate cell toxicity and impair osseointegration.The ethnopharmacological approach by using natural phytochemicals isolated from medicinal plants are considered to be a safe, effective, and good alternative to synthetic drugs.

When compared machined titanium surfaces to the calendula hydrogel treated discs, the hydrogel showed a significant increase in the cell attachment and cell proliferation of osteoblast like cells with a greater effect size.

With an aim of improving the success rate in cases of bone deficit and in systemic conditions like osteoporosis these hydrogels would expedite the bone healing thereby enabling immediate or early loading protocols along with stimulating bone growth to permit implant placement in sites that lack sufficient residual alveolar ridge. With these advantages, calendula officinalis hydrogel can be considered as a beneficial, promising and alternative treatment to prevent periimplantitis and help in early osseointegration.

CONCLUSION

Within the limitations of this in vitro study the following conclusions were drawn.

The *Calendula officinalis* hydrogel showed antimicrobial efficacy against few most common periimplant pathogens namely *Porphyromonas gingivalis, Aggregatibacter Actinomycetemcomitans and Staphylococcus aureus.*

The *Calendula officinalis* hydrogel showed higher antibacterial activity against *Aggregatibacter Actinomycetemcomitans* compared to *Porphyromonas gingivalis* and *Staphylococcus aureus*. However, the antimicrobial efficacy was lesser compared to routinely used Chlorhexidine Gluconate gel. The Calendula can be used as a natural therapeutic option in early periimplantitis conditions. On comparing the osteogenic potential, the cell attachment was higher on titanium surfaces which were treated with hydrogel of Calendula officinalis at all the different time intervals tested. The effects size of cell attachment was found to be significantly higher compared to machined titanium surface. The cell proliferation was higher on titanium surfaces coated with hydrogel of Calendula officinalis at different time intervals studied compared to machined titanium surface. However, there was no significant difference in the cell proliferation among the different time intervals tested.

SUMMARY

The present in vitro study was carried out to evaluate the effect of antimicrobial efficacy and osteogenic potential of Calendula officinalis hydrogel coated on titanium surfaces.

A total of 270 identical titanium disc shaped specimens measuring 10 mm in diameter and 2 mm in thickness were procured. 90 titanium discs were used for antimicrobial efficacy which were divided as study group (n=45) and control$_{MA}$ group (n = 45). The resultant specimens were subdivided into (n=15) in each subgroup to assess the antimicrobial efficacy against *Porphyromonas gingivalis*, A*ggregatibacter actinomycetemcomitans* and *Staphylococcus aureus* strain. To assess the osteogenic potential 180 titanium discs were used and divided as study group (n=90) and control$_{OP}$ group (n=90) which was further divided into two groups for cell attachment assay (n=45) and cell proliferation (n=45). These specimens were subdivided into (n=15) in each subgroup for different time intervals studied i.e. 24, 48 , 72 hours.

All the specimens from each group were subjected to Surface roughness evaluation quantitatively using Profilometer and one specimen from each group was subjected to Scanning Electron microscope to assess the surface roughness qualitatively. A mean roughness profile was evaluated for each specimen to describe overall roughness of the surface. SEM evaluation was done at 100x, 500x and 1000x magnification to visualize and compare the surface profile of all the groups qualitatively so as to provide a constant surface roughness for evaluation

After surface roughness evaluation, for the antimicrobial analysis the discs in the study group were coated with a Calendula officinalis hydrogel using dipping method while those in control$_{MA}$ group were coated with 1 % Chlorhexidine gel.Three bacterial strains were used to assess the antimicrobial efficacy on the test materials namely; *Porphyromonas gingivalis* strain ATCC 33277, A*ggregatibacter actinomycetemcomitans* strain ATCC 33384 and Staphylococcus aureus strain ATCC 25923. Antimicrobial efficacy was assessed using Zone of Inhibition.

Osteogenic Potential was evaluated by assessing the Cell Attachment and Cell Proliferation of MG-63 cells on Calendula officinalis hydrogel coated surfaces and machined titanium which was the $control_{OP}$ at 24 , 48, 72 hour time interval. The cell Attachment was evaluated using hemocytometer while Cell Proliferation was assessed using MTT Assay.

The resultant data were tabulated and then subjected to statistical analysis to draw conclusion from experimental data. ($p<0.05$)

The results showed that the hydrogel was effective against the three organisms tested. Aggregatibacter actinomycetemcomitans showed the highest zone of inhibition as compared to the other two organisms for the hydrogel treated group. The cell attachment was higher for the hydrogel treated group at all three time intervals as compared to machined titanium discs. There was a statistically significant difference in the cell attachment between the time intervals for the study group. The cell proliferation was higher in the study group with statistically significant results at all three time intervals. Hence, Calendula officinalis hydrogel could be used as surface coating which has antibacterial and osteogenic properties to prevent periimplantitis and to aid in early osseointegration.

BIBLIOGRAPHY

1. Sullivan, R.M. Implant dentistry and the concept of osseointegration: A historical perspective. J. Calif. Dent. Assoc. 2001, 29, 737–745.
2. Oshida, Y., Tuna, E.B., Aktören, O., Gençay, K. Dental implant systems. Int. J. Mol. Sci. 2010, 11, 1580–1678.
3. Bauer, S., Schmuki, P., von der Mark K., Park J. Engineering biocompatible implant surfaces: Part I: Materials and surfaces. Prog. Mater. Sci. 2013, 58, 261–326.
4. Zafar MS, Fareed MA, Riaz S, Latif M, Habib SR, Khurshid Z. Customized Therapeutic Surface Coatings for Dental Implants. Coatings. 2020; 10(6):568-72.
5. Jayesh RS, Dhinakarsamy V. Osseointegration. J Pharm Bioallied Sci. 2015;7(Suppl 1):S226-S229.
6. Liu Y, Rath B, Tingart M, Eschweiler J. Role of implants surface modification in osseointegration: A systematic review. J Biomed Mater Res A. 2020 Mar;108(3):470-484.
7. Mandracci P, Mussano F, Rivolo P, Carossa S. Surface Treatments and Functional Coatings for Biocompatibility Improvement and Bacterial Adhesion Reduction in Dental Implantology. Coatings. 2016; 6(1):7-18
8. Accioni F, Vázquez J, Merinero M, Begines B, Alcudia A. Latest Trends in Surface Modification for Dental Implantology: Innovative Developments and Analytical Applications. Pharmaceutics. 2022;14(2):455-67

9. Caló, E., Khutoryanskiy, V. Biomedical applications of hydrogels: A review of patents and commercial products. European Polymer Journal, 2015 ; 65, 252–267.

10. Mombelli A. Microbiology and antimicrobial therapy of peri-implantitis. Periodontology 2000 2002;28:177-189.

11. Kulkarni Aranya A, Pushalkar S, Zhao M, LeGeros RZ, Zhang Y, Saxena D. Antibacterial and bioactive coatings on titanium implant surfaces. J Biomed Mater Res A. 2017 Aug;105(8):2218-2227.

12. Smeets R., Stadlinger B., Schwarz F., Beck-Broichsitter B., Jung O., Precht C., Kloss F., Grobe A., Heiland M., Ebker T. Impact of Dental Implant Surface Modifications on Osseointegration. BioMed Res. Int. 2016; :6285-6290.

13. Rossiter SE, Fletcher MH, Wuest WM. Natural Products as Platforms To Overcome Antibiotic Resistance. Chem Rev. 2017;117(19):12415-12474.

14. Shankar SM, Bardvalli SG, Jyotirmayee R, Chethana, Bhushan K, Kumar S. Efficacy of Calendula officinalis extract (marigold flower) as an antimicrobial agent against oral microbes: An invitro study in comparison with chlorhexidine digluconate. J Clin Diagn Res. 2017;11(10) : ZC05-ZC10

15. Alexandre JTM, Sousa LHT, Lisboa MRP, Furlaneto FAC, do Val DR, Marques M, Vasconcelos HC, de Melo IM, Leitão R, Castro Brito GA, Goes P. Anti-inflammatory and antiresorptive effects of Calendula officinalis on inflammatory bone loss in rats. Clin Oral Investig. 2018;22(6):2175-2185

16. Tanideh N, Ghafari V, Ebrahimi R, Habibagahi R, Koohi-Hosseinabadi O, Iraji A. Effects of Calendula Officinalis and Hypericum Perforatum on Antioxidant, Anti-Inflammatory, and Histopathology Indices of Induced Periodontitis in Male Rats. J Dent (Shiraz). 2020;21(4):314-321.

17. Misch C.E. Contemporary Implant Dentistry. 3rd ed. Mosby. St. Louis. 2007.

18. Guang Zhu,Guocheng G , Wang A, Jiao J Advances in implant surface modifications to improve osseointegration Mater. Adv., 2021(2) ; 6901-27

19. Weigel PH, Fuller GM, LeBoeuf RD. A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. Journal of theoretical biology. 1986. 21;119(2):219-34

20. Jaini JL, Jeeva PP, Raj RS, Mohan SG. Ethnopharmacological Reflections in Oral Health: A Review on Current Concepts. Int J Oral Care Res 2017;5(4):310-316

21. Patil K, Sanjay CJ, Doggalli N,Devi KR, Harshitha N. A Review of Calendula Officinalis Magic in Science. Journal of Clinical and Diagnostic Research. 2022 16(2). ZE23-ZE27.

22. K. J. Bundy, M. F. Butler, R. F. Hochman. An Investigation of the Bacteriostatic Properties of Pure Metals. Journal of Biomedical Materials Research 1980;14:653-663.

23. Parr GR, Gardner LK, Toth RW. Titanium: the mystery metal of implant dentistry. Dental materials aspects. J Prosthet Dent. 1985;54(3):410-4

24. Mombelli A, Van Oosten MAC, Schurch E, Lang NP. The Microbiota Associated with Successful or Failing Osseointegrated Titanium Implants. Oral Microbiol Immunol 1987;2:145-151

25. Sennerby L, Ericson LE, Thomsen P, Lekholm U, Åstrand P. Structure of the bone-titanium interface in retrieved clinical oral implants. Clin oral impl res. 1991;2;103-11

26. ESM Ong, H.N. Newman, M. Wilson, J.S. Bulman. The Occurrence of Periodontitis-Related Microorganisms in Relation to Titanium Implants. J Periodontol 1992;63:200-205

27. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clinical microbiology reviews. 1999;1;12;147-79.

28. Lauk L, Lo Bue AM, Milazzo I, Rapisarda A, Blandino G. Antibacterial activity of medicinal plant extracts against periodontopathic bacteria. Phytother Res. 2003 ;17(6):599-604

29. Basch E, Bent S, Foppa I, Haskmi S, Kroll D, Mele M, Szapary P, Ulbricht C, Vora M, Yong S; Natural Standard Research Collaboration. Marigold (Calendula officinalis L.): an evidence-based systematic review by the Natural Standard Research Collaboration. J Herb Pharmacother. 2006;6(3-4):135-59.

30. Lindhe J, Meyle J, Group D of the European Workshop on Periodontology.Peri-implant diseases: consensus report of the sixth European workshop on periodontology. Journal of clinical periodontology. 2008;35:282-5.

31. Renvert S, Roos-Jansåker AM, Claffey N. Non-surgical treatment of peri-implant mucositis and peri-implantitis: a literature review. J. Clin. Periodont. 2008;35;305-15.

32. Fernández-Barbero A et al. Gels and microgels for nanotechnological applications. Adv. Coll. Int. Sci. 2009(1);147:88-108.

33. Wennerberg A., Albrektsson T. On implant surfaces: A review of current knowledge and opinions. Int. J. Oral Maxillofac. Implant. 2010;25:63–74.

34. Ungvári K et al. Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis. J Biomed Mat Res. 2010;94;222-9.

35. Faria RL, Cardoso LM, Akisue G, Pereira CA, Junqueira JC, Jorge AO, Santos Júnior PV. Antimicrobial activity of Calendula officinalis, Camellia sinensis and chlorhexidine against the adherence of microorganisms to sutures after extraction of unerupted third molars. J Appl Oral Sci. 2011;19(5):476-82.

36. Saini P, Al-Shibani N, Sun J, Zhang W, Song F, Gregson KS, Windsor LJ. Effects of Calendula officinalis on human gingival fibroblasts. Homeopathy. 2012 Apr;101(2):92-98

37. Parente LM, Lino Júnior Rde S, Tresvenzol LM, Vinaud MC, de Paula JR, Paulo NM. Wound Healing and Anti-Inflammatory Effect in Animal Models of Calendula officinalis L. Growing in Brazil. Evid Based Complement Alternat Med. 2012; 3756-71

38. Napimoga MH, Clemente-Napimoga JT, Macedo CG, Freitas FF, Stipp RN, Pinho-Ribeiro FA, Casagrande R, Verri WA Jr. Quercetin inhibits inflammatory bone resorption in a mouse periodontitis model. J Nat Prod. 2013;76(12):2316-21.

39. Jemat A, Ghazali MJ, Razali M, Otsuka Y. Surface Modifications and Their Effects on Titanium Dental Implants. Biomed Res Int. 2015 :791-725

40. Xuereb M., Camilleri J., Attard N. Systematic Review of Current Dental Implant Coating Materials and Novel Coating Techniques.Int J Prosth, 2015 : 28(1), 51–59.

41. Mendoza-Arnau A, Vallecillo-Capilla MF, Cabrerizo-Vílchez MÁ, Rosales-Leal JI. Topographic characterisation of dental implants for commercial use. Med Oral Patol Oral Cir Bucal. 2016;21(5):631-36

42. Uribe-Fentanes LK, Soriano-Padilla F, Pérez-Frutos JR, Veras-Hernández MA. Action of Calendula officinalis essence on bone preservation after the extraction Rev Med Inst Mex Seguro Soc. 2018;56(1):98-105

43. Yadav SC et al. Calendula : A Blooming Alternative Against Periopathogens. J Pharm Bio Sci. 2017 ; 12(2) : 36-39

44. Chowdhary R, Pachimalla P ,Mishra S. An in vitro Study to Evaluate the Bioactivity of Osteoblast Cells on the Titanium Disk Coated with the Hydro Gel formulated from Acemannan and Curcuminoids. Int J Prosthodont Restor dent. 2018(8). 22-27.

45. Gleiznys D, Gleiznys A, Abraškevičiūtė L, Vitkauskienė A, Šaferis V, Sakalauskienė J. Interleukin-10 and Interleukin-1β Cytokines Expression in Leukocytes of Patients with Chronic Peri-Mucositis. Med Sci Monit. 2019 ;25:7471-7479

46. Yin Y, Hu B, Yuan X, Cai L, Gao H, Yang Q. Nanogel: A versatile nanodelivery system for biomedical applications. Pharmaceutics. 2020;12;290.

47. Camargo SEA, Roy T, Carey Iv PH, Fares C, Ren F, Clark AE, Esquivel-Upshaw JF. Novel Coatings to Minimize Bacterial Adhesion and Promote Osteoblast Activity for Titanium Implants. J Funct Biomater. 2020;11(2):42.

48. Milutinovici RA, Chioran D, Buzatu R, Macasoi I, Razvan S, Chioibas R, Corlan IV, Tanase A, Horia C, Popovici RA, Dinu S, Dehelean C, Scurtu A, Pinzaru I, Soica C. Vegetal Compounds as Sources of Prophylactic and Therapeutic Agents in Dentistry. Plants (Basel). 2021;10(10):2148.

49. El-Sayed MK, Hommos AM, Kotry GS, Labib GS. The effect of a calendula based topical formulaversus oxidized regenerated cellulose on palatal wound healing: A randomized controlledclinical trial. Alex Dent J. 2021;46(2):45-53

50. Ayyanahalli Matta BK, Kumar S, Mehta CH, Nayak UY, Rodriguez PG. Comparative Evaluation of the Effectiveness of a Combination of Absorbable Gelatin Sponge and Calendula officinalis with Absorbable Gelatin Sponge Used Alone as a Hemostatic Agent-An In-Vitro Study. Dent J (Basel). 2022;10(5):76

51. Khairnar MS, Pawar B, Marawar PP, Mani A. Evaluation of Calendula officinalis as an anti-plaque and anti-gingivitis agent. J Indian Soc Periodontol. 2013 ;17(6):741-7

52. Liu N, Wang H, Fu Z, Zhang C, Hui W, Wu J, Zhang Y, Zhang S. Quercetin-Coating Promotes Osteogenic Differentiation, Osseointegration and Anti-Inflammatory Properties of Nano-Topographic Modificated 3D-Printed Ti6Al4V Implant. Front Bioeng Biotechnol. 2022 ;10:933-950.

53. Rizo-Gorrita M, Luna-Oliva I, Serrera-Figallo MA, Torres-Lagares D. Superficial Characteristics of Titanium after Treatment of Chorreated Surface, Passive Acid, and Decontamination with Argon Plasma. J Funct Biomater. 2018;9(4):71

54. Lukaszewska-Kuska M., Leda B., Gajdus P., Hedzelek W. Evaluation of modified titanium surfaces physical and chemical characteristics. Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. Atoms. 2017;4(11):94–99.

55. Da Costa L, Valente M., Shimano A.C., Marcantonio Junior E., Reis A.C. Relationship between the surface chemical composition of implants and contact with the substrate. J. Oral Implant. 2015;41:17–21.

56. Oshida Y, Tuna EB,Aktoren O, Gencay K. Dental Implant Systems. Int J Mol Sci. 2010 ; 11 : 1580-1678

57. Tomisa AP, Launey ME, Lee JS, Mankani MH, Wegst UG, Saiz E. Nanotechnology approaches for better dental implants. Int J Oral Maxillofac Implants 2011 ; 26 : 25-49

58. Esteves GM, Esteves J, Resende M, Mendes L, Azevedo AS. Antimicrobial and Antibiofilm Coating of Dental Implants-Past and New Perspectives. Antibiotics (Basel). 2022;11(2):235.

59. Heitz-Mayfield L.J.A., Salvi G.E. Peri-implant mucositis. J. Periodontol. 2018; 89: S257–S266.

60. Salvi G.E., Aglietta M., Eick S., Sculean A., Lang N.P., Ramseier C.A. Reversibility of experimental peri-implant mucositis compared with experimental gingivitis in humans. Clin. Oral Implants Res. 2012;23:182–190.

61. Santiago-Medina P., Sundaram P.A., Diffoot-Carlo N. Titanium Oxide: A Bioactive Factor in Osteoblast Differentiation. Int. J. Dent. 2015;2015:357-653.

62. Janson O., Gururaj S., Pujari-Palmer S., Karlsson Ott M., Stromme M., Engqvist H., Welch K. Titanium surface modification to enhance antibacterial and bioactive properties while retaining biocompatibility. Mater. Sci. Eng. C Mater. Biol. Appl. 2019;96:272–279.

63. Boyan BD, Hummert TW, Dean DD, Schwartz Z. Role of material surfaces in regulating bone and cartilage cell response. Biomaterials. 1996;17(2):137-46.

64. Max A. Listgarten. Microorganisms and Dental Implants. J Periodontol 1999;70(2):220-222.

65. Angie Lee, Hom-Lay Wan. Biofilm Related to Dental Implants. Implant Dentistry 2010;19(5):387-92.

66. Masahiro E, Tadashi M, Tetsuo K, Atsushi S, Masao Y. In Vitro Adherence of Periodontopathic Bacteria to Zirconia and Titanium Surfaces. Dental Materials Journal 2013;32(1):101-106.

67. Barnard, R. T. The Zone of Inhibition. Clinical Chemistry. 2019 : 65(6), 819–820.

68. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016 Apr;6(2):71-79.

69. Schmidgall J, Schnetz E, Hensel A. Evidence for bioadhesive effects of polysaccharides and polysaccharide-containing herbs in an ex vivo bioadhesion assay on buccal membranes. Planta Med. 2000;66:48–53

70. Yusoff S, Kamin S. The effect of a mouthwash containing extract Calendula officinalis on plaque and gingivitis. J Clin Periodontal : 2006;33:118-127.

71. González-Blanco C, Rizo-Gorrita M, Luna-Oliva I, Serrera-Figallo MÁ, Torres-Lagares D, Gutiérrez-Pérez JL. Human Osteoblast Cell Behaviour on Titanium Discs Treated with Argon Plasma. Materials (Basel). 2019;12(11):1735.

72. O'Doherty M et al. Improving the Intercellular Uptake and Osteogenic Potency of Calcium Phosphate via Nanocomplexation with the RALA Peptide. Nanomaterials (Basel). 2020;10(12):2442

73. Chacon E., Acosta, D., & Lemasters, J. J. . Primary Cultures of Cardiac Myocytes as In Vitro Models for Pharmacological and Toxicological Assessments. In Vitro Methods in Pharmaceutical Research, 1997 : 209–223.

74. Parfitt, A.M. Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. Bone. 2002 : 30: 5–7.

75. Zhao, G et al. High surface energy enhances cell response to titanium substrate microstructure. J Biomed Mat Res. Part A. 2005 : 74: 49–58.

76. Qu, Z., Rausch-Fan, X., Wieland, M., Matejka, M., Schedle, A. The initial attachment and subsequent behaviour regulation of osteoblasts by dental implant surface modification. J Biomed Mat Res. Part A 2007 : 82: 658–668.

77. Wang XC, Zhao NJ, Guo C, Chen JT, Song JL, Gao L. Quercetin reversed lipopolysaccharide-induced inhibition of osteoblast differentiation through the mitogen-activated protein kinase pathway in MC3T3-E1 cells. Mol Med Rep. 2014 : 10 ; 3320–3326

78. Zhou, Y., Wu, Y., Jiang, X., Zhang, X., Xia, L., Lin, K. The effect of quercetin on the osteogenesic differentiation and angiogenic factor expression of bone marrow-derived mesenchymal stem cells. PLoS ONE. 2015 10 :129-39.

79. Satué, M., Arriero, M., del, M., Monjo, M., and Ramis, J. M. Quercitrin and taxifolin stimulate osteoblast differentiation inMC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7 cells. Biochem. Pharmacol.2013 : 86 ; 1476–1486.

80. De Almeida LF et al. Effect of collagen matrix saturation on the surface free energy of dentin using different agents. J Contemp Dent Pract. 2015; 16: 531–536.

ANNEXURE – I

ETHICAL CLEARANCE

2021

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University

Accredited 'A' Grade by NAAC   Placed in Category 'A' by MHRD (GoI)

Nehru Nagar, Belagavi - 590 010, Karnataka State

☎: 0831-2470362   Web: http://www.kledental-bgm.edu.in
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Sl. No. : 1457

CERTIFICATE

This is to Certify that the synopsis titled Evaluation of antimicrobial efficacy and osteogenic potential of titanium coated with hydrogel of Calandulla officinalis - An invitro study *Submitted by Dr.* REG. NO- IM0220006 *P. G. Student / Staff, Guided by* _______ *from Department of* Prosthodontics & Crown & Bridge *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

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

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE- II

CERTIFICATE OF ANALYSIS OF CALENDULA EXTRACT

Kuber Impex Ltd

Certificate of Analysis

Product Name	**: Calendula Extract 1%**
Botanical name	**: Calendula officinalis**
Batch Number	**: DSHE125/S/CE.50**
Plant parts used	**: Flower**
Solvent used	**: Hydro Methonolic**
Principal l Consti.	**: Flavones 01.0%**
Mfg. M	**: APRL,2019**
Exp. M	**: MAR,2022**

Tested Parameters	Specifications	Results
D. Physical Parameters		
Description	Light Brown Colour Powder .	Complies
Identification	Flavones By TLC	Complies
Odour & Taste	Slight Biter & salty	Complies
LoD as par IP	NMT 5.0 %w/w	3.35%
P^H 1% Solution	4 –7	4.5
Solubility in water	NLT 80%w/v	89.65% w/v
B. Chemical Parameters		
1. Heavy Metal	NMT 10ppm	Complies
Arsenic (As)	Less than 2ppm	Complies
Lead (Pb)	Less than 1ppm	Complies
Mercury (Hg)	Less than 1ppm	Complies
Cadmium (Cd)	Less than 0.5ppm	Complies
2. Active ingredient – Assay		
Total Flavones	NLT 01.0% w/w	1.87%w/w
C. Microbiological Tests		
Total Aerobic Plate Count	NMT 5000Cfu/gm	700fu/gm
Total Yeast & Mold	NMT 100Cfu/gm	20cfu/gm
E. Coli	Absent	Negative
Salmonella	Absent	Negative
S. Aureus	Absent	Negative
D. Others		
Packaging	Packed in Food grade HDPE Bags sealed into HDPE Containers	
Storage	Air tight container, protect from heat & moisture	

KUBER IMPEX LTD. INDIA INDORE

CIN:-U52311MP1997PLC011825

304-305, The Magnet Tower,
16/1 New Palasia, Indore 452001 (MP) INDIA
+91 731 4727800, 4727999.
contact@kuberimpex.com

WEBSITE:-WWW.KUBERIMPEX.COM | WWW.HERBSEXPORTER.COM

ANNEXURE – III

CERTIFICATION OF GRADE V TITANIUM DISCS

UKASCB®
UNITED KINGDOM APPROVAL
ISO 9001:2015 Certified
- Optical Emission Spectrometry
- PMI
- Hardness Testing
- Ultrasonic Flaw Detection
- Ultrasonic Thickness Gauging
- Dye Penetrant Testing

MTL

METAL TEST LAB

(Recognised By Government Deptts & Undertakings)

Office : Gr. Flr. Bhavnagari Bldg., 72, Nanubhai Desai Rd., Khetwadi Main Road, Mumbai - 400 004.
Phone : 6743 7546 • **Mobile :** 9224778882 / 9223371637 • **E-mail :** metaltestlab2016@gmail.com

TEST REPORT

T/C No : G/ 40321 DATE 04/03/2021

PARTY NAME : SPECIAL METALS

REFERENCE : -

MATERIAL DESCRIPTION: TITANIUM DISC

GRADE : TI GR.5

%	C %	Si %	Mn %	P %	S %	Cr %	Mo %	Ni %	Al %
COMP	0.0580	--	--	--	--	--	--	--	6.00
REQD	--	--	--	--	--	--	--	--	5.5000
	0.0800	--	--	--	--	--	--	--	6.7500

%	Co %	Cu %	Nb %	Ti %	V %	W %	Pb %	Fe %	N %
COMP	--	--		87.88	5.00 --	--	--	0.069	--
REQD	--	--	--	--	3.5000	--	--	--	--
	--	--	--	--	4.5000	--	--	0.40 00	--

REMARK: THE ABOVE MATERIAL CONFIRMS TO TITANIUM GR.5 W.R.T. ELEMENTS SPECIFIED.

FOR METAL TEST LAB

AUTHORISED SIGNATORY

1. The above Test Reports relate only to the sample submitted.
2. The above samples are not drawn by the laboratory.
3. The company or its partners shall in no way responsible for any financial liability due to any act of omission or error made.
4. No part of this Test Report shall be reproduced without the written permission of this laboratory.

QUALITY IS OUR MOTTO